Mahabaleshwar V. Hegde
Anand Arvind Zanwar
Sharad P. Adekar *Editors*

Omega-3 Fatty Acids

Keys to Nutritional Health

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Preface

This book, "*Omega-3 Fatty acids: Keys to Nutritional Health*," is the product of our sincere effort, to provide scientific evidence for the extraordinary power of nature's wonder molecules —omega-3-fatty acids. Chapters by experts in different specific aspects of omega-3 fatty acids for human health, have been presented to our wide spread readers, nutritionists, dieticians, clinicians, and all health conscious readers and health professionals. There is no exaggeration if we state that man owes his very existence on this planet to omega-3 fatty acids, as these molecules are largely responsible for the creation human brain. It is the brain that gives man the extraordinary power to sense the nature and its environment and enable him to adapt, to live in more comfort. Omega-3 fatty acids, besides being the hardware of the brain, take active part in almost every aspect of life reactions in health and disease.

Our Chap. 1 on "*Nutrition, Life, Disease, and Death*" narrates the importance of supply of all essential nutrients in adequate quantities including omega-3 fatty acids and Chap. 36 of Dr. M. Jeganathan on "*Role of Antioxidants*" which are also needed not only as anti-stress, anti-aging nutrient, but also to prevent oxidation of omega-3 fatty acids in human body's hostile environment.

It is unequivocally established that recent rise in the incidences and severity of several diseases, including diabetes, heart disease, obesity, pregnancy complications, alzheimer, psoriasis and aging, can be primarily attributed to the paucity of omega-3 fatty acids in modern human diet. Hence, "*Bring Back Omega-3 Fatty acid into Food Chain*" has been aglobal cry. Therefore, our Chap. 2 on "*Flax Biovillage*" and Chap. 3, "*Linseed Agriculture*" by Dr. P.K Singh aim at unleashing the power of linseed, for omega-3 nutritional security. Chap. 21 by Dr. Scott Doughman presents a case of "*Microalgae oil*" and Dr. Rafael Zarate's in Chap. 9, that of "*marine algae*,"as safe and effective vegetarian food. Authors argue that different biotechnological approaches can boost fatty acid yield in microalgae, and thereby, microalgae may become important attractive, continuous, sustainable good omega-3 source, to satisfy the increasing world demand. In Chap. 34, Georgia Lenihan-Geels discusses the prospects of bioengineering of plant seed oils for docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), algae aquaculture and enhancement of LC-PUFA in meat, and dairy products through plant-derived livestock feeds. In addition to increase the world omega-3 supply, for actually attaining omega-3 nutritional security and better health for one and all, another attractive complementary strategy is to fortify food, resourcing omega-3 fatty acid from flaxseed, marine algae, or fish oil sources. Manohar Panse in Chap. 8, while discussing the "*Omega-3 fatty acid Food Fortification*," also highlights the importance of compliance of regulatory guidelines, required for marketing such products. Further, Manohar Panse has also discussed *Development of omega-3 eggs* in Chap. 5 and *World Market of Omega 3 Fatty Acids* in Chap. 7. Most importantly, the *pharmacokinetics of safety of omega-3 fatty acids* has been reviewed by Dr. Juan Tamargo in Chap. 39. Dr. Puranik in Chap. 10 describes *omega-3 oil emulsion* that prevents lipid peroxidation and also offers not only increased stability and shelf life but also better bioavailability. In our Chap. 4 on "*Omega-3 Milk*," with our colleague Dr. P.B. Ghorpade and Dr. S.L. Bodhankar, we narrate the importance of incorporating omega-3 fatty acid in milk and its utility for human health.

The major problem today is the severe imbalance of the two essential fatty acids namely omega-3 and omega-6 fatty acids. Omega-3 fatty acids being primarily anti-inflammatory and omega-6 being proinflammatory, too much omega-6 and very little of omega-3 fatty acids in modern human diet, the disease-prone inflammatory pathway is dominant in the modern man. Dr. Kadooin in Chap. 15 discusses how omega-3 to omega-6 ratios can be manipulated in oilseeds to achieve balance of omega-3 and omega-6 fatty acids.

Most interesting thought-provoking chapters (Chaps. 27–32) have been provided by Robert Brown. His concern about the linoleic acid/alpha-linolenic acid imbalance and its influence on various aspects of ill health today is evident from the inferences drawn by him.

One of the major well-acknowledged effects of omega-3 fatty acids are their ability to prevent heart diseases. Dr. Manohar Garg in Chap. 6 discusses how omega-3 fatty acids control *hyperlipidemia*; Dr. Jubbin Jacob in Chap. 37, *cardiovascular disorder*; Dr. Quian Gao in Chap. 25, *cardiovascular events*; and Dr. Sang Lee in Chap. 33, *Myocardial Infarction*.

Dr. Sayed Ahmed in Chap. 11, discusses the mechanism by which EPA- and DHA-derived eicosanoids and lipid mediators contain chronic inflammation and prevent degenerative diseases.

Effects of omega-3 fatty acids on immune system in reducing the pathological manifestation especially in diseases related to inflammation, allergy, and autoimmunity have been discussed by Dr. Sudha Gangal in Chap. 26.

Dr. Vikas Kumar in Chap. 38 on *Psoriasis*, a multifaceted autoimmune disorder discusses the potential benefits of omega-3 fatty acid, their metabolites, and the mechanisms involved in psoriasis treatment.

Role of omega-3 fatty acids in mitochondrial diseases and its profound effects on muscle, brain, heart, liver, nerves, eyes, ears, kidney functions, involvement in CVD, and diabetes have been discussed by Dr. S. Katyare in Chap. 17. Dr. Katyare in chapter on diabetes shows the link between omega-3 fatty acids in diabetic complications, neuropathy, retinopathy, nephropathy, and angiopathy, and the beneficial effect of omega-3 fatty acid supplementation in Chap. 16, and Dr. Katyare further in Chap. 18 on Alzheimer argues that omega-3 fatty acid supplementation may be safe and prophylactic for Alzheimer's disease.

Oxidative stress and inflammation are the major mechanism that contributes to the pathogenesis of degenerative diseases including neurotraumatic, neurodegenerative, and neuropsychiatric diseases. Dr. Akhlaq A. Farooqui, in Chap. 19, concludes that increased consumption of omega-3 fatty acids may result in retardation of oxidative stress and neuroinflammation due to the production of resolvins, neuroprotectins, and maresins.

Dr. Tassos Georgiou in Chap. 20 on the role of omega-3 fatty acids on eye health describes how omega-3 fatty acid supplementation can result in regression in some type of retinopathies, including age-related macular degeneration, macular dystrophies, and also some form of drying eye.

Importance of omega-3 fatty acids in maternal nutrition in growing fetus, reducing the risk of adverse pregnancy outcome, has been reviewed in Chap. 35, by Dr. Sadhana Joshi.

Dr. Gabriel Fernandes, in Chap. 40, describes effect of fish oils on pain resolution, achieving prolonged disease free life.

Obesity leads to several chronic morbidities including type 2 diabetes, atherosclerosis, and hypertension, which are major components of the metabolic syndrome. In chapter 14, Dr. Maria J. Morena Aliaga reviews randomized controlled trials that evaluate the effect of supplementation of EPA and DHA on weight loss, insulin sensitivity, lipid metabolism, blood pressure, and inflammation in subjects with metabolic syndrome characteristics. Dr. Lindsay Brown in Chap. 13 describes linseed as a functional food for the management of obesity. He concludes that there is considerable evidence that the constituents in flaxseed especially ALA and probably also secoisolariciresinol diglucoside and fiber to a lesser extent, either separately or combined, can be defined as functional food, as they may improve the multiorgan changes induced by obesity.

Decrease in the brain DHA content causes number of neurobiological effects including depression. Dr. Beth Levantin in Chap. 22 discusses the evidences that support the involvement of decreased brain omega-3 fatty acids in the etiology of postpartum depression and other depressive disorders and their implications in prevention and treatment.

Dr. Julio Ochoa in Chap. 23 summarizes the role of omega-3 fatty acids in bone health and turnover. In Chap. 24, Dr. Julio Ochoa summarizes the interactive role of Fe and DHA in physiologicaland nutritional deficiency situations, revealing that DHA stimulates Fe metabolism.

In Chap. 12 on cancer, we discuss the anticancer action of omega-3 fatty acids that may counter the proinflammatory, proangiogenic, and prometastaic and cell proliferative actions of AA eicosanoids and induce apoptosis.

It is no wonder that omega-3 fatty acids are very crucial for our health as they constitute the functional structural component of the membrane and also the precursors of hundreds of eicosanoids and lipid mediators controlling thousands of reactions in human body. Therefore, it is not surprising that the omega-3 deficiency has wide range of adverse effects on different organs and tissues aggravating each and every disease. Therefore, the book also focuses on the means of urgently bringing back omega-3 fatty acids into food chain. These aspects have been very well illustrated by the contributory authors and co-authors of the chapters of the book. We would like to profusely thank them all, being the part of this useful exercise.

Finally, volume editors would like to extend their appreciation to Springer and their staff for providing professional platform for communication with the experts in the field.

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We would like to express deep gratitude to Prof. Dr. Shivajirao S. Kadam—honorable vice chancellor of Bharati Vidyapeeth Deemed University (BVDU), Prof. S.F. Patil—Executive Director Research BVDU, Dr. Ulhas Wagh-—former director, and Dr. A.C. Mishra—the current director of Interactive Research School for Health affairs, BVDU, Pune, for their unstinted support and encouragement for successful completion of the book. Lastly, we would like to express heartfelt thanks to our family members for their understanding and support.

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Editors and Contributors

About the Editors

Prof. Mahabaleshwar V. Hegde earned his B.Sc. (Biology), B. Sc. (Hons) (Chemistry), M.Sc. (Biochemistry), and Ph.D. (Enzymology), all from University of Pune. India. Dr. Hegde gained molecular biology research experience at Prof. Julius Marmur's Laboratory at Albert Einstein College of Medicine, New York. He was visiting scientist at the Virology Department of Sloan Kettering Cancer Center, New York.

He taught biochemistry at postgraduate level as demonstrator, lecturer, reader, and professor of biochemistry for 33 years at University of Pune and retired as the Head of the Department of Chemistry.

Thereafter, he was coordinator of omega-3 projects at Biochemical Division of National Chemical Laboratory, Pune, for three years.

Dr. Hegde later shifted to Bharati Vidyapeeth Deemed University's (BVDU) Interactive Research School for Health Affairs (IRSHA), to continue focused research on "Role of Omega-3 Fatty acids in Human Health." Realizing the adverse effects of precariously low levels of omega-3 fatty acids on human health, Dr. Hegde developed "Flax Biovillage concept." The idea was validated with the help of two National Agricultural Innovation Projects funded by Indian Council of Agricultural Research, on flaxseed, 1) Linseed Agriculture for Rural Livelihood Security, 2) Linseed Value Addition, for providing better price and buy back guarantee to the farmers. Dr. Hegde's team developed many omega-3-enriched products including omega-3 egg, omega-3 milk, and omega-3 chicken with the aim of achieving omega-3 nutritional security in the country, to reduce morbidity and mortality. In order to take the initiative forward and for promoting linseed agriculture, ICAR instituted Linseed Value Addition Center at BVDU, to connect to the remaining 13 ICAR linseed research centers for promoting Linseed Agriculture in the country.

Currently, Dr. Hegde is the director for Center for Innovation in Nutrition Health Disease established within IRSHA. He has also established Real World Nutrition Laboratory Foundation, a not-for-profit company, for validating the omega-3-enriched products in the market and gets industrial linkage for scaling up.

Dr. Hegde has guided 16 students for Ph.D., several M.Phil., M.Pharm., M.D., M.S., and M.D.S. dissertations. He has published over 75 papers in diverse fields and authored textbook in biochemistry for undergraduates, written several chapters in books. Dr. Hegde is member of several scientific societies, society of biological chemists, microbiology, nutrition, and poultry science, and fellow of society of Indian Agriculture Biochemists.

Dr. Hegde is recipient of DST-Lockheed Martin Innovation gold medal and received Rs. one lakh cash award and special appreciation award of Bharati Vidyapeeth for conducting research program, directly relevant to society.

Dr. Hegde is also recipient of research grants from several funding agencies, besides ICAR, Department of Science and Technology, Department of Biotechnology, BIRAC Biotechnology Ignition Grant (BIG), Council of Scientific and Industrial Research, Khadi Village Commission, etc.

In the last 15 years, Dr. Hegde has focused his research efforts to establish the role omega-3 fatty acids in health and disease, to verify and confirm the efficacy of omega-3 fatty acids and antioxidant supplementation in disease outcome along with usual treatment regimen, to develop technologies/protocol to enrich egg, milk, and chicken with omega-3 fatty acids, and to stabilize omega-3 fatty acids in omega-3 enriched functional foods.

Dr. Anand Arvind Zanwar M. Pharma,. Ph.D. is currently working as scientist at Interactive Research School for Health Affairs, Bharati Vidyapeeth Deemed University, India. The main research focus includes preclinical pharmacology and toxicology. He has been working mainly on pharmacological and nutritional aspects of flaxseed and omega-3 fatty acid. Further fortification of various kinds of food with omega-3 fatty acid and its pharmacological evaluation has been his important focus. Dr. Zanwar is currently scientist in charge for the newly established ICAR-AICRP-Linseed Value Addition Centre at Bharati Vidyapeeth Deemed University, India.

Dr. Zanwar worked on various projects related to anti-inflammatory, antidiuretic, immunomodulatory, hepatoprotective, diabetes, wound healing, antihypertensive, cardioprotective activities, and complication of cardiovascular disease in preclinical screening of various medicinal plants/herbal extracts. He has experience in evaluating drug safety and efficacy as well as surgical techniques necessary for animal model research of cardiovascular pharmacology. Further, he has experience in various techniques related to phytochemistry and natural product in order to isolate the active compound from extract.

Dr. Zanwar received Ph.D. in pharmaceutical sciences form Bharati Vidyapeeth Deemed University, Pune, India. He has recently received, "Young Investigator Award" by the Asia Pacific Federation of International Atherosclerosis Society at Hong Kong and "International Travel Grant Award" from Science and Engineering Research Board, Department of Science & Technology, New Delhi. He has also received best paper award by Indian Atherosclerotic Society.

Dr. Zanwar has several publications and patents to his credit. He has authored several book chapters published by Humana press-Springer Science and Academic Press-Elsevier publisher. He is currently acting as reviewer board member for many peer-reviewed international journals.

Dr. Sharad P. Adekar M.D., Ph.D. is a physician scientist with experience in Immunology, Infectious diseases, and antibody discovery. Currently, he is working as medical vice chair at WIRB, Puyallup, WA, USA. Prior starting at WIRB, Dr. Adekar was working as consultant in antibody discovery and development area. He also worked as director of antibody research at Immunome, Inc., in Pennsylvania. He received his medical degree (M.B.B.S., MD) from B.J. Medical College, Pune, and worked on his Ph.D. thesis at Thomas Jefferson University, Philadelphia, PA ,and received final Ph.D. from University of Pune, India. In India, he also worked as scientist at IRSHA, Bharati Vidyapeeth Deemed University's, Medical College Campus, Dhankawadi,

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Dr. Adekar has invented a novel method to make human monoclonal antibodies and **received US patent for `Fusion partner cell line that are used for preparation of hybrid cells that express human antibodies" in October 2013**. He has also applied patents for antibody technology as well as number of human antibodies.

Dr. Adekar has extensive experience in human antibodies in terms of discovery and leads optimization and preclinical development of monoclonal antibody therapeutics. **He has created human monoclonal antibodies in oncology, infectious diseases, and autoimmune and neurological diseases.**

He has published various peer-reviewed articles and participated in several conference presentations that include panel discussions, invited presentations, and peer-reviewed oral presentations. He has also served as Principal Investigator on SBIR grant applications and generated funds.

Dr. Adekar has demonstrated ability to develop and optimize novel research approaches and successful implementation of those ideas while collaborating with different groups. He has skills in translating business objectives into action plans and scientific methods necessary to drive a team.

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Nutrition, Life, Disease, and Death

1

Mahabaleshwar V. Hegde, Anand Arvind Zanwar, and Sharad P. Adekar

Introduction

The main aim of the chapter was to emphasize the extraordinary importance of omega-3 fatty acids for human health today. However, we cannot undermine the importance of the rest of the essential nutrients. All the essential nutrients in adequate quantities and their bioavailability have also to be ensured for good health. It is also important to be conscious of anti-nutrients [1, 2] including long-term use of drugs, tobacco, toxic pollutants, heavy metals and our ability to completely detoxify and protect our body from any harmful effects [3]. In this chapter, we will attempt to briefly discuss these confounding factors on which the ultimate beneficial effects omega-3 fatty acids actually rests.

We Are What We Eat

The living organisms are unique in that they are self-replicating, self-adjusting, self-repairing, and self-evolving systems. We are what we eat. Daily what we eat makes our body, robust or fragile. So it is obvious that we have to pay proper attention to what we eat. For this, we have to fully understand and appreciate what our body daily needs are. You must know whether or not you are providing in your daily diet all that you need. Eat right and stay healthy and fit. More often than not our health problems originate because we have not given proper attention to what we eat. Often the question is asked are we living to eat or we are eating to live. We often blame our genes that we inherited from our parents. None of us have any choice of choosing our parents. Like it or not, we have to live with it. Accept it as our fate. No one is born with perfect set of all healthy genes. There will be some defect somewhere which might crop up and start affecting our health sooner or later. If the defect happens to be in some vital genes, one can be born within born errors [4] that become apparent almost immediately. If detected early and attended early, for some, it may be possible to control and reduce the sufferings [5]. But there are many so-called diseases of civilization [6] that do not become apparent immediately but show up later in life and become a chronic degenerative disease. All of us know that these types of health problems that manifest as chronic diseases, such as heart disease, diabetes, arthritis, mental illness, and cancer because of sudden drastic lifestyle changes, are now surfacing sooner than later. Again one can blame our genes that we inherited from our parents. Although we can do nothing about the imperfect genes that we may have inherited, we have to blame ourselves for having precipitated the disease early, mostly due to unhealthy diet and partly due to lifestyle. It is therefore now these degenerative diseases are being regarded as lifestyle disease. Modern stressful lifestyle, junk food, pollutants, tobacco, overuse of drugs, alcohol, along with total negligence to the actual nutritional needs of the body, all cumulatively contribute to the early occurrence and increase in severity of degenerative diseases. It is our wrong lifestyle, bad eating habits that make us vulnerable to get the genetically predisposed disease, early and more aggressively. We must understand and appreciate that genes do not function on their own. Genes need basic ingredients, essential nutrients, incessantly supplied from our food, to do their assigned job properly. Genes can work efficiently, only if we supply these basic ingredients adequately, daily in our food, only then they are capable of giving us a healthy body.

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Of the many essential nutrients, the deficiency of omega-3 fatty acids, a crucial nutrient in modern man's diet, and also the ratio of omega-6/omega-3 [7] are largely responsible for our health problems, hence the emphasis on omega-3 fatty acids in this book.

Our body is dynamic; it continuously renews itself from the food that we eat. Different parts of the body are programmed by the genes to be renewed at different rates. Old cells die by a natural process called apoptosis and are replaced by new cells. This creation of new cells happens with the help of ingredients provided by food constituents. Old cells must die as they are no longer able to carry out their functions efficiently and have to be replaced by new cells. So it becomes apparent that without the continuous supply of proper and complete nutrients through our food, body cannot create optimally functional new healthy cells in each and every part of our body. So it is clear that perfect understanding of body's needs and their adequate supply through food is basic to our good health. Improper and incomplete nutrients in our food will result in creation of unhealthy new cells, ultimately resulting in unhealthy body. Conversely, if one gives good food, complete in all respects in quantity and quality, we can transform our body from ill health to good health.

Human Body Knows no Pathies but Understands Nutrition

Allopathy, ayurveda, unani, homeopathy, and traditional or modern medicines are different pathies or different modes of treatment. However, each one of these pathies just provides chemicals resourced from natural or synthetic sources to treat diseases through their unique treatment modalities. These chemicals have to interact with abnormal metabolism, characteristic of the disease, in a favorable way to normalize metabolism and to bring about curing process. However, human body knows no pathies but understands nutrition. These chemicals, being not natural and not normal constituent of our metabolism, can have some side effects. On the other hand, nutrients are part and parcel of the normal metabolism and hence nutrients are primary for the maintenance of good health. Actually, in most of the times, inadequate or improper intake of nutrients is the root cause of our disturbed metabolism and illness. Obviously medicines coming from modern medicine or alternative complimentary medicine cannot constitute a substitute for essential nutrients needed by the human body, in the food. This being the case, it is nearly impossible to normalize the metabolism by the chemicals coming from traditional or modern medicines, unless supported by the judicious nutritional support of essential nutrients.

Entropy and Nutrition

According to the second law of thermodynamics, cosmic force drives the whole universe toward increasing entropy, to move toward disorder and destruction. Life is order. Death is disorder. Life is defeating entropy and death is victory of entropy [8]. Hence, death is imminent and more natural. Life is therefore a miracle, and aging, disease, and death are certainty.

Thus, eating is as much a way of acquiring order, as it is a way of gaining energy for defeating entropy, to sustain life. Living organisms are not in equilibrium, rather they require a continuous influx of free energy to maintain order in a universe bent upon maximizing disorder. Metabolism is an overall process through which living systems acquire and utilize the energy they need to carry out their various functions. They do so by coupling the exergonic reaction of nutrient oxidation to the endergonic reactions, required to maintain the living state [8].

It is very difficult to imagine how life could have been formed on our planet, against all odds. It is more difficult to imagine that the life continues and evolve on this planet. However, by some miracles of circumstances, the life got formed and has sustained over four billion years. For creating and sustaining life, the life forces have to work against the nature's law of entropy, the ever increasing disorder (entropy), and against the basic laws of thermodynamics. However, not surprisingly therefore, the nature has the last laugh and ultimately entropy wins and death ensues (Fig. 1.1). What all this simply means is that for the life to sustain well, remain in a healthy state, we have to continuously help our body to manage to work against the second law of thermodynamics. So the nutrition has to fulfill two independent functions: firstly, to provide all the ingredients needed to feed our body (with basic macro and over 40 essential micronutrients) daily to renew our body (Fig. 1.2);

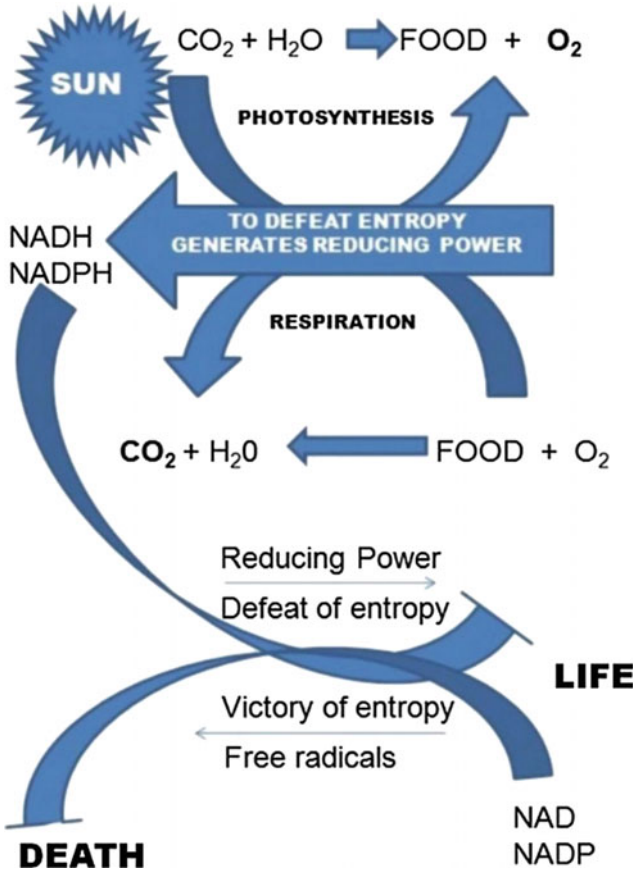

Fig. 1.1 Life is created and sustained by reducing power, defeating entropy

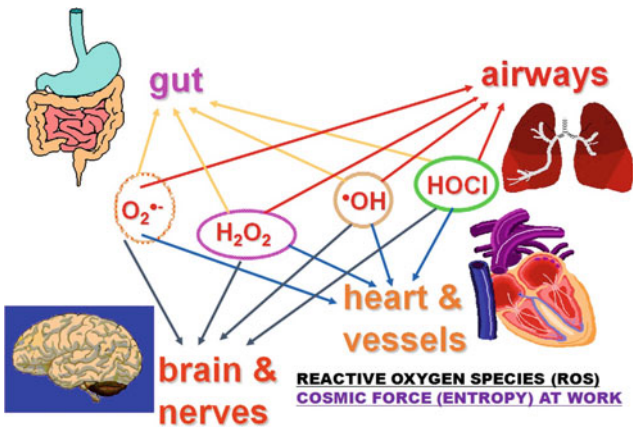

Fig. 1.2 Oxygen for life and oxygen for death

secondly, to provide enough antioxidants from food along with antioxidant defense of the body to neutralize the inevitable entropy forces such as oxidative stress and free radicals (Fig. 1.3), to protect us from disease and death. So it becomes obvious that first we have to understand the basics of nutrition and the basis on which we can live a long healthy life.

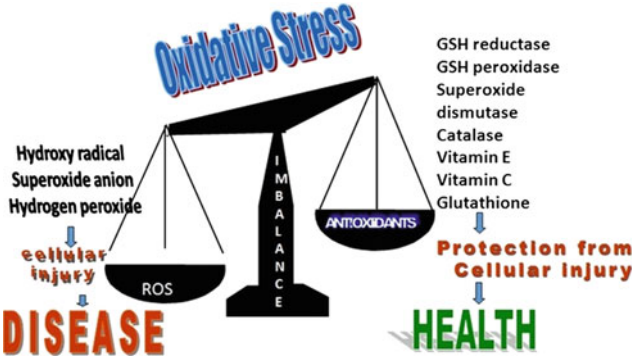

Fig. 1.3 Managing oxidative stress

Origin of Life, Chemical and Early Biological Evolution

Before we discuss the basics of nutrition and the philosophy of living, healthy long life, it may be prudent to discuss how and under what circumstances life got formed on this planet, overriding the second law of thermodynamics and the subsequent biological evolution that led to the creation most astonishing life-form, we the human being.

It is reliably estimated that life originated on our planet about four billion years ago [9], when there was no oxygen in earth's atmosphere. Carbon, hydrogen, oxygen, and nitrogen were the chosen primary elements by nature to create life on this planet. Primordial earth had all these four basic elements, carbon, hydrogen, oxygen, and nitrogen in reduced form, carbon as methane CH_4, hydrogen as H_2, oxygen as water OH_2, and nitrogen as ammonia NH_3. Urey made the case that reducing atmosphere was ideal for the origin of life [10]. Perhaps this was an ideal condition for life to originate. Oparin and Haldane [11] and Urey and Miller, by mimicking the primordial condition of the earth [12] in their laboratory, showed that CH_4, H_2, H_2O, and NH_3 can generate the fundamental life-forming molecules, amino acids, sugars, fatty acids nucleotides, which is the basis of chemical evolution, to later initiate biological evolution [13] (Fig. 1.4).

There are two main characteristics of living state that distinguishes it from lifeless state, viz. growth and replication. Today we know that deoxyribonucleic acid (DNA) has attained that ability not only to store all the information of a life-form, but also to express the information, transmit to the next generation, and evolve to survive and adapt to any environment to the extent possible. The information stored in DNA is expressed through the formation of variety of very versatile protein molecules that can take up several responsibilities to keep the life in healthy living state, including the function of replication of DNA. So it is very intriguing whether DNA has to be replicated by the DNA polymerase, a protein, the product of DNA itself. So

Fig. 1.4 Important ingredients needed in food for good health

naturally we wonder, what came first DNA or protein? DNA that codes for protein or protein that replicates DNA? This leads to famous dilemma chicken or egg what came first? However, this riddle has been fairly well resolved by assuming that RNA is the first living molecule, a molecule that had originally the ability to replicate and grow. Initially, RNA [14] could take up the responsibility of both, of a genetic material, that can store information and also transfer information to next generation, as well as act as an enzyme replicase. However, RNA could not carry out either of these functions very efficiently. Therefore, the nature evolved two distinct more efficient molecules, deoxyribonucleic acid (DNA) as genetic material and protein as enzyme, to act as catalytic agent to carry out all life reactions.

Originally, under primordial conditions, the energy was derived from the reduced molecules, but soon a way was found to derive the energy from the sunlight, through a process of photosynthesis, that again produced reduced molecules $NADH^+$ and $NADPH^{+,}$ to provide the most basic support to sustain life, for maintaining reducing conditions within each and every cell. $NADH^+$ is primarily used for generating ATP, a universal ubiquitous energy currency of all life-forms on this planet. On the other hand, $NADPH^+$ is the driving force to build up our body matter from the building blocks supplied by food ingredients. It seems this initial process of photosynthesis became so overwhelming and self-perpetuating that whole earth got engulfed by green plantation. The process also led to the evolution of large amount of oxygen by splitting water. The oxygen later converted into O_3 (ozone) and formed the outermost cover of the earth that filtered some radiations, and the photosynthesis was modified to suit this new situation. Animal forms were later evolved to keep the life in balance. Photosynthesis and respiration are same reactions but occurring in reverse. While in respiration process, glucose and oxygen yield carbon dioxide and water. In photosynthesis, organic carbon (glucose and starch) are produced from inorganic carbon (carbon dioxide). On the one hand in respiration, NADPH and ATP are derived via oxidation of organic carbon (food), and on the another hand, the photosynthesis generates fundamental reduced molecules NADH, NADPH, and ATP by capture of sun energy and manufacture food and in respiration NADH, NADPH, and ATP are produced, via oxidation of food. Ultimately it is the reduced internal cellular environment that supports life, although differently produced in plants and animals. All these discussions are to emphasize the importance of antioxidants (reduced molecules) in our food besides essential nutrients.

Vegetarianism Is Healthier

It can be noted that the plant forms derive energy from the sun through photosynthesis and by fixing carbon dioxide CO_2 from air, by reductive synthesis form biomolecules and evolve oxygen. This became a perfect platform set for the nature to experiment and to develop very complementary animal life-forms that can derive energy from biomolecules in plant forms by oxidation process that uses oxygen from atmosphere and evolves carbon dioxide. The nature thereby acquired balance between the two opposing ways of living. We the human, the animal forms, derive energy by oxidative process that uses oxygen and evolves carbon dioxide. The oxidation–reduction process produces again the same reduced molecules NADH and NADPH. So it seems the reducing power is the driving force for the life that defeats

the entropy. This oxidation process also inevitably produces some highly reactive oxygen species (ROS), free radicals. ROS are primarily now recognized as agents of aging disease and death. So these free radicals can be considered as agents of entropy at work. Therefore, the major approach to protect ourselves from disease and death would be to effectively neutralize the free radicals by consuming sumptuous amount of food rich in antioxidants. Between the vegetarian and non-vegetarian, obviously the vegetarian would be rich in reduced molecules (antioxidants) as during the process of photosynthesis oxygen evolves and more reduced molecules are left behind. It is understandable that vegetarian food has more power of neutralizing free radicals defeating entropy and can support healthy long life.

Man Is Crippled in Evolution

Everyone is aware of human genome project [15]. The project on which very huge amount of money was spent to get the nucleotide sequence of all 23 pairs of human chromosomes. The project was completed ahead of time. When the project started, it was thought that man is the most complex organism on the planet and possibly has more than hundred thousand genes. But at the end of the project, it became evident that man has less than twenty thousand genes [16]. This is very much off the expected mark, particularly when even the simple-looking rice plant has forty-five thousand genes [17], twice that of human genome. Although this finding seems paradoxical, it is not surprising if we recognize the fact that man is crippled in evolution. Now this seemingly paradoxical situation can be explained by the difference in synthetic capabilities of rice versus man. Rice has ability to synthesize all its needs, namely all vitamins, all 20 amino acids, and the two essential omega-3 and omega-6 fatty acids from carbon dioxide from the air, minerals, and water from the soil with the help of energy obtained from sunlight. However, man is not endowed with this extraordinary synthetic power. He has lost them during evolution. He is not provided with the genes to make these nutrients. He is crippled in evolution. Therefore, he needs to obtain these nutrients, which are regarded as essential nutrients, readymade, daily from the food. However, we have been provided with the brain instead, with the ability to think. Now that we know that we are deprived of these genes and have lost the genes and as we have no ability to synthesize these nutrients, it should be our endeavor to ensure that our daily diet has the quality proteins that can provide all the essential amino acids adequately, all the vitamins, minerals, and also two essential omega-3 and omega-6 fatty acids. Only if we take care of these, we can have healthy body and any ill health that arises out of this negligence or ignorance may possibly be managed to some extent, for some time, by medicines, but cannot be corrected. Nutrition is vital and primary for good health; this seems to be the hidden message from the human genome project. Food for thought, think of food, seems to be a revelation from the human genome project [18].

Essential Micronutrients in Human Nutrition

Human food must contain six categories of ingredients: water, proteins, carbohydrates, fats, vitamins, and minerals. Although fiber cannot be regarded as required food ingredient, but is extremely important in prevention of diseases as it is helps in digestion and absorption and the bioavailability of micronutrients, about 60 % of the human body is composed of water. Nearly all the life-sustaining chemical reactions are carried out in aqueous medium. All the three bulk foods, namely proteins, carbohydrates, and fat can be sources of caloric energy. Carbohydrates and proteins provide four calories per gram. Carbohydrates are primary source of energy, and proteins and fat are used sparingly as source of energy or under emergency. Fat provides nine calories per gram. The human body is capable of manufacturing saturated and monosaturated fats, but not the essential omega-3 and omega-6 polyunsaturated fat.

Given enough calories, human body is capable of manufacturing thousands of chemical compounds from the food ingredients called metabolites by a process called metabolism to sustain life. However, unlike plants, as mentioned above, human body is incapable of synthesizing several nutrients, and hence they are called essential. These are some 40 micronutrients (Fig. 1.2).

Digestion Absorption, Critical in Nutrition

For the bioavailability of the nutrients, proper digestion and absorption are the critical factors. The diet is largely determined by the availability, processing, and palatability of foods. A healthy diet includes preparation of food and storage methods that preserve nutrients from oxidation, heat or leaching, and that reduce risk of food-borne illnesses. Bioavailability of nutrients is very vital as it actually determines the proportion of a nutrient that is absorbed from the diet and used for normal body functions. There are several steps in the metabolic pathway that can affect the ultimate nutrient bioavailability. Nutrients are first processed in mouth by process of chewing and get mixed with acid and enzyme in gastric juice. Finally released into small intestine.

Here with action of more enzymes, supplied by the pancreatic juice, food matrix is broken down to make the nutrients bio-accessible. Bioavailability is influenced by minerals and other ingredients in food. For example, vitamin C can enhance iron absorption, and phytosterols [19] can inhibit cholesterol absorption. The host factors, for example, intrinsic factor (IF), are needed for the absorption of vitamin B_{12} in lower intestine [20].

Survival of the Sickest

The two guiding force of evolution is to survive and reproduce. All kinds of environmental factors, weather patterns, changing food supplies, and even dietary preferences, have affected our evolution. In the course of evolution, mutations that are bad do not survive; when they are good, they lead to the evolution of a new trait through the process of natural selection. Obviously, DNA does not determine life, it shapes it; environment and your choice of environment and food surely does. Referring to Triage theory put up by Bruce Ames, we stated that nature favors short-term survival over the long-term survival. This aspect is discussed at length in the book entitled "Survival of the Sickest" by Maolem [21]. This very nature of nature selects you to suffer from deadly disease (thalassemia) to save you from deadly disease (malaria), immediate death, and suffering for long time.

Similar principle also seems to be true even with respect to nutrition. This aspect has been convincingly demonstrated by the Triage theory put up by Bruce [22]. The theory is based on the fact that "nature favors short-term survival over long-term survival." Nature strives to keep you alive today, even if the action taken is responsible for your long-term suffering later.

Very important aspect of nutrition, that we often tend to neglect, is the need of completeness, need of every essential nutrient in adequate quantity. All nutrients are important in its own right. Every one of the 40 nutrients mentioned has a well-defined role in the metabolism. If the diet is deficient in any one of them, body will not be able to function optimally and it is certain to have adverse effect on health. According to Bruce Ames, our body prioritizes the supply of micronutrients to vital organs (heart, for example) and for vital functions (blood coagulation) to ensure our immediate survival. In the event of suboptimal supply of micronutrients, only vital organs and vital functions will get the micronutrients on priority. The rest of the organs (liver, kidney, and the organs that are not as important as heart for immediate survival) get starved of these nutrients and eventually succumb to this continued discrimination, so also less vital functions not important for immediate survival such as DNA repair gets compromised. Not surprisingly, inability of our body to repair the DNA damage that inevitably occurs because of the want of enough micronutrients can be the primary cause for developing cancer later.

Triage literally means sorting out patients based on the possibility of their survival and prioritizes treatment, particularly during war. Bruce Ames worked with vitamin K [23] as micronutrient to prove his triage theory. Vitamin K works in our body with five gene products to control blood coagulation, a vital function for our immediate survival. Vitamin K also works with 12 more gene products for bone health, not vital, not so important for immediate survival. Knocking vital blood coagulation genes in mice experiment was lethal and knocking out bone health genes was not lethal. In the event of suboptimal supply of vitamin K, vitamin K would be prioritized for blood coagulation function and vitamin K availability for bone health gene functions will be compromised. This suboptimal vitamin K supply can eventually lead to bone diseases. Bruce Ames further suggests that our present recommended daily allowance for micronutrients must be reviewed in light of triage theory. The present RDA for vitamin K, 90 mcg per day, may be just enough for managing blood coagulation function and not enough for bone health. The take away home message from the theory is that we must ensure the adequate intake of all the 40 essential nutrients daily for good health.

Omega-3 Fatty Acid Crucial Nutrient

We have above argued that intake of all 40 nutrients daily is a must to keep the human body in a healthy state. Available evidence from recent published meta-analyses indicates that omega-3 fatty acids plays a crucial role in the prevention of non-communicable diseases such as cardiovascular disease, breast cancer, and colorectal cancer. [24].

Detailed account of the role of omega-3 fatty acids in health and disease has been mentioned in a number of chapters that follow. Here, it is suffice to say that because of paucity of omega-3 in the modern diet and also because of excessive omega-6 fatty acid intake, there is an imbalance, resulting in highly inflammatory conditions, prevailing globally in modern man. The recent rise of degenerative diseases is attributed to this imbalance and dominance of inflammatory condition in modern man.

Omega-3 Index

The omega-3 index is the combined percentage of eicosopentanoic acid and docosohexanoic acid of total fatty acids in red blood cell membrane [25]. It is inferred that man

as hunter–gatherer consumed almost equal amount of omega-3 fatty acid; the omega-3 index was close to 1:1. But in recent times, his food has very little of omega-3 and is excessively loaded with omega-6 fatty acid; the omega-3 index is 1:10, 1:20, or even 1:50, but ideally the healthy omega-3 index suggested to be 1:5. This ratio 1:5 has been thought to be ideal because omega-3 fatty acid (ALA) and omega-6 fatty acid(LA) are processed by the same set of elongase and desaturases to highly unsaturated fatty acids (HUFA), EPA and DHA (both omega-3), and AA (omega-6). Five times higher levels of omega-6 do not affect the conversion of omega-3 PUFA to omega-3 HUFA. This is because the elongases and desaturases have higher affinity to omega-3 than omega-6 fatty acids. Therefore, even at five times less level of omega-3 fatty PUFA's can be equally converted. Omega-3 fatty acids are one of the most sought after essential nutrient. In order to cut down omega-6 fatty acid consumption, omega-9 (MUFA olive oil) oleic acid is being promoted all over the world [26].

Free Radicals

Free radicals are molecules, such as reactive oxygen species (ROS), that have lost an electron and have become very unstable and become highly reactive and can rob electrons from neighboring vital molecules [27]. They act as terrorists in the body, attack vital protein, DNA, leading to dysfunction, mutation, and cancer. They attack proteins and enzymes, inactivate them, and thereby disrupt the normal activities they perform. Free radicals attack cell membranes, in cells that line our blood vessels, and hardening and thickening of the arteries eventually resulting in heart attacks and strokes. Free radicals attack on collagen can cause cross-linking, resulting in the stiffness of joints. Thus, ROS contributes to both initiation and promotion of many major diseases [27]. This constant attack of ROS is also referred to as oxidative stress [28]. Actually, the clinical presentation of various diseases, the way illness finally appears, can be the representation of variation in the individualistic protection provided by the body's antioxidant defenses [29] and also the antioxidants in the food. Under oxidative stress, the weakest link in the body may be the first to give way to a specific disease, partly determined by genetic predisposition.

It would be wrong to infer that free radicals are always bad. Free radicals and antioxidants play a dual role both toxic and beneficial compounds, since they can be either harmful or helpful to the body [30]. However, free radicals may create a chain reaction that can damage the body. It must be noted that the production of free radicals in the body is continuous and inescapable.

Oxidative Stress

It is well known that chronic, persistent stress triggers numerous illnesses. However, exactly how that occurs is, although, not fully understood; there are some ideas and theories explaining the stress and its relation to illness. Oxidative stress is a condition wherein ROS is in excess of the available antioxidant buffering capacity [30].

Human body has trillions of cells of over 200 different kinds as a part of various organs, heart, brain, kidney, liver, and lungs. performing various important functions and working in unison to keep our body in healthy state. In every cell, there are numerous smaller organelles, mitochondria, power house of the cell. Every cell generates ROS if it cannot neutralize it effectively with its available antioxidant buffering capacity; oxidative stress will be induced potentially injuring the cell and inevitably can initiate disease process [31].

Autophagy

Autophagy is a self-degradative process that is important for balancing sources of energy, in response to nutrient stress. Free radicals change cellular responses. At low level, they may act like signaling molecules but at high levels can damage the organelles, particularly mitochondria [32]. Associated mitochondrial dysfunction may result in energy depletion, redox changes, and cell death. Autophagy (or self-eating) is a lysosome-mediated degradation process for nonessential or damaged cellular constituents. Physiologically autophagy serves to preserve the balance between organelle biosynthesis, protein synthesis, and their clearance. Oxidative stress is inseparably linked to mitochondrial dysfunction. Oxidative stress normally implies that ROS/RNS are toxic species because of their highly reactive nature. Similarly, autophagy may be regarded as bad for the cell since an unhealthy cell has multiple vesicles. However, under some situations, "self-cleansing" can be good for the cell. Generally, autophagy can be regarded as the activity that decreases ROS/RNS damage, as autophagy plays an important role in both sensing oxidative stress and removing oxidatively damaged proteins and organelles.

Integrating Homeostasis Allostasis and Stress

Homeostasis [33] means staying the same. The enzymes work best at optimum temperature and pH. Internal conditions allowed to vary within narrow limits. Human body temperature, for example, varies between 36.1 and 37.8 °C.

So a better definition of "homeostasis" is the maintenance of the internal environment within narrow limits. Homeostasis consist of number of cooperating mechanisms acting simultaneously or successively. Homeostasis is the result of organized self-governance. Actually many pharmaceutical interventions work for homeostasis by suppressing extreme reaction. Although body tries to keep metabolites normally within normal limits by the process of homeostasis, sometimes the body may need to readjust and cross the normal limits and remain stable by changing to maintain stability by a process called allostasis [34]. Allostasis is an adoptive change, usually temporary, to deal with emergency situations. However, in the long run, allostatic change, so-called allostatic load, may fail to be adaptive as the maintenance of allostatic changing over a long period may result in wear and tear. Human body is adaptable, but it cannot maintain allostatic overload for very long time, without consequence of adverse effect on health.

Life, Disease, Cancer, and Death: Simple Play of Redox

Redox homeostasis [35] actually governs the life, disease, death, and cancer. Cell maintains redox balance under physiological conditions. This is achieved through generation and elimination of free radical. Free radical includes reactive oxygen species (ROS) such as superoxide, hydroxyl radical and non-radical species such as hydrogen peroxide and nitrogen species (RNS) comprised of nitric oxide, peroxynitrite. For maintaining redox homeostasis cells are equipped with enzymatic and non-enzymatic antioxidant system. Superoxide dismutase is major class of enzymatic antioxidant system that catalyses dismutation of O_2 to H_2O_2. H_2O_2 is further broken down by catalase. H_2O_2 can also be eliminated by glutathione peroxidase by converting reduced to oxidized glutathione.

There is a view that cancer is an epigenetic disease. Normal cells in response to chronic stress, in desperate attempt to survive, undergo many different adaptive mutations, to bring about malignant transformation, to survive and multiply indefinitely. It must be noted that the cancer cells have abnormal energy metabolism that depends heavily on aerobic glycolysis, known as Warburg effect.

Telomers and the Lifespan

Telomers are repeated nucleotide sequences located at the ends of linear chromosomes. It is believed that telomers shorten with each somatic cell replication. Telomers are essential for the chromosomal stability and replication. Enzyme telomerase is important in telomere formation, restoration, and maintenance [36]. Telomers can be maintained or lengthened [37] by adding telomeric DNA to shortened telomers [38]. Telomere length is also linked to and likely regulated by exposure to pro-inflammatory cytokines and oxidative stress [39]. Higher n-6 intake is associated with shorter telomere length [40] Inflammation triggers T cell proliferation, one known cause for T cell shortening [41]. Oxidative stress promotes telomere erosion during replication. Oxidative stress stimulates the synthesis of pro-inflammatory cytokines. Although telomers shorten with aging, shortening is not inevitable. PUFAs in blood may prevent telomere shortening. Omega-3 fatty acids can reduce inflammation and decrease oxidative stress [42]. Lower n-6/n-3 ratios were associated with longer telomere length. N-3 supplementation lowered F-2 isoprostane (oxidative stress). Although shortening of telomers with age is a natural phenomenon, the dietary intervention that reduces the joint burden of oxidative stress and inflammation can have positive impact on telomere length within few months [43]. Circulating PUFA levels are not always correlated with dietary intake that depends on absorption and metabolism. There is a compelling evidence that lower n-6/n-3 ratio slows biological aging. It is obvious that omega-3 fatty acids, possibly other essential nutrition, provide long healthy life.

Role of DHA in Human Brain, Adaptability to the Diverse and Adverse Environment for Survival

We now refer to ground-breaking, courageous, and visionary book of Bruce Lipton "Biology of Belief" [44] that provides solid evidence that membrane directly in contact with internal microenvironment or with external macroenvironment decides and dictates the fate of the cell and it is not the DNA, decorated master molecule that governs life. All function that our body carries out are also carried out by every single cell in our body and these cells also learn from their microenvironment and create their own cellular memories.

Most importantly, Bruce Lipton vehemently argues that it is the membrane, may it be of a prokaryote without any organelles or an eukaryotic cell with nucleus, mitochondria, and other well-defined organelles, with well-defined functions within the cell, which governs life within the cell, as dictated by the environment, and therefore, it can be regarded as the brain of the cell.

Fossil evidence reveals that single-celled life-forms originated within 600 million years after the earth was first formed, and for the next 2.75 billion years, only free-living single-celled organisms—bacteria, algae, protozoans populated the earth. However, around 750 million years ago,

these single-celled organisms became smarter, intelligently chose to live as community of cells, socially interactive millions, billions, and even trillion cells, as biological compulsion to survive. For efficient living and to survive in harmony, individual cells became tissues, assumed specialized task, and became organs. These organized collection of cellular communities formed organism: plants, animals including most sophisticated complicated humans.

Bruce Lipton surmises that "*the cells operations are primarily molded by its interaction with the environment not by its genetic code.*" Although the DNA in the nucleus are remarkable molecules and carry the blueprint of all life-forms, however, they do not control the operations of the cell. Therefore, the membrane with their receptors, interacting intelligently with the environment and efficiently governing the life functions inside the cell, constitute the true brain.

Darwin's theory of evolution proposes that animals well suited to their environment survive. It is clear now that environment is driving force behind evolution, which in turn decided by ability of organism to process information about its environment. Therefore development of brain that has ability to receive, process, respond to environmental challenges faster has increased chances of survival [44–46]. Therefore, a complex human brain that contains about 100 billion neurons, more than 100,000 km of interconnections, and having an estimated storage capacity of 1.25×10^{12} bytes [47] was evolved.

Omega-3 fatty acid, particularly DHA, has an important role in the development of human brain. If you regard human brain as super computer, it would be appropriate to regard DHA as the hardware of the brain.

DHA has special significance in evolution of human brain [48, 49].

DHA is abundantly present in the human brain and is an essential requirement in every step of brain development such as neural cell proliferation, migration, differentiation, and synaptogenesis. The multiple double bonds and unique structure of DHA allows imparting special membrane characteristics for effective cell signaling. Evidences indicate that DHA accumulates in areas of the brain associated with learning and memory. Many development disorders such as dyslexia, autism spectrum disorder, attention deficit hyperactivity disorder, and schizophrenia are causally related to decreased level of DHA.

Therefore it is clear that Omega-3 fatty acids have not only played a major role in evolution of man, providing him the smartest brain, but also have provided with increased ability to interact with the environment and better adaptability to very diverse, adverse, environment and survive.

Concluding Remarks

Although we regard omega-3 fatty acids as most crucial nutrient today, we do not in any way wish to undermine the importance of other nutrients. In fact, we advocate that our diet should be complete in all essential nutrients, free of anti-nutrients, and high in antioxidants to ensure good health.

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Flax Bio-village Concept

2

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Introduction

The Flax Bio-village Concept (FBC) was formulated with holistic approach, its primary objective being to attain omega-3 nutritional security, at the same time promote flaxseed (linseed) agriculture. FBC researches and develops methods, to add value to linseed, by resourcing omega-3 fatty acid from linseed and enriching commonly consumed food, including egg milk chicken, for public health.

Functional Foods and Public Health

The focus of public health has to be to improve health and quality of life through prevention and treatment of disease and other physical and mental health conditions. Food is one of the most important and modifiable lifestyle determinants of human health. Under-nutrition and over-nutrition are both crucial determinants of morbidity and mortality and therefore nutritional interventions are needed to reduce morbidity and mortality through dietary changes. There are two approaches for micronutrient intervention, direct supplementation, or fortification (functional foods). Supplementation involves supplying the essential micronutrient nutrients in capsule form and requires a commitment of the consumer to take them regularly and religiously. On the other hand, functional foods provide the essential micronutrients as natural ingredients of the food. The latter, functional food approach is particularly suitable for developing countries like India, wherein a sea of illiterate masses divided on caste, religion, and regional basis, no one would understand the language of reason. Better way to tackle the problem is to provide functional foods that simultaneously attain food security as well as nutritional security.

There is a distinction between nutritional problems of developing and developed countries. Developed countries may suffer from over-nutrition and developing countries mostly with under-nutrition. Today, industrialized societies are characterized by increase in energy intake and decrease in energy expenditure; excessive increase in saturated fat, omega-6 unsaturated fat, and trans fat, along with drastic decrease in omega-3 fat intake; a decrease in complex carbohydrate and fiber; an increase in cereal grains; and a decrease in fruits and vegetables. On the other hand, developing countries, while struggling to catch up with developed world, have not only got the health problems of industrialized world, associated with affluent lifestyle, but at the same time a large section of the society face the problems of under-nutrition, malnutrition, with associated health problems like low birthweight, premature birth, infant mortality, and other pregnancy complications. Humans evolved on a diet in which the ratio of omega-6 to omega-3 essential fatty acids (EFAs) was about 1, whereas it is now become 15:1 or more [1], because the modern human diet is precariously low in omega-3 and harmfully excessive in omega-6 fatty acid. The phenomenon of omega-3/omega-6 imbalance exists in almost all parts of the world, equally in both developed and developing countries. Omega-3 functional food intervention would benefit both developed and developing countries.

Therefore, "bring back omega-3 fatty acids into food" chain has been a global cry.

These functional foods are responsible for overall well being and also protects from several disorders such as cancer, cardiovascular, inflammation, diabetes and many other degenerative diseases. It is well established that there is strong correlation between active constituent in food and its efficacy in controlling the progression or prevention of disease [2]. For the maximum reach, it is desirable to

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incorporate the most crucial omega-3, functional components, in daily consumed foods, such as egg, milk, and chicken.

Crucial Role of Omega-3 Fatty Acids in Public Health Today

Our health is very largely governed by nutrition in food. Health of our people is closely linked to our economy and progress. Today, we are faced with a very peculiar situation of malnutrition, under-nutrition, and over-nutrition. It is becoming increasingly evident from recent research that our health problem primarily originates from the inadequate supply of essential nutrient to our body. We need over 40 essential nutrients—vitamins, essential amino acids, minerals, and omega fatty acids. Of these essential nutrients, deficiency of omega fatty acids in modern human diet is responsible in a very major way to our disease-prone health status today.

Why omega-3 is so important? Omega-3 is part of cell membrane. They are responsible for hormonal regulation that regulates blood clotting, contraction and relaxation of artery walls, and inflammation. Both omega-6 and omega-3 are essential. However, most of us get too much omega-6 and very little of omega-3 fatty acid. Here is a situation of over-nutrition of omega-6 and under-nutrition of omega-3 fatty acid. This imbalance is largely the root cause of the increase in severity and incidences of several diseases, including heart disease, diabetes, arthritis, cancer, mental disorders, pregnancy complications, infant mortality, and child health. Role of omega-3 in well known during pregnancy in particular for infant nerve and eye function. Therefore it is critical to provide adequate supply of omega-3-poly unsaturated fatty acid during last trimester. It is therefore important to ensure adequate and balanced supply of EFAs, particularly to the nations with emerging economies and this would be the most prudent public health strategy for improving the health of the populations [3].

The ratio of omega-6 to 3 2.3:1 is recommended so as to maximize ALA to DHA conversion. This is because both omega-3 and omega-6-fatty acid compete for the same desaturase and elongase. Higher than this ratio of omega-6 (Linoleic acid) in the diet can affect ALA to EPA, DHA conversion in vivo. Kinetic studies conducted in vivo [4] have shown that ≈15 % of dietary ALA is converted to the long-chain omega-3 fatty acids, of which eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6) predominate at typical intakes of both linoleic acid (LA; 18:2), 15 g/d (5 % of energy) and alpha-linolenic acid (ALA; 18:3) 2 g/d (0.6 % of energy). Quantitatively, this conversion results in ≈300 mg of n-3 long-chain fatty acids being derived via conversion from ALA. When dietary linoleic acid is increased to 30 g/d, conversion of ALA to the long-chain omega-3 fatty acids is reduced by ≈40 % [5]. Thus, the conditions that favor maximal conversion of ALA to EPA and DHA are critically dependent on the amount of linoleic acid in the diet [6].

Inuit metabolism study revealed fish derived omega-3-fatty acids are protective. It seemed that Inuit were protected from cardiovascular disease and low incidence was attributed to their fat rich in traditional marine mammal diet. This findings led to recommendation, resulting in millions of westerners consuming fish oil to prevent heart disease. The rarity of ischemic heart disease in Greenland Inuits, once known as Eskimos, may partly be explained by the antithrombotic effect of the long-chained diets rich in marine oils [7]. On their traditional diet, rich in fat from marine mammals, Inuit seemed to be quite healthy with a low incidence of cardiovascular disease, so fish oil must be protective. Those conclusions eventually led to the recommendation that Westerners eat more fish to help prevent heart disease and sent tens of millions scrambling for fish oil pills.

Recently, it has been shown that Greenland Inuits show genetic signatures of diet and climate adaptation [8]. The adverse health effects of a high-fat diet are counterbalanced by high omega-3 (EPA and DHA) diet. They have unique mutations, nearly in 100 % of the Inuit, which is only 2 % in Europeans and 15 % in Han Chinese. The strongest signal of signature of adaptation was found on chromosome 11 in the cluster of fatty acid desaturases. Two genes FADS1 and FADS2, coding for delta 5 and delta 6 desaturase (D5D, D6D), are rate limiting steps and have been selected for adaptation to Inuit diet. It seems obvious that this mutation is vital for their survival of Inuit on high-fat diet. It was also noted that the mutation was found to be strongly associated with height because growth is in part regulated by person's fatty acid profile, which also affect the regulation of growth hormones. So it seems that what is true for Inuit, high EPA, and DHA fish diet may not be straightaway true for everyone else. Difference in the type omega-3 intake of vegetarian and non-vegetarian is depicted in Fig. 2.1.

Fish oils provide a source of EPA and docosahexaenoic acid (DHA), two fatty acids now recognized to be important for human health [9]. The increasing demand for EPA and DHA containing fish oils is putting pressure on fish species and numbers [10]. Fisheries provide fish for human consumption and supplement production, at a maximal historical rate, suggesting mass-scale fishing is no longer sustainable. High rate of fishing is resulting in a substantial effect on fish levels with the possibility of extinction [11]. The world's fish stocks are fast declining and it has been estimated that 100 % of the world's fish taxa will have collapsed by 2048 [12]. The major sources of these omega-3 fatty acids are oily fish species including salmon, mackerel, and herring [13].

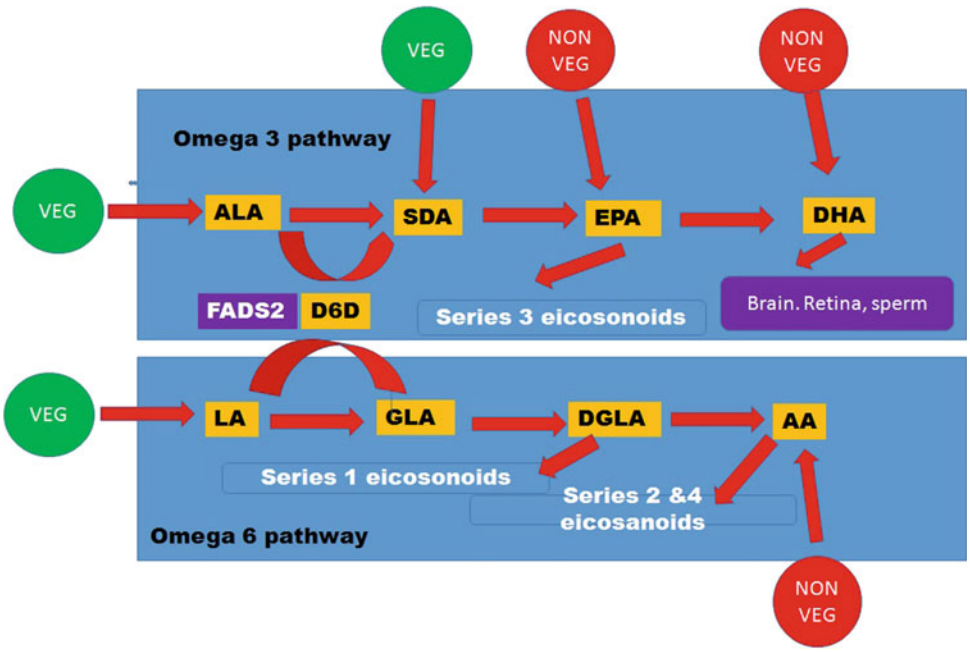

Fig. 2.1 Difference in the type of omega-3 and omega-6 fatty acids by vegetarian and non-vegetarians

The numerous health benefits provided by fish consumption may be compromised by the presence of toxic metals and metalloids such as lead, cadmium, arsenic, and mercury, which can have harmful effects on the human body if consumed in toxic quantities [14].

Health properties of omega-3 fatty acids have now been well established. High prevalence of degenerative disease, primarily attributed to the paucity of omega-3 fatty acid in human diet. In order to protect fish species and the ocean ecosystems, alternative sustainable sources of omega-3 fatty acids are need of the day.

Fish oil and algal DHA oil has problem of patient compliance in high dose therapy because of fishy taste and gastrointestinal complaints. The benefits and risks of algal, fish oil, plant, omega-3 fatty acid-enriched dairy products, animal-derived food, krill oil, and seal oil have been reviewed [3]. Algae are the primary producers of the ocean's ecosystem, providing the foundation of the oceanic food chain. Specifically, algae synthesize omega-3 fatty acids that are subsequently consumed by other marine life. Cost of extraction and purification methods are currently limiting the potential of using micro-algal oils on a large scale [15].

Admittedly, all omega-3 fatty acids are not equal (Fig. 2.2) ALA is available from plant sources and linseed is the richest vegetarian source of ALA. ALA cannot be synthesized in human body and therefore the essential omega-3 fatty acid. ALA is a precursor for EPA and DHA, which are physiologically more potent omega-3 fatty acids. Fish is a good direct source of EPA and DHA and hence regarded as more effective. There is a notion that ALA is not efficiently converted to EPA and DHA and therefore EPA and DHA are conditionally EFAs. ALA is the only form of omega-3 fatty acid available to vegetarians and hence there is a controversy as to whether ALA can really fulfill the omega-3 needs of vegetarians adequately. As fractional conversion of DHA from ingested ALA represents only the proportion of the dose that is found in the blood compartment, which is a very small portion of the DHA synthesized from ALA, these estimates are likely underestimates of actual DHA synthesis from ALA in humans [16]. When ALA is administered orally, it is absorbed into the lymphatic system and then deposited into systemic circulation. This is problematic for human tracer studies that administer ALA orally and measure appearance of labeled omega-PUFA products in the plasma, as a large portion of the trace will have taken up by tissues and adipose tissue and do not reach liver and then to plasma. In 2009, Barcelo-Coblijn and Murphy [17] have elegantly argued that dietary ALA is a significant contributor of tissue DHA. The pertinent issue raised is whether a terrestrial animal (humans) that is an omnivore truly requires dietary DHA of marine origin, in order to have optimal physiological performance, despite the true rarity of DHA in the world's terrestrial food web, where ALA exists in abundance? Dietary ALA is critical for maintaining tissue LC-PUFA levels. Absence of ALA results in omega-3 deficiency including that of DHA. There are three basic fates of ALA in human body: (A) ALA undergoes beta-oxidation and provides energy, (B) ALA gets converted to EPA and DHA in a tissue dependent manner, and (C) ALA is stored in adipose tissue and mobilized as and when required. Vegans and vegetarians have similar prevalence of neurological disease as compared with omnivores, suggesting that any

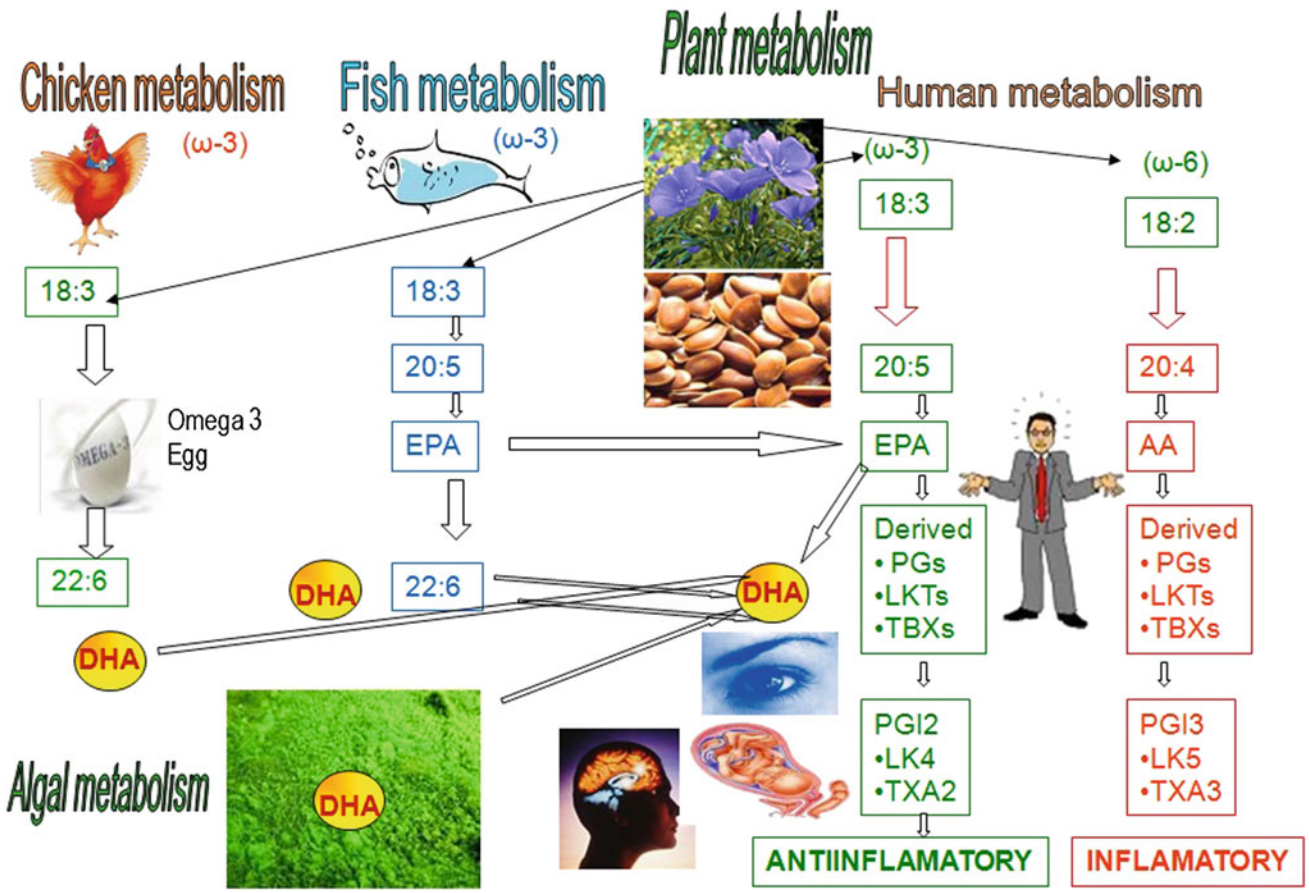

Fig. 2.2 Origin, supply, and utilization of omega-3 PUFAs

altered brain DHA metabolism in these individuals does not manifest neurologically [18]. The need-based regulation of conversion of ALA to LC-PUFA is also evident. Sex hormones may influence the enzymatic synthesis of LC-PUFA, which may lead to need-based sex-specific differences in LC-PUFA supply [19].

Both ALA and LA constitute the 18 carbon EFAs, precursors of physiologically potent 20 carbon eicosapentaenoic acid (EPA; 20:5) and arachidonic acid (AA; 20:4). The desaturation and elongation of both LA (18:2) and ALA (18:3) to their corresponding LC-PUFAs, arachidonic acid (AA; 20:4) from LA and EPA (20:5) and DHA (22:6) from ALA, are performed by the same sets of common desaturases and elongases. Fortunately, these enzymes have 10 times more affinity for ALA than for LA. Therefore, taking ALA instead of preformed EPA and DHA has additional benefit of reducing the conversion of LA to AA, which is responsible for the production of inflammatory eicosanoids. Further, a recent article provides evidences that ALA to DHA conversion is sufficient to maintain DHA levels in adult brain [20].

The epidemiologic evidence suggests comparable benefits of plant-based and marine-derived omega-3-PUFAs [21].

It is clear from the above account that the main three omega-3 fatty acids available from different sources have different effects on the omega-3 metabolism in humans (Fig. 2.2) Fish would provide EPA and DHA, non-vegetarian food like chicken would provide ALA, EPA, and DHA, and algae would provide DHA or EPA, whereas linseed, which is basic raw material being promoted through FBC, provides only ALA.

Although all omega-3 fatty acids are not equal, each one of them ALA, EPA, and DHA are all uniquely bioactive. DHA is considered most potent, main omega-3, fatty acid as it is concentrated in the brain, and constitutes 10–15 % of the brain fatty acids. Dietary DHA is known to downregulate enzymes involved in its own synthesis [22].

In view of the above considerations that promoting non-fish sources of omega-3, such as linseed, is particularly rewarding for vegetarians, we put forth "Flax Bio-village" Concept to resource omega-3 from linseed and enrich egg, milk, and chicken. We felt that although linseed is a source of ALA, a shorter chain omega-3-PUFA would be ideal as a sustainable, renewable, economical, and land source, and would substantially reduce the impact on fish levels.

ALA is the essential omega-3 fatty acid, as it cannot be synthesized in human body. Evidences of potential benefits of omega-3 for human health have largely come from the sea food omega-3 fatty acids, particularly EPA and DHA. However, fewer studies have evaluated the plant-derived omega-3 fatty acid ALA [23]. Because EPA and DHA are rapidly incorporated into plasma and membrane lipids, the efficacy of EPA and DHA is are higher than that of DHA. However, ALA being the primary essential omega-3 fatty acid, for the long-term beneficial effect, ALA may be more important in human nutrition [24]. ALA is beneficial as a nutraceutical/pharmaceutical candidate and is safe for use as food ingredient [25].

Data on beneficial effects of ALA in flax are not as mature as fish data. Nine major studies revealed inverse relation between ALA levels and cardiovascular disorders [26–34]. The results are persuasive as most of these studies are from large sample populations and/or over relatively long period [26–34]. Therefore, our initiative to implement "Flax Bio-village Concept" to promote linseed agriculture, resource omega-3 from linseed to enrich various food products, for omega-3 nutritional security, is justified.

Health benefits of plant-derived ALA have been recently been reviewed [35]. It has been reported that the rise in blood pressure during mental stress is ameliorated by flaxseed [36].

Flaxseed is the richest vegetarian source of omega-3 fatty acid (ALA). Omega-3 fatty acid being from plant source has wider acceptability to both vegetarians and non-vegetarians. If properly exploited flaxseed has the potential to meet the crucial need of omega-3 fatty acid nutritional security and good health for all. But there are many challenges that need technological solutions to exploit the full potential of flaxseed to achieve omega-3 nutritional security and good health for all.

However, flaxseed is not regarded readily edible because of cyanogenic glycosides and phytic acid, but processing of flaxseed for food stuff reduces its toxicity [37]. Flaxseed meal also contains 2.3–3.3 % phytic acid that can interfere with the bioavailability of micronutrients. Flaxseed meal also contains 10 mg/100 g linatin (gamma glutamyl-1-amino-D proline), which induces vitamin B_6 deficiency [38]. However, it has been observed that consumption of up to 50 g of flaxseed per day did not affect vitamin B_6 level. Flaxseed is also rich source of lignan which has antioxidant and weak estrogenic activity. Lignan (secoisolariciresinol diglucoside) content in flaxseed is 800 times more than other plant foods [39]. Secoisolariciresinol diglucoside is converted by bacteria in the gut to enterodiol and enterolactone which can provide health benefits due to their weak estrogenic and anti-estrogenic effects [40]. However, raw flaxseed can act as anti-nutrient, as it can interfere in postnatal development such as the estrous cycle [41]. In India, as the flaxseed is generally regarded as inedible, and the flaxseed oil has been mostly used in varnish and paints and there has not been much demand for flaxseed consequently, farmers do not get good price for their produce, and hence flaxseed has remained a neglected crop. The area under flaxseed has decreased from 20 lakh ha in 1980s to mere 2.9 lakh ha today [42].

Omega-3 fatty acids, being polyunsaturated fatty acid, are very unstable and they need to be stabilized to have better shelf life. Lastly in developing countries, like India, there has to be awareness about omega-3 fatty acids and their health benefits, the crucial need of these vital nutrients. It has also to be appreciated that in a sea of illiterate masses, divided on caste, religion, and regional basis, no one understands a language of reason, under the circumstances, to reach out to the masses faster, and it is better to provide omega-3 fatty acids through commonly eaten daily food such as egg milk and chicken.

Flax Bio-village Concept

Based on the above facts and recognizing that the incorporation of omega-3 fatty acid into our daily consumed food would be immensely rewarding for the health of our people, we decided that linseed, a naturally rich vegetarian source of omega-3 fatty acid, would be ideal to meet the challenge. However, this had several hurdles as mentioned above. We put forth a unique, innovative idea called it "Flax Bio-village Concept" [43]. We would like to briefly narrate the success story, wherein we have systematically tackled each one of the hurdles and have succeeded in developing a replicable model for resourcing omega-3 fatty acid from linseed and enrich egg, milk, and chicken meat besides other food products.

Omega-3 intake of mother is reflected in breast milk and omega-3 in breast milk is very low in populations living mainly on plant-based diet and high in fish-eating countries. The total n-3 fatty acid supply is below the recommended range in nine countries with the lowest GDP. The supply of n-3 fatty acid needs to be increased by using vegetable oils with higher ALA content [44]. Flax oil is richest in n-3 fatty acid. It is interesting that the conversion of ALA to EPA DHA is better in women than in men. Of course their need is higher. Generally, it can be stated that estrogen stimulates, whereas testosterone inhibits, the conversion of EFA to LC-PUFA [45].

Flaxseed (Linseed)

Flaxseed is one of the most important oilseed crops for industrial as well as food, feed fiber purposes [46]. Flaxseed, also called linseed, this is very traditional crop and because

of the presence of omega-3-fatty acid, fiber, phytoestrogen, protein, it has status of good nutritional functional food helpful in reducing cardiovascular diseases, decreases risk of cancer, anti-inflammatory activity, laxative effect, and alleviation of menopausal symptoms and osteoporosis [38].

Flaxseed is mainly cultivated in Canada followed by China, USA, India and Ethopia [47].

Based on the above facts and recognizing that the incorporation of omega-3 fatty acid into our daily consumed food would be immensely rewarding for the health of our people, we, at Bharati Vidyapeeth University, set out to take up this onerous task in right earnest. However, this had several challenges to be tackled. We put forth a unique, innovative idea called it "Flax Biovillage Concept" (FBC) [43]. We will briefly narrate the success story. We have systematically tackled the problems and have succeeded finding solutions and have developed a replicable model for resourcing omega-3 fatty acid from linseed and enrich egg, milk, and chicken meat besides other food products. FBC envisages backward linkages with linseed growing farmer and forward linkage with the consumer. From farm to fork at various stages of its implementation, FBC has tackled several hurdles which needed innovative solutions.

Linseed Is a Neglected Crop

Although linseed is an important high value oilseed crop, however, in India, it is a neglected crop. As the oil was mostly used in varnish and paints, linseed therefore did not fetch much value in the market and the crop has not been found to be lucrative to the farmer. The area under linseed and the production has been continuously decreasing in last three decades. The area under linseed cultivation was 19.51 lakh ha in 1980. Under All India Coordinated Research Project (AICRP) on Linseed, Indian Council of Agriculture has developed more than 58 varieties of linseed and has released for commercial cultivation, in various parts of the country in last three decades. In spite of this persistent effort by the government, there has been a continuous decline in area under cultivation in linseed from 19.51 lakh ha to 3.5 lakh ha today. The linseed cultivation is not lucrative due to low price, less market demand, lack of processing, and value addition. Linseed is an important bioeconomy crop as it is the richest source of omega-3 fatty acid and lignan. It is extremely important to promote linseed agriculture. For that, it is important to develop technologies to resource omega-3 from linseed and enrich commonly consumed food and thereby add value to linseed to provide better price to linseed growing farmer and at the same time provide omega-3 nutritional security to the people.

The efforts initiated by Dr. PDKV, Akola, Bharati Vidyapeeth Deemed University, Pune (BVDU), BAIF Development and Research Foundation, Pune (BAIF-DRF), and M/s. Ensign Diet Care, Pune, as an Industrial Partner with support from two NAIP Projects under ICAR in Component III & Component II are successful to establish public–private partnership (PPP) value chain model to create market demand, better price to farmers, thereby increasing area under linseed and sustainability under rainfed farming system.

With the introduction of high-yielding, disease-resistant linseed PKV-NL-260 developed by Dr P.B. Ghorpade (AICRP-Linseed, Nagpur), the farmers in Vidharbha are now harvesting more yield. With the linseed value addition efforts of BVDU, farmers now get buyback guarantee and good price (5 % over and above the market price). This has a positive impact on the area under linseed in Vidharbha, Maharashtra, and the farmers are finding linseed farming lucrative.

Omega-3 Fatty Acids Are Very Unstable

This has been the real bottleneck in taking omega-3 to the people. We have developed a formulation (a universal omega-3 fortifier, an emulsion completely miscible with water) in honey [48] with antioxidants that is stable for over nine months. This enables us to enrich any food product with omega-3 fatty acid. Omega-3 fortifier, as it is completely miscible in water, can be used to enrich milk and other dairy products.

Linseed Is not Readily Edible and Has Anti-nutrients

Although linseed is now regarded as super food as it contains very high amount of omega-3 fatty acid (55–60 %) and is very rich in soluble fiber, it is not readily edible as it contains high levels of anti-nutrients, such as cyanogens glycoside, linatin (anti-vitamin B-6) [49]. This problem has been successfully resolved by cold press extraction [50] of omega-3 oil free from all the above anti-nutrients. The cake formulated in omega-3 chicken feed for layers produces omega-3-enriched egg. Chicken feed [51] developed for broilers in such a way that it not only produces omega-3 chicken meat [52–54], but also gives better health to the birds [55]. So the anti-nutrients are regulated in the feed such that it does not affect the birds' performance or health.

Awareness of Importance of Omega-3 for Public Health

This is really a major hurdle. However, as the "Flax Bio-village" Concept envisages enriching the commonly consumed food including egg, milk, and chicken, it is hoped

that it would have better consumer acceptability and with the experience of health benefits to the consumer, omega-3 egg, omega-3 milk, and omega-3 chicken, will eventually become popular.

Convergence of Linseed Agriculture to Health and Wealth

Figure 2.3 depicts how FBC is able to bring about convergence of linseed agricultureto health and wealth. FBC develops methods of processing linseed to maximize the value of linseed plant and linseed. FBC provides better price to the linseed grower to increase linseed agriculture. Finally FBC provides high health value product to the consumer by processing linseed.

Many processing techniques have been found to lower the concentration of functional components in food. Omega-3 fatty acids are notoriously unstable. They are susceptible to oxidation when subjected to heat, light, or when in contact with air moisture, metal. Particular care has to be taken while extracting oil from linseed, to avoid or minimize these oxidizing factors by reducing the time of contact while processing.

Linseed has about 35–40 % oil of which 55–60 % is ALA. Omega-3 oil can be extracted by either solvent or cold press extraction. Solvent extraction is harsh but more efficient, although results in almost complete extraction of the oil. Cold press extraction is therefore preferred. The process developed by us involves cold press extraction with a cooling jacket and under inert nitrogen atmosphere. This being inefficient, only 75–80 % of oil is extracted and the rest remains in the cake. The unique innovative process has been developed to use the high-grade omega-3 oil for the production of omega-3 soft gels, omega-3 oil, and the oil is further processed to produce water miscible emulsion, i.e., omega-3 fortifier. Omega-3 fortifier is particularly suitable for enriching milk and other dairy products.

As the inefficient cold press extraction of the oil from linseed leaves ~25 % of oil still in the cake, cake can be further processed suitably for producing omega-3-enriched feed mix formulated appropriately to enrich layer feed for the production of omega-3 eggs and also for the enrichment of broiler feed for the production of omega-3 chicken.

Flax Lignan for Pharmaceutical Application

Bakke and Klosterman in 1956 isolated SDG lignan [56] from flaxseed which is also known as SECO, the aglycone of SDG, depending on the method of analysis. Apart from SDG, flaxseed also contains matairesinol, isolariciresinol, lariciresinol, demethoxy-secoisolariciresinol, and pinoresinol in smaller quantities. Apart from flaxseed lignans are present in legumes, cereals, vegetables, berries, seaweed, tea, and alcoholic beverages. SDG lignan has array of pharmacological actions.

Linseed lignan (SDG) has estrogenic [57] and strong antioxidant activities [58]. SDG exhibits cardioprotective, anti-atherosclerotic [59, 60], and anti-diabetic [61–63] activity. Linseed has about 1–3 % SDG which has cardioprotective [64], antihyperlipidemic [65] activity. Active metabolite of SDG lignan namely enterolactone has anti-metastatic activity [66]. Penumathsa et al. reported the efficacy of SDG lignan in hypercholesterolemic myocardial infarction [67]. Apart from lignan, some phenolic butanol

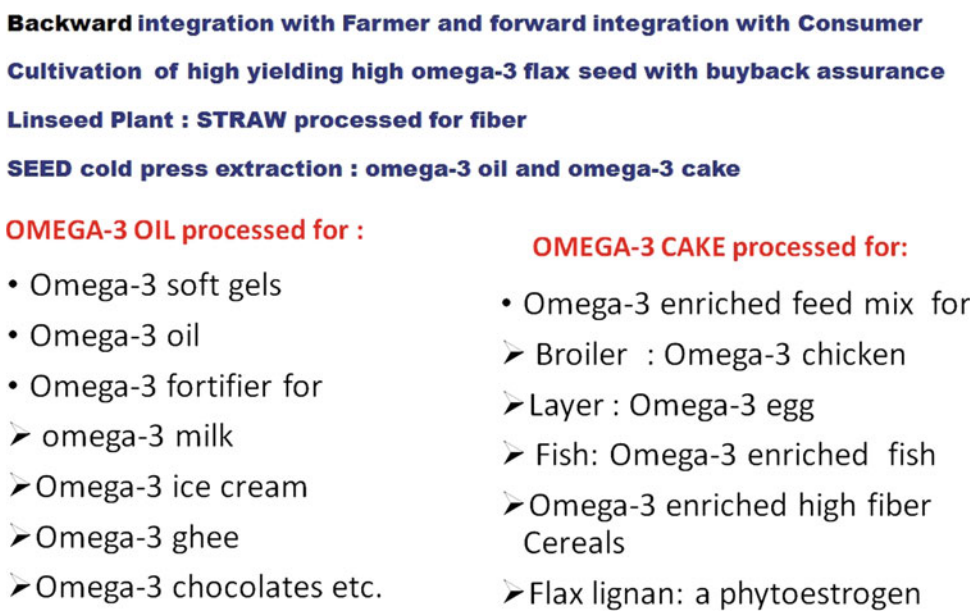

Fig. 2.3 Flax bio-village concept (FBC)

soluble non-lignan components extracted from defatted flax meal have hepatoprotective [68], immunomodulatory activities [69]. In nutshell, bioactive lignan in linseed has potential activities which further add value.

Summary

Flax Bio-village Concept envisages creating wealth to the linseed growing farmer by adding value to linseed, and at the same time, it provides technologies to develop omega-3-enriched functional food for better health of the consumer.

- FBC can revive the linseed crop and lure the farmers for linseed cultivation to meet the challenge of attaining omega-3 nutritional security.
- FBC will provide region-wise state released high-yielding omega-3 seed to the farmer and hence farmer will get higher production and also higher returns.
- FBC provides technology to cold press extract linseed under mild non-oxidizing condition to get high-quality omega-3 oil.
- FBC provides technology to tailor-made omega-3 fortifier to enrich any food products in bakery, dairy, confectionary industries
- FBC processes the cake with still leftover omega-3 in it to produce enriched feed mix (EFM) to feed layer and broiler chicks to produce omega-3-enriched egg and chicken meat.
- The birds consuming omega-3 also enjoy better health with reduced morbidity and mortality increases profitability.
- FBC aims to achieve omega-3 nutritional security and good health for all.

FBC mission is "**Harvesting wealth and cultivating Health**."

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Status Paper on Linseed/Flax Agriculture

3

P.K. Singh

Crop Description

Linseed, an oldest oilseed crop of the world, is considered to be domesticated in north-east region 1000 years ago and serving as a source of both oil and fibre from prehistoric times until present [1].

Ancient Greeks and Romans cultivated linseed for fibre and seed as also substantiated by remains left by Stone Age people. Linnaeus (1857) first time gave botanical name *Linum usitatissimum* to the cultivated species [2]. Carbonized seeds of *Linum* spp. are known from 1600 to 1400 BC at Navdatoli, Madhya Pradesh, India [3]. Its uses were also inferred from the discovery of spun fibres in a string of beads at Chandote in 1440–1200 BC [4].

Linseed is an annual, herbaceous winter crop with short and slender taproot that grows vertically downwards into numerous lateral branches. However, depth of the root is dependent upon the plant type, climate and soil factors. Primary branching in linseed is mostly found on main shoot very near to radicle–plumule junction (near the ground level). Two distinct morphological types namely seed type and flax/dual-purpose type are recognized in this crop. The linseed varieties grown in India are mainly utilized for oil extraction. Seed type linseed is generally short statured (30–85 cm), whereas dual-purpose/flax type is tall in height (80–130 cm). Linseed plant has one stem whose thickness ranges from 2 to 4 mm. The main stem and basal branches give rise to primary, secondary and tertiary branches that bear the leaves and flowers. Accordingly, profuse and bushy secondary and tertiary branches are found in seed type; top secondary and tertiary branches are there in DP/flax type. Leaves are simple, linear, lanceolate, 3-nerved sessile, entire and without stipules. The basal leaves are in alternate pairs and those above the fourth node are spirally arranged. Leaf length varies in size (3–4 cm) with smooth upper surface, and the colour ranges from fresh green to bluish green.

Origin

Initially two theories were propounded.

Polyphyletic origin/convergent evolution. 2. Origin from *Linum angustifolium.*

The available evidence indicates that Central Asia is the primary centre of origin, whereas Mediterranean region is the secondary centre of origin [5, 6]. There is clear evidence in the archaeological record that linseed was domesticated for both oil and fibre use more than 8000 years ago in the Near East [7]. Morphological [8], genetic [9] and molecular [10] evidence suggest that the wild progenitor of cultivated flax is pale flax (*Linum bienne)*, with which it is interfertile.

It has been under discussion whether oil or fibre was the primary reason of domestication and whether domestication took place once or happened several times in independent domestication events in different diversity regions of flax [9]. Analysis of genetic diversity in the stearic acid desaturase locus sad 2 suggests a single domestication event of cultivated flax from its progenitor *L. angustifolium* Huds. Moreover, an oilseed type of flax is proposed as the first domesticate, while fibre flax appears as a later descendant from oilseed flax [1].

Importance

Linseed is basically an industrial oilseed crop, and its each and every part is endowed with commercial or medicinal importance. Of its 2.27 million hectares global area, 40.19 % belongs to Asian region with 34.29 %

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(0.717 million tonnes) contribution to the total production of world (2.24 million tonnes). Productivity of this region (728 kg/ha) is merely 85 % of the world productivity of 986 kg/ha. The reason for the lower productivity in Asian region is abysmally low productivity of 435 kg/ha of India, which occupies the major chunk of acreage nearly 14.88 % of the world acreage.

India is the third largest linseed growing countries in the world and production wise it ranks fourth (6.57 %) in the world after Canada (40.51 %), China (18.68 %) and Kazakhstan (10.89 %). At present, linseed is cultivated on about 0.338 million hectares with a contribution of 0.148 million tonnes to the annual oilseed production of the country. Presently, linseed is under cultivation in as many as 13 states of the country, viz. Madhya Pradesh, Maharashtra, Chhattisgarh, Uttar Pradesh, Bihar, Odisha, Jharkhand, Karnataka, Assam, Nagaland, West Bengal, Rajasthan and Himachal Pradesh. Productivity of the states like Nagaland (803 kg/ha) and Bihar (850 kg/ha) is approaching the world average productivity (986 kg/ha). The increase in production and productivity indicated the impact of improved varieties coupled with technologies and favourable environments.

In India, linseed is a *rabi* oilseed crop predominantly grown under rainfed (63 %), *utera* (25 %) and irrigated (12 %) and input-starved conditions due to which the national average productivity (435 kg/ha) is very low as compared to that of world average (986 kg/ha). The major impediments for the lower national average productivity have been the area of production under major linseed producing states, i.e. Madhya Pradesh, Chhattisgarh, Maharashtra, Uttar Pradesh and Orissa (about 83 % area) basically lie under sub-marginal, un-irrigated, input-starved and poor crop management conditions. In India, rainfed agriculture occupies nearly 58 % of the cultivated area, contributes 40 % of country's food production, and supports 40 % of the human and 60 % of the livestock population. Continuous decline in groundwater levels, growing efficiency of major and micronutrients, declining factor productivity and looming threat of climate change are some of the issues which will have a bearing on food production in the near future. Thus, rainfed/linseed agriculture assumes importance from the consideration of growth, equity and sustainability.

Kingdom	Plantae
Phylum	Angiospermae
Division	Magnoliopsida
Order	Malpighiales
Family	Linaceae
Genus	*Linum*
Species	*L. usitatissimum*

Scientific Name and Species Relationship

The genus *Linum* has been divided into five sections, i.e. *Linum, Linastrum, Cathartolinum*, *Dasylinum* and *Syllinum*. The phylogeny of the genus *Linum* can be illustrated as follows:

Hooker (1985) reported the occurrence of five wild spp., viz. *L. perenne, L. strictum, L. mysorense, L. angustifolium* and *L. grandiflorum* out of which *L. grandiflorum* is mostly grown as ornamental plant and non-crossable with the cultivated linseed *L. usitatissimum*, the only species with economic importance [11].

The basic relationship between various sections of the genus, *Linum,* is not clear, and several species were classified at one time or the other as members of various subsections. *Linum* is the largest genus of the family Linaceae and consists of nearly 200 species spread over the temperate and warm temperate zones of the Northern Hemisphere. Wide range of species diversity within the genus along with diverse basic chromosome number ranging from $n = 8$ to 43 continues to challenge/elude botanists as they develop systematic treatments. Winkler (1931) [12] divided the genus on the basis of morphological characters into six taxonomic sections, i.e. *Linum, Dasylinum, Linastrum, Cathartolinum, Syllinum* and *Chiococca.* Chennaveeraih and Joshi (1983) [13] described the evolutionary relationships of 19 *Linum* spp. based on chromosomes numbers and karyomorphological similarities. Gill (1987) reported the chromosomes no. of 41 species of the genus ranging from $2n = 16$ to 80 [14].

Interspecific hybridization between cultivated flax and *L. angustifolium* was first time reported by Tammes, 1928 [15]. Later on, Sharma and Khanna (1964) [16] reported crosses between *L. usitatissimum* and three wild spp. *L. marginale, L. hirsutum* and *L. africanum* without any cytological information. Gill and Yermanos (1967) [9] attempted 84 interspecific crosses among 27 *Linum* spp., out of which only 24 crosses were found successful. In most of the unsuccessful crosses, ovaries did not develop. Crosses among species with $n = 15$ chromosomes, i.e. *L. usitatissimum, L. africanum, L. angustifolium, L. corymbiferum* and *L. decumbens* were highly successful. Interspecific hybridization among cultivated and wild species have played an important role in the search for an introgression of desired traits of economic importance from closely related wild species into cultivated flax [17, 18]. Meiotic behaviour of interspecific hybrids between species having 9 and 15 haploid chromosomes numbers has revealed that speciation in the genus *Linum* has occurred probably in two steps, i.e. polyploidy followed by chromosomal interchanges.

Fu and Allaby (2010) [19] assessed the phylogenetic relationships among 29 *Linum* accessions representing 16

species including cultivated flax and its progenitor pale flax (*L. bienne*) based on four non-coding regions of chloroplast DNA sequences for understanding evolutionary pathways and the history of flax domestication. The results revealed that evolutionary pathways are consistent with those derived from morphological and cytological data. These relationships also support an earlier hypothesis that cultivated flax is descended from a single domestication of pale flax plants, apparently for oil usage. The exotic gene pool for linseed improvement should consider phylogeny and phonetic relatedness to guide the assessment of species crossability, as interspecific hybridization has been successfully made only among some species having either $n = 15$ or 9, not among those of different chromosome numbers.

Floral Biology

Inflorescence is a loose terminal raceme or cyme. The pedicles are 1.0–1.5 cm long and erect. Linseed flowers are hermaphrodite, hypogyny with 5 sepals, 5 petals, 5 stamens and a compound pistil of 5 carpels in a symmetrical arrangement. The sepals are 5 in number, ovate, ciliate three-nerved, acuminate and persistent. The flowers are terminal with about 2.0–3.0 cm in size and are blue, white, violet and red violet in colours.

Corolla is polypetalous, deciduous and is responsible for different flower colours. Petals remain twisted at the base into hypogynous discs and fall before 2 PM. Gynoecium is superior with 5 united carpels and 5 styles. Ovary is completely divided into two by a false septum, resulting into 10-roomed capsule each with one ovule/embryo. The five long styles are free to the base with loose union at the stigmatic ends. The styles are white with varying amounts of blue or purple shades. The stigma remains receptive for a period of 5–6 h after the opening of the flower.

The androecium has 5 stamens arranged alternately with petals and is monadelphous. The filaments are white, blue or violet, whereas anther's colour varies from cream, grey, violet to blue. The anthers are two celled, introse and dehisce longitudinally. Pollen grains are blue, yellow or white and may accentuate by the colour of the anther.

Fruit/capsule is somewhat round in shape and surrounded by persistent sepals. It possesses 10 locules each with one seed, and its size (10–12 × 7–16 mm) may vary from variety to variety. The mature capsule is generally non-dehiscent, but in some varieties, semi-dehiscent condition may prevail. Fruits exhibit variations in colour light brown to dirty creamy at maturity. The seed is oval, lenticular and pointed at one end. Its length and breadth varies from 3 to 5 mm and 2 to 3 mm, respectively. The seed surface is smooth and generally shinning, but it may be dull in some varieties. Ten seeds are expected in one capsule, but the number depends upon the nutritional condition of the flowers. The predominant seed colour is light brown to dark brown, but in some varieties, yellow or fawn colour exists. The main constituent of seed is oil (35–45 %) and protein (20–28 %).

The flowers open in the early morning (8 am) and remain open till midday after which it begins to fade, and by about 4 pm, the petals fall down in sunny winter days. The rapid growth of the filament soon brings the anther to the same level as the stigma and this position heralds the opening of flowers. The stigma in the bud stage protrudes over the anther lobes, but as the bud expands and the petals begin to unfold, the anthers gradually overtake the stigma and by the time the flower is in full-bloom stage, the anthers surrounded/enveloped the stigma completely. The dehiscence of anthers takes place before the complete opening of the flower, and self-pollination occurs with the help of expansion of corolla. At a little later stage, the burst anthers often fall together forming a cap over the stigma and ensure self-pollination a practical certainty. However, 0.43–3.4 % natural out-crossing has been reported [20]. Linseed plants usually blossom over a long period (25–40 days) and influenced by temperature and moisture regime.

Nutritional Values

The major nutritional components of flax are oil (fat), protein and dietary fibre. Milled flax provides about 36 kcal/tbsp. Flax oil provides about 124 kcal/tbsp. Ground flax is very low in carbohydrates (sugars and starches), providing only 0.1 g/tbsp—one reason why flax is popular with people following a high-protein, low-carbohydrate weight-loss diet.

Composition of flax as a food	
Fat	41 %
Total dietary fibre	28 %
Protein	20 %
Moisture	7 %
Ash	4 %

A Unique Mix of Fatty Acids

Flax is naturally low in saturated fat and has a moderate amount of monounsaturated fat. Most of the fatty acids in flax are polyunsaturated. Flax is particularly rich in alpha-linolenic acid (ALA), the essential omega-3 fatty acid. As little as one tbsp of ground flax provides 1.8 g ALA, more than enough to meet the daily recommended intake for this nutrient.

Fatty acid composition of flax oil	
Polyunsaturated fatty acids omega-6s	16 %
Polyunsaturated fatty acids omega-3s	57 %
Monounsaturated fatty acids	18 %
Saturated fatty acids	9 %

A Low Omega-6/Omega-3 Fatty Acid Ratio

Because of its high ALA content, flax has an omega-6/omega-3 fatty acid ratio of 0.3:1. Consuming flax, flax products, and omega-3 enriched eggs derived from hens fed flax or other similarly enhanced foods increases the omega-3 fatty acid content of the diet and improves the dietary omega-6/omega-3 fatty acid ratio. Consumers are advised to increase their omega-3 fat intake because the typical Western-type diet is high in omega-6 fats and low in omega-3 fats compared with the Palaeolithic diet on which humans evolved. Eating more omega-3 fats and less omega-6 fats may lower the risk of chronic diseases such as heart disease, stroke and cancer.

Essential Fatty Acids

Flax contains two essential fatty acids (EFAs)—alpha-linolenic acid (ALA), the parent fatty acid of the omega-3 family, and linoleic acid (LA), the parent fatty acid of the omega-6 family. EFAs are those not synthesized in human body but required for maintaining the structure of cell membranes and the health of the skin, and they are involved in cholesterol transport and metabolism. EFAs can be converted to compounds called eicosanoids, which play a role in inflammatory reactions.

Lignans

Lignans are both antioxidants and phytoestrogens. Antioxidants are compounds that work to keep oxygen from reacting with and damaging proteins, fats and other compounds in our tissues. Phytoestrogens are compounds found in plants that can have weak oestrogen activity in animals and humans. The main lignan in flax is secoisolariciresinol diglucoside (SDG). The SDG found in flax and other foods is converted by bacteria in the gut to the lignans found in humans and other mammal—enterodiol and enterolactone. The level of enterodiol and enterolactone in blood and urine reflects the lignan content of the diet. In one study of nine healthy young women, for example, eating milled flax for seven days produced significant increases in the plasma and urinary concentrations of enterolactone and enterodiol. Lignans protect against cancer by blocking certain enzymes involved in hormone metabolism and interfering with the growth and spread (metastasis) of tumour cells. Indeed, populations with high intakes of lignans, antioxidants and phytoestrogens from fruits, vegetables, nuts and wholegrains have low rates of cancer of the ovaries and the gastrointestinal (GI) tract—including cancer of the mouth, oesophagus, stomach, colon and rectum compared with those who have low intakes of these foods.

Dietary Fibre

Flax is a source of dietary fibre, providing about 2.2 g/tbsp of ground flax. It contains both insoluble and soluble fibre. Insoluble fibre helps improve laxation and prevent constipation, mainly by increasing faecal bulk and reducing bowel transit time. In a recent study of elderly residents in a long-term care facility, adding 1 tbsp of milled flax to the daily diet resulted in a 32 % increase in bowel frequency by the end of the 4-month intervention. The use of suppositories decreased 50 % in this population over the course of the study. The water-soluble fibre fraction of flax makes up about one-third of total dietary fibre. The main soluble fibre in flax is mucilage gum. Water-soluble fibre helps maintain blood glucose levels and lower blood cholesterol levels.

Edible Linseed Oil—"Linola"

To diversify the uses of linseed oil, it is imperative to evolve linseed varieties for cooking purpose. In India, few germplasm lines with 25–30 % linolenic acid have been identified. Breed linseed varieties for edible oil are a new development. Australia and Canada have achieved success in isolating low linolenic acid lines through mutation breeding. For reducing the linolenic acid content in linseed oil, EMS in the cultivar "Glenelg" was used and obtained two mutants, viz M 1589 and M 1722 with 50 % reduction in linolenic acid [21]. By recombining the two mutants, the line "Zero" with less than 2 % linolenic acid in the seed oil was obtained [22] with the same oleic acid content as in cv. "Glenelg". These so-called Linola quality lines have been patented by Green (1995) [23].

A Canadian programme identified an EMS-induced low linolenic acid mutant in the cultivar "Mcgregor" [24]. Low linolenic trait was determined by two genes (Ln1 and Ln2) located on separate chromosomes and acting additively.

Linseed Cake—Quality and Economic Aspects

The linseed cake is the product left after the extraction of oil from its seeds, which may be further ground to give linseed meal. The approximate composition of the linseed cake is moisture 11 %, protein 32 %, oil 10 %, carbohydrates 32 %, fibre 9 % and minerals 6 %.

Since the linseed cake contains more than 30 % protein, it is an important source of protein for livestock particularly milch cattle. It has higher phosphorus content and also good amount of total minerals and calcium. The cake is hard and considered to be safe for the dairy cattle, sheep and horses, but unfit for poultry birds as it reduces their growth rate and may also cause the death of birds. It absorbs large amounts

of water and increases the bulk proportionately. No other feed has this water absorbing quality to the same degree as that of linseed cake. It also has a laxative effect, which aids in keeping the live stock healthy. This effect is especially important when the cattle are fed with very low grade roughage. However, it should not be fed in large quantities to cows, since it makes the butter soft and greasy. Apart from being an animal feed, linseed cake is also a very good source of organic manure. It contains about 5 % nitrogen, 1.5 % phosphorus and 1.8 % potash.

Important Zones for Cultivation in India

There are three agro-climatic zones for linseed cultivation in India

Zone I—Himachal Pradesh, Punjab, Haryana and Jammu & Kashmir
Zone II—Uttar Pradesh excluding Bundelkhand, Bihar, Jharkhand, West Bengal, Assam and Nagaland
Zone III—Bundelkhand area of Uttar Pradesh, Madhya Pradesh, Rajasthan, Chhattisgarh, Odisha, Maharashtra, Andhra Pradesh and Karnataka.

Comparative Analysis

Table 3.1 represents area, production and yield of major linseed (Rabi) growing states (2009–2010 to 2013–2014).

Table 3.2 represents area, production and yield of major linseed growing countries (2013–2014).

Gap of Yield with Other Countries

The average yield of linseed in India is lowest among the major linseed growing countries of the world.

Export–Import Status of the Crop Produce

Table 3.3 represents trends in India's import and export in linseed commodities from 2007 to 2012.

Varietal Development

Important varieties released and notified by CVRC

Till date 62 improved varieties of linseed have been released and recommended for general cultivation in different agro-ecological regions of the country as per details enumerated in Tables 3.4, 3.5 and 3.6.

Climatic Requirement

Linseed is an annual herbaceous plant grown as winter crop in warm climates of India. Moderate temperature (21–27 °C) is ideal during vegetative and reproductive development. Flowering and grain filling duration are the most critical growth stages of the crop. High temperature (above 32 °C) accompanied with moisture stress during flowering stage reduces the seed yield, oil content and quality of oil. Flowering duration is also adversely affected by low temp (below 10 °C) as the crop is susceptible to frost. Germination is rapid at a temperature range of 18–24 °C.

In India, linseed is grown under varied soil and climatic conditions. Its production depends principally on rainfall and moderately cool climate. The crop is grown in Bundelkhand region of Madhya Pradesh and Uttar Pradesh and Maharashtra in heavy, deep, moisture retaining black cotton soils, whereas in Uttar Pradesh and Bihar, it is grown in the lighter Gangetic alluvium. In Kangra (Himachal Pradesh), Orissa and eastern part of Uttar Pradesh and Madhya Pradesh, it is grown in paddy land. The crop makes its best growth on well-drained, fertile, medium heavy soils, especially silty clays. Light soils are unsuited for the seed crop particularly in the regions of deficient rainfall. However, it is better to grow rainfed linseed on soils having high moisture retention capacity in the upper layers. Linseed is tolerant to a wide range of pH. It thrives well on soils with pH ranging between 5.0 and 7.0; however, a pH 6.0 was reported to be the best. It is also reported that yield and quality decreased significantly at the electrical conductivity of 17.5 and 6.1 mmhos/cm, but the iodine value increased with an increase in salinity.

Genetic Potentiality Advancement

Improvement in linseed breeding has been very successful during the last century, particularly in last four decades spectacular progress has been achieved regarding yield and quality through conventional breeding. Further progress can certainly be made through application of tissue and cell culture and molecular techniques as supplementary aids: regeneration of plants from hypocotyl [25, 26] and from cotyledons [27] and induction of large number of shoots from stem explants.

Anther culture In these species, it is possible now to obtain "haploid" plants reproducibly through microspore—or anther—culture [28].

Haploid plants carry a single set of chromosomes in their somatic cells. By doubling this chromosome set artificially, e.g. by colchicines, homozygous diploid, i.e. doubled haploid, lines are obtained. Such inbred lines are an unalterable

Table 3.1 Area, production and yield of major linseed (Rabi) growing states (2009–2010 to 2013–2014)

Sl. no.	State		2009–2010	2010–2011	2011–2012	2012–2013	2013–2014
1	Madhya Pradesh	A	1.179	1.247	120.3	109.7	110.4
		P	0.514	0.422	57.0	57.4	55.0
		Y	436	338	474	523	498
2	Maharashtra	A	0.36	0.39	31.0	27.0	31.0
		P	0.09	0.1	8.0	8.0	8.0
		Y	250	256	258	296	258
3	Chhattisgarh	A	0.4	0.384	39.0	31.2	26.2
		P	0.12	0.1	14.5	8.9	11.1
		Y	300	260	372	285	424
4	Uttar Pradesh	A	0.34	0.65	31.0	30.0	26.0
		P	0.15	0.294	14.0	13.0	10.0
		Y	441	453	452	433	385
5	Jharkhand	A	0.198	0.159	24.2	25.7	25.5
		P	0.085	0.082	12.9	16.6	14.5
		Y	427	516	531	645	568
6	Bihar	A	0.244	0.234	23.4	22.0	18.7
		P	0.206	0.208	20.3	19.1	15.9
		Y	846	889	865	867	850
7	Orissa	A	0.264	0.207	24.6	24.5	22.9
		P	0.119	0.095	11.6	11.8	11.0
		Y	451	459	471	482	478
8	Karnataka	A	0.12	0.11	9.0	7.0	6.0
		P	0.04	0.04	2.0	2.3	2.0
		Y	333	364	222	333	333
9	Nagaland	A	0.128	0.057	5.7	5.7	5.8
		P	0.088	0.046	4.6	4.6	4.6
		Y	689	507	807	807	803
10	Assam	A	0.077	0.071	7.2	6.7	6.0
		P	0.04	0.037	3.7	3.8	3.9
		Y	517	521	520	568	643
11	West Bengal	A	0.047	0.041	4.1	3.8	10.1
		P	0.014	0.014	1.5	1.3	2.0
		Y	295	341	359	349	200
12	Himachal Pradesh	A	0.018	0.011	1.1	1.1	1.1
		P	0.004	0.03	0.3	0.4	0.3
		Y	225	273	286	327	255

(continued)

Table 3.1 (continued)

Sl. no.	State		2009–2010	2010–2011	2011–2012	2012–2013	2013–2014
13	Rajasthan	A	0.033	0.017	1.2	1.1	2.1
		P	0.065	0.021	1.3	1.0	2.9
		Y	2006	1235	1066	962	1351
	India	A	3.42	3.592	322.6	395.8	292.1
		P	1.537	1.465	152.5	148.4	141.2
		Y	449	408	473	502	484
	Asia	A	8.79	10.6			
		P	5.35	6.11			
		Y	608	575			
	World	A	21.12	22.19			
		P	21.23	19.23			
		Y	1006	867			

Source Directorate of Oilseeds Development, Hyderabad
A Area in lakh ha, *P* production in lakh tonnes and *Y* yield in kg/ha

Table 3.2 Area, production and yield of major linseed growing countries (2013–2014)

Sl. no.	Countries	Area (000 ha)	Production (000 t)	Yield (kg/ha)
1	India	338	147	435
2	Canada	412	712	1728
3	China	330	330	1000
4	Americas	502	832	1659
5	Russian Federation	410	325	794
6	Kazakhstan	384	295	767
7	Asia	1087	792	728
8	Europe	556	485	872
9	World	2270	2238	986

Source: FAO Stat

Table 3.3 Trends in India's import and export in linseed commodities since 2007–2012 (Rs. in crores)

	2007–2008	2008–2009	2009–2010	2010–2011	2011–2012	2012–2013
Import	225.50	231.38	269.24	334.14	396.36	397.88
Export	106.01	105.43	95.43	114.61	106.36	131.72

(i) Yarn and fabric imported from China (85 %)
(ii) Fibre imported from Belgium (60 %)
(iii) 75 % export made on fabric

prerequisite for breeding F_1-hybrids. For self-pollinating species like linseed (flax), inbred lines are still the ultimate product of long-term breeding and selection process. Therefore, with the aid of the above-mentioned "haploid technique", a gain of several years can be achieved in a breeding programme [29]. Haploid breeding is based on the gametes instead of sporophytes; hence, probability of obtaining a desired genotypes in haploids is much higher than that of the diploids.

Marker-assisted Breeding Marker-assisted selection (MAS) can provide an effective and efficient breeding tool for detecting, tracking, retaining, combining and pyramiding resistance genes [30]. Significant advance made in this field includes the identification of markers linked with resistance genes against Fusarium and scorch disease [31], rust [32] in linseed. These markers are being employed in breeding programmes, and the impact is felt in the

Table 3.4 Recommended for general cultivation in different agro-ecological regions of the country

Sl. no.	Name of variety	Year of release		Duration	Avg. yield kg/ha	Recommended states	Oil Content (%)
		Centre	State				
(1974–1979) Vth plan							
1	K-2	1975	–	170–175	1110 (I)	Punjab, Haryana and U.P.	40.04
2	LC-185	–	1975	150–160	500 (U)	Punjab	38.89
3	Hira	–	1978	130–135	1200 (R)	Bundelkhand and U.P.	36.36
4	Mukta	–	1978	127–132	1200 (I)	Eastern U.P.	41.40
5	Chambal	–	1978	125–130	900 (I)	Rajasthan	40.11
6	Neelum	1978	–	140–145	1500 (I)	Mid-Central and Western U.P.	43.00
(1980–1985) VIth plan							
7	Jawahar-1	–	1982	116–120	900 (R)	Madhya Pradesh	38.34
8	Jawahar-7	–	1982	116–120	700 (R), 300 (U)	Madhya Pradesh	37.79
9	Jawahar-17	–	1982	117–120	1300 (I), 800 (R)	Madhya Pradesh	37.61
10	Neela	–	1982	127–135	850 (R)	West Bengal	34.93
11	LC-54	1982	–	155–165	1330 (I)	Punjab, H.P. and Haryana	42.00
12	C-429	–	1983	125–130	1000 (R)	Maharashtra	39.07
13	T-397	1984	–	120–125	1100 (I)	Bundelkhand of U.P, Bihar, Assam, M.P. and Rajasthan	44.00
14	R-552	1984	–	118–125	900 (R)	Madhya Pradesh	44.00
15	Pusa-2	1985	–	125–150	730 (R)	Punjab, H.P., Haryana and Rajasthan	38.31
16	Pusa-3	1985	–	125–150	800 (I)	Punjab, H.P., Haryana and Rajasthan	37.65
17	S-36	–	–	130–135	400 (R)	Karnataka	34.80
18	Himalini	1985	–	150–175	1310 (I)	Punjab, H.P., Haryana and adjoining of Rajasthan	42.00
19	Jawahar-23	1985	–	120–130	1000 (I)	Madhya Pradesh, Orissa, Bundelkhand of U.P., Maharashtra, Karnataka and Rajasthan	43.00
20	Garima	1985	–	120–130	1490 (I)	U.P. (Excl. Bun.), Bihar, West Bengal and Assam	42.00
21	Sweta	1985	–	130–135	880 (R)	U.P. (Excl. Bun.), Bihar, West Bengal and Assam	44.00
22	Shubhra	1985	–	130–135	1390 (I), 870 (R)	U.P. (Excl.Bun.), Bihar, West Bengal and Assam	45.00
(1985–1990) VIIth plan							
23	Laxmi-27	–	1987	110–120	1260 (I), 1020 (R)	Bundelkhand of U.P.	45.00
24	Gaurav	1987	–	137–140	1050 (S), 950 (F)	U.P. (Excl. Bundelkhand), Bihar, West Bengal and Assam	43.00
25	Kiran	1988	–	120–126	750 (R)	Madhya Pradesh, Rajasthan, BKD of U.P, Maharashtra, Karnataka and Orissa	43.00
26	Janki	–	1988	165–170	1200 (I)	Himachal Pradesh (Kangra valley)	42.00
27	Jeevan	1988	–	175–180	1090 (S), 1100 (F)	Punjab and Himachal Pradesh	45.00
(1992–1997) VIIIth plan							
28	Surabhi	–	1995	165–170	1000 (U)	Himachal Pradesh	44.00
29	Nagarkot	1995	–	165–170	1150 (S) 950 (F)	Punjab, U.P., Himachal Pradesh, West Bengal, Assam, Haryana, and Rajasthan (Kota)	43.00
30	Shikha	1997	–	135–140	1233 (S), 1033 (F)	U.P. (Excl. Bundelkhand), Bihar, West Bengal and Assam	42.00

(continued)

Table 3.4 (continued)

Sl. no.	Name of variety	Year of release		Duration	Avg. yield kg/ha	Recommended states	Oil Content (%)
		Centre	State				
(1997–2002) IXth Plan							
31	LC 2023	–	1998	158–163	1294 (I)	Punjab	38.8
32	Padmini	1999	–	120–125	943 (R)	Madhya Pradesh, Rajasthan, Bundelkhand of U.P., Maharashtra, Karnataka and Orissa	43.00
33	JLS-9	–	1999	115–125	1250 (I), 1000 (R)	Madhya Pradesh	42.00
34	Rashmi	1999	–	135–140	1003(S), 719 (F)	U.P., Bihar, West Bengal and Rajasthan (Kota)	41.00
35	Meera	2000	–	135–140	1439(S), 1011 (F)	U.P., Bihar, West Bengal and Rajasthan (Kota)	42.00
36	RL-914	–	2000	130–134	1617 (I)	Kota Command Area of Rajasthan	41.10
37	Parvati	2001	–	140–146	1600(S), 1020 (F)	U.P. Bihar, West Bengal and Rajasthan (Kota)	42.00
38	Sheela	2001	–	155–160	1379 (R)	Himachal Pradesh, Punjab, Haryana and J&K	41.00
39	Shekhar	2001	–	135–140	1555(I), 920 (R)	U.P. (Excl. Bundelkhand), Bihar, West Bengal and Assam	43.00
40	NL-97	–	2001	115–120	641 (R)	Vidarbha Region	42.00
(2002–2007) Xth plan							
41	Suyog (SLS-27)	2004	–	118–125	1509 (I)	Rajasthan, BKD region of U.P., M.P., Maharashtra, CG, Orissa, A.P. and Karnataka.	41.43
42	Binwa (KL-210)	2004	–	179–186	858 (I)	Haryana, Punjab, Himachal Pradesh and J&K.	40.00
43	Baner (KL-224)	2005	–	171–203	511 (U)	Haryana, Punjab, Himachal Pradesh and J&K.	39.70
44	Indira Alsi-32 (RLC-81)	2005	–	110–115	780 (R)	CG, Maharashtra, Karnataka and Orissa	39.18
45	Kartika (RLC-76)	–	2005	103–108	1078 (R)	Rainfed areas of Chhattisgarh	42.93
46	Deepika (RLC-78)	–	2006	110–115	1272 (SI & U)	Partially irrigated as well as *Utera* situation of CG	41.39
47	Sharda (LMS-4-27)	2006	–	100–105	762 (R)	CG, Maharashtra, Karnataka, Andhra Pradesh and Orissa	41.32
(2007–2012) XIth plan							
48	Pratap Alsi-1 (RLU-6)	–	2007	129–135	1997 (S), 834 (F)	Rajasthan Kota Command Area	41.08
49	LC-2063	–	2007	115–125	1200 (I)	Irrigated Area of Punjab state	37.40
50	Himani KL-214	2008	–	177–200	583 (U)	HP, PB, Haryana and J & K	36.40
51	Azad Alsi-1 (LMS 9-2 K)	2008	–	125–130	1610 (I)	BKD area of UP, MP and Rajasthan	39.92
52	RLC-92	2008	–	111	1196 (I)	CG, MH, Orissa and Karnataka	37.70
53	PKVNL-260	–	2009	102–106	963 (R)	Maharashtra	37.67
54	Shival (SLS-67)	2010	–	108-110	1252 (R)	BKD area of UP, MP and Rajasthan	40.16
55	Jawahar Linseed-41 (PKDL-41)	–	2010	115–120	1600 (I)	Area of MP state with limited irrigated facility	40.00
56	Jawahar Linseed-66 (SLS-66)	–	2010	109–120	1200 (R)	Rainfed areas of MP	42.80
57	Bhagsu (KL-215)	2010	–	175–201	428 (*U*)	Himachal Pradesh, J & K, Uttaranchal, Punjab and Haryana	36.38

(continued)

Table 3.4 (continued)

Sl. no.	Name of variety	Year of release		Duration	Avg. yield kg/ha	Recommended states	Oil Content (%)
		Centre	State				
58	Ruchi (LCK-5021)	2011	–	130–135	1366 (S), 1055 (F)	UP (Except BKD), Bihar, JKD, WB, Assam and NEH Region	39.84
59	NDL 2004-05	–	2011	125–130	1800 (I)	Uttar Pradesh State	42.00
60	NDL 2002	–	2011	130–135	1800 (I & R)	Uttar Pradesh State	43.00
61	SLS-73	2011	–	111–114	1090 (R)	BKD area of UP, MP and Rajasthan	38.82
62	Mau Azad Alsi-2	2011		105–110	815 (R)	CG, MH, Odisha and Karnataka	40.10
63	Pratap Alsi-2 (RL 26016)		2012	129–135	1957 (I)	Rajasthan	41.81
64	Arpita (OL 98-13-1)		2014	102–106	849 (R)	Odisha	35.67
65	Kota Barani Alsi-3 (RL-292002)		2015	119–124	1370 (R)	Rajasthan	38.73
66	Kota Barani Alsi-4(RL-10193)	2015		120–126	1100 (R)	UP, MP, Rajasthan	40.37

Table 3.5 State-wise farmers preferred/ruling varieties under cultivation

States	Name of varieties
Less than 10-year-old varieties	
Madhya Pradesh	Indira Alsi-32, Kartika, Suyog, Azad Alsi-1, JL-41
Chhattisgarh	RLC 92, Deepika, Kartika, Indira Alsi-32, Sharda, Mau Azad Alsi-2
Uttar Pradesh	Sharda, Azad Alsi-1, Ruchi, Shival, SLS-73
Rajasthan	Pratap Alsi-1, Azad Alsi-1, Shival, SLS-73, Kota Barani-2, Kota Barani-3, Kota Barani-4
Maharashtra	PKVNL-260, Sharada, RLC-92, Mau Azad Alsi-2
Karnataka	Sharada, RLC-92, Mau Azad Alsi-2
Odisha	Sharada, RLC-92, Mau Azad Alsi-2, Arpita
Bihar	Ruchi, Azad Alsi-1
Jharkhand	Ruchi, Azad Alsi-1
Himachal Pradesh	Himani, Bhagsu
More than 10-year-old varieties	
Madhya Pradesh	JLS 9, Padmini, Parvati, Suyog, J-552, Kiran, J-23
Chhattisgarh	J 552, Padmini, Suyog, Kiran, J-23
Uttar Pradesh	Shekhar, Padmini, Parvati, Garima, Shikha, Rashmi, Meera, Sweta, Shubhra, T-397
Rajasthan	Meera RL-914, Chambal
Bihar	Shekhar, Parvati, Shikha Rashmi, Meera, Sweta, Shubhra, T-397
Jharkhand	Shekhar, T-397, Padmini, Sweta, Shubhra, Shikha, Rashmi, Parvati, Meera

rapid development of cultivars insulated with defensive traits.

Oilseeds are the second most important agricultural commodity after cereals with enormous potential for further nutritional and industrial exploitation. The government support for oilseed crops and import of edible as well as petroleum oils reiterates the fact that oilseed cultivation in India demands an additional revamp by developing new crop varieties. Super varieties can be genetically engineered as a downstream process of identifying and isolating the

Table 3.6 State-wise yield potential recorded under FLDs vis-à-vis national/state average yield and yield gap analysis

State	Yield potential under FLD (kg/ha)	State average yield (kg/ha)	Yield gap
Madhya Pradesh	1667	338	1329
Chhattisgarh	643	260	383
Uttar Pradesh	1570	453	1117
Bihar	970	889	81
Jharkhand	590	516	74
Maharashtra	1000	256	744
Rajasthan	1570	1235	335
Odisha	725	459	266
West Bengal	370	341	29
Karnataka	644	364	280
Assam	1272	521	751
Nagaland	1025	807	218
Himachal Pradesh	958	273	685

candidate genes. In recent years, there has been much focused attempt on generating transgenic seed oils with novel fatty acids possessing greater nutritional benefits. Genetic engineering of linseed enables modification of plant oils which are not feasible by mere traditional techniques. These modifications have resulted in oils that are healthier and have improved functional properties in order to adapt them to a specific food, feed or industrial utilization. Gamma linolenic acid (GLA) is an important essential fatty acid predominantly found in linseed with a superfluity of health benefits and implications in recurring many reproductive and dermatological complications. Transgenic expression in linseed of the ∂-6 pathway resulted in significant production of ∂6-desaturated C 18-PUFA (17 % GLA and 11 % DHA); however, conversion of these C18-PUFA to C 20-PUFA was limited [33]. There is an urgent need to establish networks and active collaboration of molecular biologists, breeders, pathologists and entomologists to employ marker-assisted selection in regular breeding programmes.

Embryo rescue technique Laibach (1929) demonstrated the practical utility of culturing immature embryos to overcome hybrid incompatibility by successfully getting a mature plant from the 15-day-old excised embryo of *L. perenne* × *L. austriacum* [34]. For an application of the embryo culture technique, the initial steps of an ordinary sexual hybridization have to be carried out first, **i.e.** emasculation and pollination. About 7–10 days after pollination, depending on the stage of development, the young embryo has to be dissected out of the ovarium and planted on a suitable medium, which allows the embryo to grow and form shoots and roots. Therewith, early degeneration of the immature can be avoided. The success in culturing hybrid embryos of flax initiated interest among researchers to obtain interspecific hybrids in several other plant specific where embryos aborted or whose seeds were unable to support normal development of the embryo to maturity.

Protoplast fusion technique Many related species are extremely recalcitrant to hybridization because of interspecific incompatibility of pollen and stigma. In these cases, respective interspecific hybrids could possibly be recovered by protoplast fusion techniques. This fusion product, the newly formed hybrid protoplast ("heterokaryon"), can be regenerated to a hybrid plant if plated on a suitable culture medium and maintained under appropriate growing conditions. For the *Linum* species, the requirements for fusion and regeneration have recently been established [35, 36]. Another possible application of the fusion technique is the production of new "cytoplasmic male-sterile" (cms.) lines.

Seed Scenario

1 State seed agencies of MP, CG, UP and Jharkhand states are the major indenting agencies (more than 50 %) of breeder seed.
2 Seed replacement rate of linseed crop is below 10 % at national level, and as such, this is the one of major constraint for the low productivity.
3 Varieties recommended for Maharashtra, Odisha, Karnataka and Assam states are inadequately available to the farmers of these states, because breeder seed indents of recommended varieties are not being placed by these states.

Good Crop Production Practices

Linseed crop is under cultivation in three ecosystems namely *utera*, rainfed and irrigated. The state/region-wise package of practices was standardized. The improved agro-techniques for linseed cultivation are given below:

(a) *Utera* system

Growing linseed in *utera* system is the predominant practice of regions such as Darbhanga and Madhubani area of Bihar, Jharkhand, Mayurbhanj area of Orissa, Vidarbha region of Maharashtra, Balaghat area of M.P., eastern part of Uttar Pradesh and Kangra district of Himachal Pradesh. The important research achievements of this system are given below:

- High-yielding varieties especially suited to *utera* system of linseed cultivation namely R552 for zones III and IV and Bhagsu, Himani, Baner and Surabhi for assured moisture situation of Kangra valley in Himachal Pradesh have been released for general cultivation since 1984.
- To harvest higher productivity, sufficient organic manure like FYM or green manure and phosphatic fertilizers should be applied to rice crop. A dose of 10–20 kg N/ha should be applied in standing paddy 2 or 3 days before sowing linseed.
- Crack system of sowing can be followed in areas where sufficient water is available. In this method, 5-cm-deep cracks are allowed to develop in the field, when rice crop is at the boot-stage, and the field is irrigated. When 3- to 6-cm-deep cracks are developed again, the field is irrigated and water is allowed to stay for 5–7 days and *Utera* is sown thereafter as usual. This method would give 60–100 % more yield and has no adverse effect on rice yields.
- *Cuscuta* is the problem weed of Chhattisgarh, Balaghat area of M.P. and Vidarbha region of Maharashtra. To ensure clean cultivation, *cuscuta* seeds should be separated and disordered from seed lot before sowing linseed. *Rabi* season weeds could be managed by post-emergence application of weedicides at 1.00 kg/ha at 30–35 DAS. However, 2,4-D (Na) at 0.5 kg/ha may also mixed in the tank with isoproturon if broad leaf weeds are also problem.
  - Seeding linseed at 40 kg/ha with the help of desi-plough along with *Navagaon nari* attachment may be an alternative of *utera* system of cultivation in Chhattisgarh.
  - One irrigation at 55 days after germination and 100 % RDN + seed inoculation with Azotobacter + PSB in loam soil of Jharkhand and in clay soil of north Bihar, while 100 % RDN + spray of 2 % urea at pre-flowering and two irrigations at 25-30 days after paddy harvest and 25-30 days after first irrigation in sandy loam soil of eastern UP proved economically viable for *ute ra* cultivation.

(b) Rainfed ecosystem

The linseed is predominantly grown under rainfed situation. The research achievements for rainfed ecosystem are given below:

- 30 kg/ha seed rate with a row spacing of 25 cm was found to be optimum.
- Second fortnight of October for Bihar, U.P, excluding Bundelkhand and Akola areas of Maharashtra, 3rd week of October for Bundelkhand of U.P., Chhattisgarh as well as Maharashtra and mid-November for West Bengal were observed optimum sowing times.
  - Application of fertilizer at 40 kg N + 20 kg P_2O_5 and 20 kg/ha K_2O has been found quite beneficial in increasing yield of this crop in rainfed ecosystem at various locations.
  - Application of sulphur at 15 kg/ha to linseed has been observed as critical nutrients for enhancing linseed yield in many areas.

(c) Irrigated ecosystem

(i) Seed type linseed

Application of fertilizer at 60 kg N + 20–40 kg P_2O_5 and 20–30 kg K_2O per hectare has been found very effective in increasing the seed yield.

- Application of sulphur at 20–30 kg/ha to linseed and zinc through $ZnSO_4$ at 25 kg/ha either of the crop in rotation proved critical inputs to enhance seed yield of linseed.
- Post-emergence application of isoproturon at 1.00 kg/ha and 2,4-D at 0.5 kg/ha could manage weeds effectively.
- Seed inoculation with Azotobacter was found beneficial at different locations.
- Application of FYM at 5 t/ha in *kharif* crop was found quite effective to increase linseed yield at various locations.
- Integrated nutrient management module 75 % RDF + 5 t FYM + 5 kg zinc through $ZnSO_4$ + 25 kg/ha sulphur either with or without biofertilizer (azotobacter + PSB) was found quite effective in increasing linseed yield and soil health at various locations.

(ii) **Double purpose linseed**

Double purpose linseed is such type of linseed that gives seed and fibre both. It can be grown successfully in Kangra district of Himachal Pradesh, Indo-Gangetic alluvial tract and irrigated areas of Bundelkhand in Uttar Pradesh, Tawa command area of M.P. and Kota command area of Rajasthan. The recent advances for the cultivation of such type of linseed are given below:

- A seed rate of 45 kg/ha for Indo-Gangetic alluvium of U. P. and Tawa command area of M.P., 56–57 kg/ha for irrigated areas of Bundelkhand of UP and 62–63 kg/ha for Himachal Pradesh were found optimum.
- Double purpose of linseed could be sown successfully during October to first fortnight of November depending upon the soil moisture and irrigation facilities.
- Fertilizer application at 80 kg N + 30 kg P_2O_5 + 30 kg K_2O has been found quite effective in increasing seed and fibre yield both at various locations of country.
- A seed rate of 75 kg/ha of double purpose linseed and 60 kg N/ha could give higher seed and fibre yield along with better quality fibre in Himachal Pradesh. Finest fibre with flax (Vr Ariane) could be obtained when harvested at 50 % flowering stage but without seed.

Agronomical Practices for linseed in different crop growing situations is depicted in Table 3.7.

Latest Crop (linseed) harvesting calendar is depicted in Table 3.8.

Cropping Systems

(a) **Crop rotation**

- Soybean–linseed at Sagar (MP) and Raipur (Chhattisgarh) and black gram—linseed followed by paddy–linseed at Kanke (Jharkhand) were observed remunerative cropping systems under rainfed situation.
- Under irrigated situation, maize–linseed at Palampur (HP) and paddy–linseed at Kanpur (UP) were adjudged most profitable crop sequences.

(b) **Intercropping systems**

- Linseed crop may be intercropped with cereals, pulses and oilseeds of *rabi* season very well. When linseed is intercropped with chickpea, the incidence of wilt and pod borer in chickpea is reduced. Some of the efficient intercropping systems for various states are given in Table 3.9:
- Linseed variety Kiran at Raipur, Kiran and J-23 at Powarkheda, Janki at Palampur and Garima at Kanpur were observed best varieties for linseed + mustard (5:1) intercropping system.
- Fertilizer application under rainfed situation in linseed + safflower (4:2) intercropping system at 100 % RDF to main crop and 50 % RDF to sub-crop on area basis was observed remunerative at Sagar.
  - Fertilizer application under irrigated situation recorded highest net monetary returns in linseed + wheat (4:2) intercrop system at 100 % RDF to both the crops on area basis at Palampur and Faizabad and in linseed + mustard (5:1) intercrop system at 100 % RDF to main crop and 50 % RDF to sub-crop at Palampur. Intercropping of linseed with dwarf field pea was found remunerative over sole cropping in the row ratio of 4:1 and 2:3 in central and eastern part of UP and 4:4 row ratios in MP.

Efficient soil and moisture management

The role of better agronomic practices in exploiting the yield potential of available crop varieties has been widely recognized. Soil management for raising a crop is dependent upon the physico-chemical condition of soil, previously grown crop, weed infestation, situation of cropping, soil moisture, soil air, soil temperature, soil mineral matter, soil organic matter, soil organisms and soil reactions which are the factors responsible for the growth and development of the crop. Adoption of moisture conservation practices, recycling of crop residue, addition of organic matter like FYM and compost, green manuring and use of proper equipments for tillage are key factors for the efficient soil management.

Since linseed crop utilizes minimum moisture among major *rabi* crops [37], moisture content of soil is the only factor, which can force the crop not to complete its life cycle. For efficient management of soil moisture, it is essential to conserve the moisture in rainy season by ploughing and planking after each rain, ridge making, terracing, cover cropping, etc. Besides, management of moisture in standing crop is also essential. Mulching of soil or straw is of great help in moisture conservation. Straw mulching was found more useful than soil mulching. Application of straw and soil mulches in linseed produced 651 and 560 kg/ha seeds compared with 476 kg/ha without mulching [38, 39].

Integrated Nutrient Management

Linseed crop responds well to fertilizer. The recommended doses of fertilizer for rainfed condition are 40–60 kg N, 20–30 kg P, 20 kg K and 20 kg S (through gypsum or pyrite) per hectare as a basal dose, whereas for Utera condition,

Table 3.7 Agronomical practices for linseed in different crop growing situations

State	Situation	Recommended varieties	Optimum time sowing	Spacing (cm)	Seed rate (kg/ha)	Fertilizers N:P (kg/ha)
Assam	Rainfed	Shekhar, Sweta, Shubhra, T397	Ist fortnight of October	25	30	40:20
	Irrigated	Shekhar, Garima, Shubhra	IInd fortnight of October	25	25–30	60–80:30
	DP	Rashmi, Meera, Shikha, Gaurav, Parvati, Ruchi	Ist week of November	20	45	80:30
Bihar	Rainfed	Shekhar, Sweta, Shubhra, T397	Mid-October	25	30	40:20
	Irrigated	Shekhar, Garima, Shubhra	IInd fortnight of October	25	25–30	60–80:30
	DP	Rashmi, Meera, Shikha, Gaurav, Parvati, Ruchi	IInd fortnight of October	20	45	80:30
Chhattisgarh	Rainfed	Indira Alsi-32, Sharda, Deepika, Padmini, Mau Azad Alsi2	Ist fortnight of October	25	25-30	30:15
	Utera	R552, Kartika, Padmini, Kiran, T397	Ist–3rd week of Oct.	Broad cast	35–40	10–20:00
	Irrigated	Suyog, JLS-9, J23 and T397, RLC92	Mid-October	25	25–30	60–80:30
Haryana	Irrigated	Binwa, Himalini, LC-54	IInd fortnight of October–Ist week of November	25	20–25	60–80:30
	DP	Nagarkot, Jeevan	IInd fortnight of October–Ist week of November	20	45	80:30
H.P. and J&K	Rainfed	Surabhi, Janki, Sheela	IInd fortnight of October	25	25–30	40:20
	Utera	Baner, Surabhi, Himalini, Bhagsu	October	Broad cast	50–60	20:00
	Irrigated	Binwa, Janki, Himalini, LC-54	IInd week of October–Ist fortnight of November	25	25–30	60–80:30
	DP	Nagarkot, Jeevan	Mid-October	20	65–75	90:30
Jharkhand	Rainfed	Shekhar, Sweta, Shubhra, T397	Mid-October	25	30	40:20
	Irrigated	Shekhar, Garima, Shubhra, T397	Ist fortnight of October	25	25–30	60–80:30
	DP	Rashmi, Meera, Shikha, Gaurav Parvati, Ruchi	Ist fortnight of October	20	45	80:30
Karnataka	Rainfed	Indira Alsi-2, Padmini, Kiran, Sharda, Mau Azad Alsi2	Up to Ist week of October	25	25–30	40:20
	Irrigated	Suyog, J-23, RLC 92	Up to Mid-October	25	25–30	60–80:30
MP	Rainfed	Padmini, Kiran, T-397, JLS9, SLS67, SLS73, Kota Barani Alsi-4	Ist fortnight of October	25	25–30	30:15
	Utera	R-552, Sweta	Ist–3rd week of October	Broad cast	35–40	10–20:00
	Irrigated	Suyog, J-23, T-397, Azad Alsi 1, JL 41	Mid-October	25	25–30	60–80:30
Uttar Pradesh	Rainfed	Shekhar, Sweta, Shubhra, T397, SLS67, SLS73, Padmini, Kota Barani Alsi-4	Mid-October	25	30	40:20
	Irrigated	Shekhar, Garima, Shubhra, T397, Azad Alsi 1, Suyog	Ist fortnight of October	25	25-30	60–80:30
	DP	Rashmi, Meera, Shikha, Gaurav Parvati, Ruchi	Ist fortnight of October	20	45	80:30

Table 3.8 Latest crop (Linseed) harvesting calendar

State	Name of the crop	Season	Harvesting calendar			
			Starting		Ending	
			Week	Month	Week	Month
Assam	Linseed	*Rabi*	Ist week	March	IVth week	April
Andhra Pradesh			IVth week	February	IVth week	March
Bihar			Ist week	March	IVth week	April
M.P.			Ist week	March	IVth week	March
Chhattisgarh			Ist week	March	IVth week	March
Maharashtra			Ist week	March	IVth week	March
Uttar Pradesh			Ist week	March	IVth week	April
Orissa			Ist week	March	IVth week	March
Jharkhand			Ist week	March	IVth week	April
Karnataka			Ist week	February	IInd and IIIrd week	March
Nagaland			Ist week	March	IVth week	April
West Bengal			Ist week	March	IInd and IIIrd week	April
Rajasthan			Ist week	March	IVth week	March
Himachal Pradesh			IVth week	April	IInd and IIIrd week	May
J&K			IVth week	April	IInd and IIIrd week	May
Punjab			IVth week	March	IVth week	April

Table 3.9 Efficient intercropping systems for various states

State	Situation	Intercropping system
Uttar Pradesh (Excluding Bundelkhand)	Rainfed	Linseed + Chickpea/Lentil (4:2 or 2:4)
	Irrigated	Linseed + Wheat (4:2),
		Linseed + Mustard (5:1)
		Linseed + Potato (3:3)
Bundelkhand of U.P.	Rainfed	Linseed + Chickpea/Lentil (4:2 or 2:4)
		Linseed + Wheat (1:3)
Madhya Pradesh and Chhattisgarh	Rainfed	Linseed + Chickpea (4:2 or 2:4))
Bihar and Jharkhand	Rainfed	Linseed + Chickpea (4:2 or 2:4)
		Linseed + Mustard (5:1)
West Bengal	Irrigated	Linseed + Mustard (S: l)
		Linseed + Potato (3:3)
Maharashtra and Karnataka	Rainfed	Linseed + Chickpea (4:2 or 2:4))
		Linseed + Safflower
		(Different row ratio)
Punjab and H.P.	Rainfed	Linseed + Chickpea (4:2 or 2:4))
	Irrigated	Linseed + Mustard (5:1)
		Linseed + Wheat (4:2)

20 kg N/ha should be applied 2–3 days before broadcasting the seed.

According to Rawal and Yadav (1988) [40], 60 kg N + 40 kg P + 60 kg K is the optimum dose at Chittorgarh. Sandy loam soils of Ludhiana 60 kg N + 40 kg P + 30 kg S per hectare, Varanasi 40 kg N + 9 kg P [41] per hectare, Jabalpur 30 kg N + 15 kg P per hectare [42], Durgapura 20 kg N + 20 kg P + 20 kg K per hectare [43] and Rewa 45 N + 20 kg per/ha (Awasthi et al., 1989 [44]) were found to be optimum under rainfed condition. Linseed crop from Raipur (M.P.) yielded the highest with 40 kg P/ha (0.60 t/ha) and 40 kg K/ha (0.58 t/ha) [45]. Application of FYM at 5 t/ha along with inorganic fertilizer yielded more than no FYM application. Application of FYM at t/ha + 75 % recommended dose of NPK yielded equal to that of recommended dose of inorganic fertilizer [46].

It is, therefore, concluded that 40 to 60 kg N + 20-30 kg P + 20 kg K and 30 kg S per hectare in deficient soils along with 5/ha FYM can produce a bumper crop of linseed.

Weed management

This crop is usually dwarf statured and therefore suffers severe competition by weeds. Initial 3–6 weeks after sowing is critical period of crop–weed competition. The uncontrolled weeds can reduce yields by 25–40 %. The losses are more in rainfed and *utera* cropping systems primarily due to competition for moisture followed by nutrients.

The important weeds of linseed include *Anagallis arvensis, Vicia hirsuta, Fumaria parviflora, Melilotus* spp., *Chenopodium album*, *Phalaris minor,* etc. The crop is parasitized by *Cuscuta* sp. leading to heavy losses of yield. Post-emergence (2–3 weeks after sowing) application or pronomide at 1.5 kg/ha and crop rotation with cereals have been recommended for its effective management. Weeds can also be controlled by 2 weedings after 3 and 6 weeks of sowing.

Application of clodinafop at 60 g/ha in sandy loam soil of central UP, isoproturon at 1.0 kg/ha in clay loam soil of central Bihar, pendimethalin 30 EC + imazethapyr 2 EC at 0.75 kg/ha in vertisols soil of Bundelkhand region of UP; pendimethalin 30 EC at 1 kg/ha and imazethapyr 10 EC at 75 g/ha in vertisol soil of MP and clodinafop at 60 g/ha and imazethapyr 10 EC at 100 g/ha in sandy loam soil of eastern UP proved economically viable alternative of hand weeding twice under irrigated condition.

Harvesting, drying and threshing

The crop should be harvested crop should be left in the field for a few days for drying. Threshing is performed by beating the plants with the help of sticks or by treading of cattle. Winnowing to remove the seed from the straw is carried out with the help of a winnower or in the natural wind.

Post-harvest technology

The post-harvest technologies include storage, processing and marketing of the produce. The seeds and oil of linseed by virtue of their chemical composition is liable to deteriorate during storage. The loss in storage can be minimized by providing proper storage conditions [47]. The most important factor, which affects the stability of linseed in storage, is initial moisture and fatty acid content of linseed besides the storage conditions, viz. humidity, temperature design and construction of godown and the method of storage. The upper safe limit of 70 % relative humidity with 8 % moisture content is the best condition. There is no serious insect pest, which attack linseed in storage except those seeds which got adhered to the web produced by the larvae of rice moth (*Corcyra cephalonica* Station) and almond moth (*Cadra cautella Walk.*) For its control, suitable fumigants may be used.

The oil may be better protected in sealed containers than open ones [48]. In India, linseed oil is extracted successfully with the help of power-driven mills, but cold press extraction is suggested for better stability of oil.

Integrated pest management

More than 36 species of insect–pests are associated with linseed/flax crop throughout the world, but only small number of the major cosmopolitan insect–pests attacks this crop. Bud fly (*Dasyneura lini* Barnes) is the only national key pest of regular occurrence, which is responsible for the vertical reduction in linseed productivity, whereas other pests are sporadic and location specific. It is a serious pest of linseed in Asia particularly India, Bangladesh and Pakistan [49, 50], while it appears as a pest of lesser economic importance in Europe [51]. In India, this pest may cause up to 90 % losses in seed yield and attacks the crop during flowering stage by infesting the flower buds. Incidence of bud fly in linseed was reported first time in India [49] from Pusa, Bihar. Yield losses in linseed due to bud fly have been estimated to the tune of 90 % in Maharashtra followed by UP (80 %), MP (75 %), Odisha (62 %), Bihar (60 %), Rajasthan (46 %), West Bengal (35 %) and Haryana (27 %).

Linseed bud fly

Bio-ecology

A single female lays 22–103 whitish, minute curved eggs singly or in small loose batches of 3–5 eggs underneath the buds with an average of 5–10 eggs/bud, which hatch in 2–5 days. Neonate grub is whitish, which turns to deep pinkish at full grown stage. The larval period is 5–14 days with four larval instars. Pupation takes place generally in soil about 5–7 cm below the soil surface, but occasionally in fallen leaves also, which lasts about 5–11 days. There are

four generations of the pest on linseed in a season, and the life cycle is completed in about 1427 days [52, 53]. The best multiplication of this pest occurs at 16–20 °C temperature and 60–70 % relative humidity. Temperature is negatively associated with the pest multiplication, whereas positively, with the relative humidity [54].

IPM schedule

Cultural control

Solarization by summer ploughing of the linseed fields is very useful as the larvae of bud fly aestivate in off-season inside the soil about 5–7 cm below the soil surface [53]. A non-host crop should be sown after one to two years of cultivation of linseed in a particular field.

Sowing time and intercropping

Planting of linseed in the first fortnight of October in Madhya Pradesh, Maharashtra and Karnataka; second fortnight of October in Himachal Pradesh, Punjab, Gangetic alluvium of U.P. and Bihar; and first fortnight of November in West Bengal and Orissa provided higher yield with moderate bud fly infestation [53, 54]. Intercropping of linseed with chickpea, lentil and safflower in different row ratios enhances the net monetary return with reduction in pest infestation [55].

Host–plant resistance cultivation of resistant/moderately resistant varieties like Neela, Kiran, Shubhra, Garima, Laxmi-27, Janki, Himalini, Surabhi, Mukta, Sweta, RLC 92, Padmini, Shekhar, Sharda, Pratap Alsi-1, Ruchi and SLS 73 is recommended for different agro-climatic zones of the country, which may be utilized as a principal component of pest management strategy.

The resistance in linseed to bud fly is positively associated with short flowering period [56] and small size of flower buds with thin sepals [57]. The higher HCN, nitrogen and phosphorus contents in leaves at bud initiation stage favour the bud fly infestation, whereas the polyphenol content affects adversely [58].

Biological control

The biological suppression of linseed bud fly occurs through its larval parasites, viz. *Systesis dasyneurae* Mani, *Elasmus* sp., *Eurytoma* sp. and *Tetrastichus* sp. *Systesis dasyneurae* Mani is an effective larval parasite, which parasitized about 50 per cent larvae during early February [59]. The grub and adult stages of *Coccinella septempunctata* Linn. and Menochilus sexmaculatus Fabr. predate upon the fulfed maggots of bud fly outside the buds before pupation. Findings at Project Coordinating Unit (Linseed), Kanpur, have shown that the pond heron (*Ardeola grayii*) predate upon the larvae of cutworms (*Agrotis ipsilon* Hfn.) at the time of first irrigation, whereas the Black drongo or king crow (*Dicrurus adsimilis*) birds predate upon the larvae of capsule borer (*Helicoverpa armigera* Hubn.) during capsule formation stage of the crop. Bamboo pegs, used as perch (dead), increase their feeding propensity, as these predators sit upon these perches [46].

Chemical control

1. If necessary, apply methyl parathion 2 % dust at 25 kg/ha at sowing time to control termite and cutworm infestation. Spray 0.04 % monocrotophos 36 EC or 0.07 % endosulphan 35 EC or 0.03 % phosphamidon 85 SL for defoliators, leaf miner and sap sucking pests, if needed [60].
2. Two fortnightly applications of 0.03 % phosphamidon 85 SL or 0.002 % decamethrin 2.8 EC or 0.07 % endosulphan, 35 EC or 0.05 % chlorpyrifos 20 EC, starting from bud initiation stage can effectively manage the bud fly infestation [61, 62].
3. Combined application of indofil M-45 (0.2 %) with phosphamidon 85 SL (0.03 %) as first application followed by second spray of decamethrin 2.8 EC (0.02 %) effectively controls bud fly and Alternaria blight infestation, which is economical as well as eco-friendly [46].
4. Two fortnightly sprays of 2 % NSKS, starting from bud initiation stage, lowers the bud fly infestation considerably [63].
5. Linseed + gram (4:2) was best intercropping system with lowest bud fly infestation (11.5 %) and highest seed production (2057.5 kg/ha) in comparison with sole linseed.
6. Cultivation of improved variety of linseed at normal sowing time in irrigated and rainfed situation along with recommended agronomic practices and two alternate fortnightly spray of insecticides (imidacloprid 17.8 SL at 0.004 %) and botanical (nimbecidine 300 ppm at 0.5 % or vice versa) reduced 64–70 % bud fly infestation with 148–203 % higher seed yield in irrigated and 54–68 % lower bud fly infestation with 79–491 % more yield in rainfed situation.

Integrated disease management

Linseed crop suffers from several diseases (Table 3.10), but the most damaging are wilt, *Alternaria* blight, powdery mildew and rust, causing losses up to 87, 60, 60 and 100 %, respectively, in susceptible varieties in epidemic years [64]. Rust is prevalent in Jammu and Kashmir, Himachal Pradesh, Punjab, Haryana and hills of Uttar Pradesh. Alternaria blight is also severe in northern parts of the country excluding cold and low humidity areas. Wilt and powdery mildew occur throughout the country, but are more severe in central and peninsular India. Wilt is especially a problem in rainfed cropping system. *Cuscuta*, a phanerogamic plant parasite, is

Table 3.10 Diseases of linseed in India

Sr. no.	Disease	Causal organism
1	Rust	*Melampsora lini*
2	Blight	*Alternaria lini*, *A. alternate*, *A. linocola*
3	Wilt	*Fusarium oxysporum*, *F. lini Rhizoctonia bataticola*
4	Powdery mildew	*Oidium lini*
5	Root rot, stem rot and blight	*Macrophomina phaseolina*, *Rhizoctonia solani*, *Sclerotium rolfsii*
6	Leaf spot/Pasmo	*Collectorichum lini*, *Septoria linicola* (*Mycospaerella linorum*).
7	Seed root	*Asperigillus* sp., *Alternaria* sp., *Ascohyta Colletotrichum lini*, *Fusarium* sp., *Macophomina* sp., *Polypora lini*.
7	Phanerogamic parasite	*Cuscuta hylina*

severe in Chhattisgarh and adjoining areas of Vidarbha region of Maharashtra and Odisha states.

The important diseases and their management are as under

Wilt

Linseed wilt is caused by *Fusarium oxysporum* Scht. F. sp. lini (Bolley) Snyder and Hansen. Continuous cropping in the infected fields creates soil sickness. The crop losses up to 87 % due to the pathogen were reported by Sattar and Hafiz (1952) [65]. In India, it was first reported from central province [66, 67], Maharashtra [68] and Rajasthan [69]. The disease is now reported from all linseed growing areas of the country.

Disease development

The pathogen is soilborne and can survive by the infected root pieces in soil for several year. It has also been isolated from the seeds. The temperature range of 25–28 °C is most suitable with an optimum of 24 °C. In India, Goel and Swarup (1964) [70] have reported that the disease is favoured by low moisture and light sandy soils. The pathogen has several races and biotypes. In soil, it is known to persist for several years even in absence of host due to strong competitive saprophytic ability [71]. In seeds, the mycelium may persist on or inside seed coat. It may penetrate the soil up to 12 inches, but more abundantly found at a depth not exceeding 5 inches. The chlamydospores of the fungus present in soil/debris germinate in response to root exudates, and the germ tubes penetrate plants through root hairs. Cortical parenchyma is the first tissue to be invaded by the pathogen. In roots of resistant varieties, the entrance of the pathogen across vascular system is prevented by suberization of the cell wall and formation of cork layers in the cortex. Nair (1958) [72] reported that invasion of resistant host is restricted to the cortex. It can also enter into the young seedlings through epidermal cells or stomata. In seeds, it enters through mocrophyle or wounds, subsequently invading cotyledons and radicals.

Table 3.10 represents diseases of linseed in India.

Management

1. Continuous cropping of linseed on the same field should be avoided. Flooding of field and crop rotation reduces wilt inoculums in soil [73, 74].
2. Grow resistant/tolerant cultivars like K-2, LC-54, LC-185, Himalini, R-552, Kiran, Meera, Padmini, R-552, Rashmi, Sheela, Surabhi and T-397
3. To eradicate seedborne inoculums, seed treatment with bavistin at 2 g/kg seed or topsin-M or thiram at 3 g/kg seed is recommended [46].
4. Seed treatment with antagonists like Agrobacterium and Pseudomonas florescence [75] has been found effective.
5. Seed treatment with T. *harzianum* was found most effective in reducing wilt percentage followed by Th + thiram and Th + tv + thiram at Kanpur, while minimum incidence of wilt was recorded in fym-amended plots followed by tv + th, th, tv + thiram at Raipur centre.

Alternaria blight

The disease is caused by *Alternaria lini* Dey, *Alternaria alternate* (Fr.) Kigesten and *Alternaria linicola*. The disease was reported for the first time from India from Kanpur and then from Gorakhpur, Uttar Pradesh [76]. Later, it was reported from IARI, Delhi [77], Punjab [78] and Jabalpur [42]. Now it is reported from all linseed growing areas. It is a serious disease in areas where high humidity persists especially in Indo-Gangetic tracts.

The disease is serious in northern parts of the country favoured by high humidity coupled with temperature around 20–30 °C. The leaf infection causes damage from 27 to

60 %, while bud infection can cause losses up to 90 %. It is most harmful when both buds and capsules are affected [79]. Yield loss can be estimated through regression equation, Y = 733.35−8.24X [80]. The disease affects seed weight, fibre quality and oil percentage.

Disease Development

The pathogen perpetuates through seeds and diseased plant debris. The temperature range of 26 °C and humidity above 75 % are most favourable for disease development [80]. Singh et al. (2008) [81] reported that most favourable period for disease development was between end of January and February, and the max. and min. temperature ranged between 24.9–31.2 °C and 8.4–15.4 °C which was most favourable.

Management

1. Grow resistant/tolerant cultivars like LC-54, Himalini, Surabhi, Nagarkot. Rashmi, Sheela, Shubhra, Sweta and Surabhi
2. Sowing between second fortnight of October to first fortnight of November should be preferred [55].
3. Seed treatment with topsin-M (2.5 g/kg seed) or thiram at 3 g/kg seeds or iprodione at 2 g/kg [82] reduces disease intensity.
4. Need-based sprays (2–3) of Rovral (0.2 %) or Indofil M-45 (0.25 %) at 15-day interval are very effective in disease control [46].
5. Resistance-inducing chemicals, namely benzoic acid (0.1 %), naphthalene acetic acid (naa 0.1 %) and bion (acibenzolar-s-methyl 0.05 %), were found effective in reducing Alternaria blight infection.
6. IPM module including tolerant variety, sown during first week of November with optimum dose of fertilizers, seeds treated with thiram or thiophanate methyl (2 g/kg) sown during first week of November and one or two sprays of 0.25 % indofil m-45 (mancozeb) significantly reduced disease intensity over module including farmer's traditional practice.

Powdery mildew

Powdery mildew of linseed is caused by *Oidium lini* Skoric. The disease is prevalent throughout the country, but it is more severe in central and southern parts. Yield losses up to 60 % have been reported [64]. The crop suffers from heavy losses, when disease appears at the early stages of growth. Affected plants produce poor-quality seed and fibre. Powdery mildew of linseed is a disease of wide occurrence, but it is more serious in central and peninsular regions of the country. The first symptom of the disease is appearance of small white floury patches on the upper surface of leaves, which enlarge and cover the entire plant surface including stem, leaves and capsules. It results into increased respiration and decreased photosynthesis. The leaves covered with thick powdery masses show twisting and drooping symptoms, which ultimately dry up. Early-infected plants remain small in size and produce less number of capsules and small-sized seeds.

Disease development

The pathogen perennates in soil on diseased plant parts through the formation of perithecia, which initiate disease in the next season when favourable weather conditions prevail. The temperature range of 20–25 °C is favourable. The pathogen consists of several pathotypes.

Management

1. Use resistant cultivars like K-2, LC-54, LC-185, Himalini, Surabhi, Nagarkot, R-552, J-23, Padmini, Parvati, Ruchi, Meera, Rashmi and Kiran.
2. Early sowing is the best method for disease escape.
3. 2-3 sprays of bavistin (0.2 %) or karathane (0.2 %) or wettable sulphur (0.25 %) at 15-day interval were found very effective [46].
4. Foliar spray of sulphur at 45 and 60 DAS at of 0.4 % (Sulfex) and/or soil application of gypsum at 30 kg/ha + one spray of sulphur at 45 DAS at of 0.3 % (Sulfex) and another spray at the time of disease initiation for the control of powdery mildew enhanced yield, oil content and linolenic acid in north-eastern part of Karnataka.

Rust

Linseed rust caused by *Melampsora lini* (Ebrenb.) Lev. Is a serious disease in northern parts, causing losses between 70 and 100 % during epidemics. The infected plants are easily identified from the presence of bright yellow or orange coloured uredia on leaves, stems and capsules. Under favourable conditions, the entire plant is covered by yellow orange pustules and such plants are very conspicuous in the field. Severely infected plants die prematurely, and if leaves persist and the occurrence of the disease is late, the telia become apparent. In light infection, there is no loss in oil content, but in heavy infection, it is reduced by 10–34 % [83]. Singh et al. (1978) [84] found 13.1 % loss in oil content in heavily infected linseed variety artificially infected with *M. lini*. However, the oil quality and iodine number were not affected [85].

Disease development

Linseed rust is autoecious. Misra and Sethi (1962) [86] and Prasada (1948) [87] have reported the active role of teleutospores in initiating the disease. The pathogen survives in uredial form on self-sown plants and among seeds in the form of stem buits bearing tilia as contaminant in Simla and Kangra hills of Himachal Pradesh, which initiate the

outbreaks. From there, the uredospores are blown to plains by wind [87]. A temperature range of 13–250 °C is optimum for uredial development [87–89]. The disease is greatly influenced by duration of relative humidity of 90 % or above [90]. Physiological specialization in linseed rust has been established [88]. Later, rust differentials were identified by Flor [91–93]. His contributions on pathogenic variability are pioneering and outstanding.

Management

1. Destory diseased plant debris in hills to reduce source of primary inoculums [73, 74].
2. Grow resistant cultivars like K-2, LC-54, LC-185, R-552, Himalini, Surabhi, Nagarkot, Jeevan, Jawahar-23-10, Garima, Shubhra, Sweta, Meera, Padmini, Sheela and Rashmi.
3. Spray Mancozeb at 0.25 % or Cuman or benomyl or calixin at 0.05 % [93–95].

Cuscuta (Doddar)

Dodder is a major parasitic weed infesting linseed in Madhya Pradesh, Chhattisgarh and adjoining areas of Bihar, Jharkhand, Maharashtra and Odisha. *Cuscuta* spp. are annual parasitic plants, which have no roots or leaves and live entirely at the expense of their host plants. Reproduction occurs by seeds. Dodder seeds sprout at or near the soil surface. Germination can occur without a host, but it has to reach a green plant quickly. Dodder seedling will die if it does not reach to a host plant within 5–10 days of germination. The appearance of this parasite in severe form is due to use of contaminated seeds. The seeds are minute with hard coating which are produced in large quantities. Survival of the seeds in the soil remains for 5–10 years or more. Seeds are dispersed through trade of contaminated seed lots, forage and fodder. Cuscuta stressed crops are more often attacked by insect–pests and diseases.

Management

1. Remove the parasite vines from fields and use clean seeds free from parasitic seed.
2. Restrict the flow of irrigation water from infested areas to clean area.
3. Prevent movement of grazing animals from infested fields to healthy fields.

For seedling rots, root rots and stem rots, field sanitation, crop rotation, seed treatment with benomyl, thiram and organomercurials and treatment of soil with thiram or captan are the effective control measures. Meagre literature is available on host resistance. Anthracnose has also been managed through seed treatment or dusting with organomercurials or thiram.

Crop Products

Industrial Uses: About 80 % of oil goes to paints, varnishes, a wide range of coating oils, linoleum pad and printing inks, leather and soap industries and rust preventive.

Nutritional Value: Linseed is naturally highly nutritious. It is a source of complete protein (all 8 essential amino acids), linolenic acid (an essential polyunsaturated omega-3 fatty acid), carbohydrates, vitamins and minerals; all in one package.

Medicinal Value: Linseed is best herbal source of omega-3 and omega-6 fatty acids, cholesterol lowering cardiovascular benefits by affecting prostaglandins and leukotrienes related to blood clotting and inflammatory disorder such as rheumatoid arthritis. Seed is bowel clearing fibre and anti-cancerous related to colon, prostate and breast tumours. Gamma linolenic acid found in concentrated form in flax seed showed astounding effect in diabetes by normalizing the faulty fatty acid metabolism responsible for this disease. Linseed antibiotic Linatine found in seed. Linatine cure diseases for which no other medicine is effective. The Indian linseed oil has the good acceptability for medicinal purposes in international market; hence, many countries are exporting linseed oil of worth Rs.53.03 crores during 2006–07.>

Flax Fibre Uses: Flax is the oldest textile fibre. It is one of the most natural and eco-friendly of all the textile fibres. Flax fibre has more strength, fineness and durability than the cotton fibre. It is soft, lustrous and flexible and has high water absorbency and blends very well with wool, cotton and silk, etc., and for the purpose, fibre is being imported for the value of 350 crores every year.

Strong twines, canvas, hosepipe (water storage tanks), suiting-shirting and various indispensable products for aerospace and aeronautical and defence purposes are manufactured. Woody core of stem and short fibre used as raw pulp for making paper of quality. Rough and strong fibre is used for making low-cost roofing tiles based on convertible plastics made using unsaturated polyesters.

Animal Consumption: Oil cake is good feed for livestock, which makes them immune to certain diseases and improves their digestion.

Soil Health: Oil cake is used as manure to prevent soil from unwanted microbes due to its germicidal property.

Special Initiatives for Encouraging the Cultivation of the Crop

Government of India has so far not supported the linseed growers in terms of minimum support price, which is urgently required to make this crop remunerative.

Global output of linseed is estimated around 2.60 million ton per years with Canada, China, U.S. and India dominating the list of producers. Canada is the leading producer and accounts for nearly 80 % of the global trade in linseed. Global production of linseed oil is estimated between 600,000 and 7000,000 ton, while linseed meal ranges between 1.1 and 1.4 million tons.

Oil markets in Indore, Kanpur, Agra and Gwalior are the main trading centres of linseed oil. Paint and allied industries are the main consumers of linseed oil accounting for nearly 70 % of the total consumption. West Bengal, Maharashtra, Delhi and Uttar Pradesh are the main centres of linseed oil consumption in the country.

Important Websites

Given are name of important national and international organizations involved for crop improvement

National organizations

1. AICRP Linseed Centre, CSK HPKV, Palampur, Himachal Pradesh
2. AICRP Linseed Centre, AU, Kota, Rajasthan
3. AICRP Linseed Centre, CSAUA&T, Kanpur, Uttar Pradesh
4. AICRP Linseed Centre, CSAUA&T, Mauranipur, Uttar Pradesh
5. AICRP Linseed Centre, NDUA&T, Faizabad, Uttar Pradesh
6. AICRP Linseed Centre, BAU, Sabour, Bihar
7. AICRP Linseed Centre, BAU, Ranchi, Jharkhand
8. AICRP Linseed Centre, JNKVV, Sagar, Madhya Pradesh
9. AICRP Linseed Centre, IGKV, Raipur, Chhattisgarh
10. AICRP Linseed Centre, OUA&T, Keonjhar, Odisha
11. AICRP Linseed Centre, PDKV, Nagpur, Maharashtra
12. AICRP Linseed Centre, UAS, Raichur, Karnataka
13. AICRP Linseed Centre, AAU, Shillongani, Assam
14. AICRP Linseed Centre, CAU, Umiam, Meghalaya
15. AICRP Linseed Value Addition Centre, BVDU, Pune, Maharashtra

International organizations

1. Institute of Nature Fibres, Poland.
2. Crop Development Centre, University of Saskatchewan, Canada.
3. Commonwealth Scientific and Industrial Research Organization, Canberra, Australia.
4. International Plant Genetic Resources Institute, Rome, Italy.
5. North Dakota State University, USA.
6. Thomas Jefferson Agricultural Institute, Columbia, USA.
7. All-Russian Flax Institute, Torzhok, Russia.
8. N.I. Vavilov Research Institute for Plant Industry, St. Petersburg, Russia.
9. Research Institute for Technical cultures, China.
10. Breeding Company DSV, Lippstadt, Germany.
11. North Central Plant Introduction Centre, Ames, Iowa, USA.
12. Plant Gene Resources of Canada, Saskatoon, Canada.
13. Research Institute for Cereal and Industrial Crops, Romania.

Name and website of advisory services to farmers

1. Saskatchewan flax development commissions, Canada
2. Agriculture and Agri-food Canada
3. Manitoba flax growers association, Canada
4. Ameri flax
5. Flax Council of Canada
6. Genome Prairie/Canadian flax genomic network, Canada
7. www.flaxcouncil.ca, www.saskflax.com, www.goldenflax.com, www.healthyflax.com

Researchable Issues

a. Genetic enhancement for seed yield, early duration, quality traits and resistance against biotic and abiotic stresses.
b. Heat tolerance and development of short-duration high-yielding varieties of linseed for central and peninsular regions.
c. The use of biotechnological approaches in crop improvement with activities like developing mapping population, tagging and pyramiding of useful genes and marker-assisted selection in breeding of varieties with higher yield and insulation against major stresses.

d. For developing resistant varieties, characterization of resistance sources using races/isolates of the pathogen/pests needs to be done.
e. Development of location specific integrated crop management practices for high input use efficiency and reducing the cost of cultivation in different cropping system.
f. Development of suitable eco-friendly IPM technologies with enhanced input use. Efficiency and profitability.
g. Development of varieties and technologies for *utera* cultivation.
h. Value addition and product diversification for medicinal, industrial and textile purposes.
i. Climate change concomitant to pest and disease scenario.

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Omega-3 Milk

4

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List of abbreviations

ALA	Alpha-linolenic acid
n-3-FA	Omega-3 fatty acid
n-3 LC-PUFA	Omega-3 long-chain polyunsaturated fatty acid
LA	Linoleic acid
EFAs	Essential fatty acids
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
AA	Arachidonic acid

Introduction and Background

Human milk is first food consumed immediately after birth and also during the complete period of lactation. Naturally, it is a complete human food, provided naturally. However, there is a fundamental difference between the cow and buffalo milk that man consumes throughout his life and the human milk that infants consume.

It is proven that breast milk is ideal source of calories, essential fatty acids, vitamins, and other important nutrients for preterm and term infants [1]. Omega-3 fatty acid plays an important role in infants to grow after birth; omega-3 fatty acids are obtained from breast milk or infant formula. In particular, preterm infants require higher amount of essential fatty acids as compared to that of full-term infants [2]. Alpha-linolenic acid (ALA) is an important constituent in human breast milk. The concentration of ALA is 1.2–1.9 % (% weight of total fatty acids) in breast milk samples taken from women in Canada (1.2 %), Brazil (1.4 %), and Nepal (1.9 %). The ALA content in human breast milk is 3–10 times more than docosahexaenoic acid (DHA); however, it depends on the mother's diet [3].

Man is perhaps the only animal consuming milk throughout his life. This adoption is more by compulsion as milk is highly nutritious and provides the high-quality animal protein at an affordable price. However, as it can be appreciated by everyone, cow's milk is specifically intended for calves that need to grow strong bones with lots of calcium but cows do not need to grow large brains. Naturally, cow's milk is low in essential fatty acids. So relying on cow's milk, without omega-3 fatty acid fortification for children's nutrition and even for general human consumption, is not a good idea. So it is strongly suggested that fortification of cow's or buffalo's milk with omega-3 fat for better human nutrition is absolutely essential. Traditionally, cattle grazed in the field get some omega-3 fatty acid from the green pastures. However, now, they are fed with defined diet solely for getting high milk yield. Inevitably, like all other human food, milk has also become deficient in omega-3 fatty acid.

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There are two essential fatty acids (EFAs) in human nutrition: ALA (ALA; 18:3n-3), an omega-3 fatty acid, and linoleic acid (LA; 18:2n-6), an omega-6 fatty acid. Humans must obtain EFAs from foods because the human body cannot synthesize it. Unsaturated nature of these fatty acids help membrane functions by maintaining fluidity. ALA and LA are precursors of long chain fatty acid i.e. eicosapentaenoic acid and docosahexanoic acid which in turn get converted to eicosanoids and lipid mediators and control locally (paracrines) and within the cells (autocrines), many biological functions such as cell signaling, inflammatory actions and ion transport. They also regulate gene expression [2]. The principal biological role of ALA appears to be as a precursor for the synthesis of longer chain n-3 polyunsaturated fatty acids (PUFAs). ALA is converted to eicosapentaenoic acid (EPA; 20:5 n-3) and docosohexaenoic acid (DHA; 22:6 n-3), which are considered omega-3 long-chain PUFA (LC-n-3 PUFA) [4]. LC-n-3-PUFA play important role in modulation of many physiological processes [5]. Also blood levels of LC-n-3 PUFA are considered biomarkers of health status. However, improvement of blood LC-n-3-PUFA, cannot be immediately seen with supplementation of ALA [6]. Omega-3 fatty acid imparts positive effect on diseases such as hypertension, arthritis, atherosclerosis, depression, diabetes mellitus, myocardial infarction, thrombosis, and heart disease [7].

Large proportion of ALA undergo beta-oxidation and only small part is converted to LC-n-3-PUFA and therefore it is inferred that ALA supplementation does not show up LC-n-3-PUFA (EPA and DHA) in plasma in good amount [8]. Further, it has been reported that conversion of ALA to EPA is limited in men as compared to women and further transformation to DHA is very low [9–11]. Recently, Dyerberg et al. reported bioavailability of marine omega-3 fatty acid formulations, which indicated significant effect on the bioavailability [12]. Also recently, Schuchardt and Hahn reviewed bioavailability of long-chain omega-3 fatty acids and concluded need to systematically investigate the bioavailability of omega-3 fatty acids formulations. Various attempts have been made to fortify food products with omega-3, which includes juice, breakfast cereals, bread, butter, yoghurt drink, milk and eggs Fortification of food with n-3-FA may offer an effective way of increasing omega-3 long-chain polyunsaturated fatty acid intakes [13].

Recent data indicate that blend of dairy lipids and omega-3-fatty acid from vegetarian oil, can potentiate higher levels of n-3 LC-PUFA levels endogenously [14, 15]. Since dairy lipid is not recommended in patient of high risk, it is hypothesized that addition of omega-3-fatty acid along with dairy lipid may offer advantage by neutralizing the adverse effects caused due to dairy lipids.

The important component of human brain's structural lipids is the long-chain PUFA DHA (C22:6w3, DHA) comprised of 36 % of the solids of gray matter. These fatty acids plays a key role in nerve and eye function [16].

It has been observed that formula-fed infants showed lower cerebral and erythrocyte LC-PUFA levels compared to that of breast fed infants when consuming an unsupplemented formula or a formula supplemented only with EFA [17, 18]. Infants fed with formula supplemented with DHA and AA showed higher erythrocyte membrane omega-3 concentrations than that of unsupplemented formula [19]. It has been shown that formula containing vegetarian oil along with dairy lipids achieve long chain PUFA status in healthy full term infants similar to breast fed infants [15].

In comparative study of formula milk and human milk for elevation in the level of DHA in the plasma phospholipids, it was observed that human milk was found to be significantly more effective, although the omega-3-fatty acid level in human milk very less as compared to that of formula milk [20].

Although milk from vegan mothers had over double the LA and ALA than that of non-vegetarian mothers' milk but less than half of the DHA and co-relation was observed in EFA status of the infants reflected the levels in the milk they received [21].

However Qin et al. reported dairy fat blends high in ALA can equally well increase DHA in rat brain [22].

Biofortification Not Economically Feasible in Ruminants

Enriching cattle milk by feeding omega-3-rich cattle feed is also not straight forward. However, it is readily possible to enrich chicken egg with omega-3 fatty acid by feeding omega-3 fatty acid-enriched chicken feed, which can enrich egg yolk with omega-3 fatty acid as they are monogastric [23, 24]. However, such biofortification is not possible and is met with a biological hurdle in the cattle, as they are ruminants. In their rumen, cattle harbor variety of microorganisms. The food they eat is retained in rumen for more than 24 h and acted upon by the rumen micro flora, before the food is sent to the intestine for further processing. When omega-3 polyunsaturated fatty acids are provided in the cattle feed, they get biohydrogenated by the action of the rumen microflora [25, 26]. However, small increments in omega-3 fat in milk have been achieved by feeding derivatized fatty acids such as calcium fatty acids or fatty acid imides [27]. Small increase in omega-3 fatty acids by such innovative approach has not been found to be economically viable. Therefore, fortification of milk with vegetarian omega-3 fatty acid is economically viable alternative.

Current knowledge of maternal nutrition requirements during pregnancy and its association with birth outcome has been recently reviewed [28]. Association between maternal nutrition

and birth outcome is complex and influenced by many biological, socioeconomic, and demographic factors [29]. In case of term infants, human milk plays a key role with respect to nutrition support, which compensates for metabolic and gastrointestinal immaturity, immunologic compromise, and maternal psychosocial conditions [30]. Omega-3 fatty acid is essential and plays an important role in developing fetus. Bhutta et al. [31] has emphasized the need for food based approaches.

Omega-3 fatty acids are critical for fetal neurodevelopment and may be important for the timing of gestation and birth weight as well. Human milk is an important source of EFAs mainly DHA (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6). As cow milk is deficient in essential fatty acid hence it cannot fulfill infant's requirement. Brain has got 60 % lipid. DHA and AA together constitute about 20 % of brain lipids. DHA and AA are main fatty acids of grey matter [32, 33]. It is well known that modern food is deficient in omega-3-FA (ALA, EPA and DHA) and is insufficient for not only to children but also for adults [34]. Approximately 600 ml of human milk is consumed by nursing infant. Average amount of lipid content of the mature ranges from 3.2 to 3.5 %, which is not influenced by diet [35]. The role of essential fatty acid in regulation of gene expression in the brain synapse formation, cognitive development in child has been extensively reviewed [16, 36–43]. With the excessive intake of omega-6 and prevalence of omega-3-dificiency in modern diet, the ratio of omega-6 to omega-3 has shifted to 20-25:1 [44]. The deficiency of omega-3-fatty acid in human diet has serious implications on human health and development [45]. Many epidemiological studies revealed strong co-relation between omega-3 intake and pregnancy outcome, cognitive development in children [46–51]. ALA and DHA concentration in maternal plasma phospolipid has strong correlation with IQ and body mass index [52].

Breastfed infants show higher levels of DHA in plasma than formula fed infants, however with similar level of DHA (0.32 %), supplemented formula milk give similar level of DHA as breast milk as reviewed by Hoffman et al. [53]. In last trimester of pregnancy, accretion of DHA by fetus is maximum and therefore preterm babies will have low brain concentration of LC-n-3-PUFA. Therefore as indicated by Eilander et al. supplementing formula milk with LC-PUFA to preterm babies is important for cognitive development [37].

Role of Omega-3 Milk in Human Health

It has been reported by Molinari et al. that 400 mg of omega-3-FA in milk results in significant increment of blood fatty acid profile in clinical study. However this study could not establish significant difference in cardiovascular risk parameters but it was observed that homocysteine levels did not change in experiment group and increased in control group. This study establishes that milk is ideal source to provide omega-3-FA [54].

Human milk provides an ideal source of EFAs for premature infants [55].

Cow's milk in comparison with human milk has higher proportion of small chain fatty acid, whereas breast milk has higher levels of DHA and AA that needs to be added to commercial infant formula [56]. Extensive report arising from 65 studies worldwide on 2474 women gives an average concentration of breast milk 0.32 % DHA and 0.47 % AA. DHA was very highly variable in human milk. In north America was one of the lowest (0.17 %) and in Japan highest (0.99 %). This difference actually reflects the corresponding differences omega-3 fatty acid intakes by the mother during pregnancy and lactation [57]. The long-chain omega-3 fatty acid DHA and the long-chain omega-6 fatty acid AA are always found in breast milk and are recommended for addition to commercial infant formula [57].

Benito et al. [58] reported the control group consumed 500 cm^3 per day of semi-skimmed milk and test group consumed 500 cm^3 per day of enriched milk. The study reveals that, in case of test group, reduction in serum triglycerides, total cholesterol, Apo B, glucose, and homocysteine was observed in patients with metabolic syndrome. Further omega-enriched milk was well tolerated and accepted by all patients [58].

In a similar study of Baro et al. have also reported, omega-3-fortified milk results in, significant decrease in plasma concentration of total and LDL cholesterol accompanied by a reduction in plasma levels of homocysteine and vascular cell adhesion molecule 1 was observed [59].

Eduardo Lopez-Huertas reviewed scientific aspects of supplementation of omega-3-fortified milk and its effect on cardiovascular health. In nine controlled intervention studies of subjects with increased risk factors and cardiovascular patients, the significant reduction in blood lipids, mainly cholesterol, LDL cholesterol, and triglycerides, was observed indicating health beneficial outcome of omega-3-fortified milk in cardiovascular disorder [60].

Dangat et al. reported the effect of maternal supplementation of omega-3 fatty acids to a micronutrient (folic acid and vitamin B_{12})-imbalanced diet on gastric milk volume and LC-PUFA composition in animal model. It was observed that, imbalance in maternal micronutrients reduces gastric milk volume and milk DHA levels and omega-3-FA supplementation increases milk DHA levels which in turn have impact on infant growth and development [61].

Carrero et al. reported the cardiovascular health benefits of fortified milk with omega-3 and omega-9, folic acid, and vitamins E and B6 in mild hyperlipidemic subjects. Consumption of 500 ml/day of semi-skimmed milk for 4 week and then 500 ml/day of the omega-3-fortified milk for 8

week resulted in increasing plasma concentrations of DHA and EPA. Also, there was a significant alteration in lipid profile. However, in case of low-density lipoprotein oxidation and vitamin E, non-significant changes were observed. Further, there was a significant decrease in plasma concentrations of vascular cell adhesion molecule 1 (9 %) and homocysteine (17 %) were found, accompanied by a 98 % increase in plasma concentration of folic acid. Indicating fortified omega-3 PUFAs milk along with oleic acid and vitamins is found to be beneficial for controlling cardiovascular markers [62].

Padro et al. reported the effects of phytosterols and omega-3-fortified low-fat milk (milk) on the LDL lipidome in overweight and moderately hypercholesterolemic subjects. Consumption of milk (250 ml/day), fortified with either 1.57 g phytosterols or 375 mg omega-3 fatty acid (EPA + DHA), was provided for 28 days. Significant alternation in triglyceride and very low-density lipoprotein levels were observed after ω3-milk intake. Omega-3-fortified milk showed significant changes in the long-chain polyunsaturated cholesteryl esters and in the ratio PC36:5/lysoPC16:0, associated with a reduced inflammatory activity, indicates protective effect of omega-3-fortified milk in inflammatory and atherogenic effects apart from its low-density lipoprotein and triglyceride-lowering effects [63].

In normolipidemic volunteers supplemented with 500 ml day(-1) of partial skim milk for 1st month followed by 500 ml day(-1) omega-3-enriched milk (400 mg of n-3 fatty acids of which 300 mg were EPA + DHA and 15 mg vitamin E) for 6 weeks, it was observed that, although there no change in lipid parameters in 1st month and significant decrease in triacylglycerol (19 %) and significant increase in high-density lipoprotein (19 %), also there was a very significant increase in plasma EPA 31 % and DHA 31 % was observed at the end of 6th week [64].

Fonolla et al. reported the effect of milk enriched with EPA, DHA, oleic acid, vitamins A, B6, D and E, and folic acid compared with semi-skimmed and skimmed milk (500 ml/day) in volunteers with moderate cardiovascular risk. Supplementation was continued for 1 year. There were significant marked increases in serum folate (58 %) and high-density lipoprotein cholesterol (4 %), and plasma triacylglycerols (10 %), total cholesterol (4 %) and low-density lipoprotein cholesterol (6 %) were reduced significantly; however, serum glucose, homocysteine, and C-reactive protein remained unchanged, indicating significant improvement in the nutritional status and cardiovascular risk markers of volunteers [65].

In conclusion, it needs to be appreciated that cow and buffalo milk is actually meant for their calves. Cattle milk has high calcium and calves almost immediately stand on their legs. Man requires more than a year to stand on legs and to grow brain to full size. Human milk is lower in calcium and richer in omega-3-fatty acid. Therefore, fortifying cattle milk with omega-3 fatty acid would be healthier option. This can be accomplished by incorporating emulsified omega-3 oil in commercial dairy plant. It has been observed that, emulsification step enhances bioavailability of omega-3-fatty acid especially increases the absorption of longer chain more highly unsaturated fatty acids (i.e., EPA and DHA) and no effect in terms of absorption of shorter chain less saturated fatty acids [66]. Milk being accepted by majority of world population, both vegetarians and non-vegetarians can be the ideal medium for omega-3 nutritional security and health for all.

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Omega-3 Egg

5

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List of Abbreviations

EFAs	Essential fatty acids
n-3 PUFA	Omega-3 polyunsaturated fatty acids
n-6 PUFA	Omega-6 polyunsaturated fatty acids
EAA	Essential amino acids
AA	Arachidonic acid
ALA	Alpha-linolenic acid
LA	Linoleic acid
EPA	Eicosapentaenoic acid
DHA	Docosahexanoic acid
CHD	Coronary heart diseases
US FDA	United States of America Food and Drug Administration
CVD	Cardiovascular diseases
PCB	Polychlorinated biphenols
EW	Egg white
CLA	Conjugated linoleic acid
AHA	American Heart Association
AMA	American Medical Association
UK	United Kingdom
FAO	Food and Agriculture Association
MO	Menhaden oil
FO	Fish oil
LDL	Low-density lipoprotein
HDL	High-density lipoprotein
HMA	Heterotrophic microalgae
HUFA	Highly unsaturated fatty acids
RNI	Recommended nutrition intake
RDA	Recommended daily allowance

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Introduction

Egg is nature's life-supporting chemical storehouse. It provides a perfectly packaged, highly nutritious food containing vital nutrients including high-quality protein, vitamins, and minerals, which are essential for humans. Egg, due to its excellent nutrients profile, less cost, and versatility in food preparation, is a popular food item for all societies of the world. They are consumed worldwide, without restriction by any religious or cultural consideration [1].

Egg Nutritional Content

Eggs are one of the best low-price sources of high-quality protein, vitamins, and minerals. The comparative analysis of regular eggs and omega-3 eggs (in house data) is given in Table 5.1. Proteins are made up of amino acids. Some amino acids are essential to humans because the human body cannot synthesize them. Eggs contain all nine essential amino acids (EAA): histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. The pattern of essential amino acids in egg protein is very similar to the pattern needed by the human body; therefore, the egg is often referred as a gold standard for comparing the protein quality of other foods [2]. The Food and Agriculture Organization of the United Nations (FAO) [3] rates the biological value of whole egg protein at 93.7 (based on a 100-point scale), followed by cow's milk 84.5, fish 76, beef 74.3, soybeans 72.8, whole wheat 64, corn 60, and dry beans 58. Eggs provide considerable amounts of all vitamins (except vitamin C) and minerals known to be needed for the human body [2, 3]. They are also a good source of choline (a nutrient that is essential for normal brain development), folate, and selenium [4]. An egg can be divided into two distinct parts: the albumen (egg white) and the yolk. Both the albumen and the yolk provide important nutrients to the human body.

Health Benefits of Eggs

Young or old, men or women, wealthy or poor, for everybody, an egg is complete affordable food with numerous health benefits. The eggs are widely recommended as part of a healthy, balanced diet because of their rich nutrient content [5]. Pregnancy and weaning are life stages at which a healthy, balanced diet is important, for promoting growth and development of baby. During pregnancy, there is an increased requirement of nutrients including protein, vitamin A, vitamin C, thiamine (vitamin B1), riboflavin (vitamin B2), folate, and vitamin D. All these nutrients are found in eggs and help to support maternal health and fetal development during pregnancy. The maternal diet influences infant health. The eggs are an important source, especially of vitamin D3 (cholecalciferol), which is more bioavailable than vitamin D2 (ergocalciferol) used in certain fortified foods and supplements [6]. Two medium-sized eggs provide 32 % of the pregnancy-specific recommended nutrition intake (RNI) for vitamin D. Folate is another vital substance of egg, important during pregnancy for normal development of the neural tube. With low intake, there is increase in the risk of birth defects [7]. Eggs are one of the few natural sources of choline in the diet which is used in the body to create phospholipids, a component of cell walls and to produce the neurotransmitter acetylcholine [8]. The health benefits of choline are not understood fully, although an adequate intake appears to be important during reproduction [9]. Choline may also be important in helping to prevent neural tube defects. In a survey of 180,000 pregnant women in the US [10], those who had infants with neural tube defects had significantly lower serum levels of choline compared with women who had healthy infants. Choline may also be important in helping to prevent neural tube defects. Two medium-sized eggs provide 288 mg of choline. There are no recommended dietary allowances (RDA) for choline, but a daily requirement of 550 mg has been suggested [11]. Ruxton [12] reported a consistent beneficial effect of egg consumption on weight management and satiety, as well as concomitant changes in the gut hormones responsible for hunger control [13]. The rich micronutrient content of eggs will be beneficial in the diet once solid foods have been introduced to baby at six months of age. In childhood, vitamin A—a group of substances including retinol and the carotenoids—has a major role in the normal development of vision. Retinol helps the retina adapt to changes in light and color, helping to prevent night blindness [14]. One medium-sized egg provides 18 % of the RNI for children aged seven to twelve months or 16 % of the RNI for children aged one to three years [15]. Vitamin D has a key role in bone accretion by enabling calcium absorption in the gut and maintaining the correct ratio of calcium and phosphorus in the blood [16]. There is evidence that vitamin D status during infanthood may influence the risk of infection during childhood [17] and development of type 1 diabetes in childhood or early adulthood [18]. The RNI for children aged seven months to three years is 7 mcg [15]. One medium-sized egg provides 23 % of the RNI for children aged seven months to three years.

Global Egg Production

A hen's egg production is divided into two categories: hatching eggs and table eggs. Hatching eggs, if incubated, develop into chicks, while table eggs are unfertilized eggs

Table 5.1 Comparative analysis of regular eggs and omega-3 eggs

S. no.	Nutritional analysis	Regular eggs concentration/100 g	Omega-3 eggs concentration/100 g
1.	Water in %	75.80 %	75.39 %
2.	Energy in kilocalories	163 kcal	168.8 kcal
3.	Carbohydrates	1.08 g	1.14 g
4.	Protein	12.10 g	12.60 g
5.	Total fats	9.00 g	9.18 g
6.	Saturated fat	2.70 g	3.42 g
7.	PUFA	1.26 g	2.16 g
8.	MUFA	3.60 g	2.70 g
9.	Omega-3 fatty acids	36 mg	504 mg
10.	DHA	30.6 mg	360 mg
11.	Cholesterol	383.4 mg	324 mg
Minerals and Vitamins			
12.	Sodium	125 mg	122 mg
13.	Potassium	122 mg	124 mg
14.	Calcium	47.42 mg	49.28 mg
15.	Phosphorus	222 mg	219 mg
16.	Magnesium	9.2 mg	8.7 mg
17.	Zinc	1.02 mg	1.06 mg
18.	Chloride	168.1 mg	173.8 mg
19.	Iron	1.6 mg	2.0 mg
20.	Vitamin A	628 IU	652.9 IU
21.	Vitamin D	42.7 IU	49.6 IU
22.	Vitamin E	2.9 mg	6.3 mg
23.	Vitamin B6	0.132 mg	0.139 mg
24.	Folic acid	46.4 µg	49.2 µg
25.	Pantothenic acid	1.299 mg	1.267 mg
26.	Vitamin B1	0.059 mg	0.053 mg
27.	Niacin	0.08 mg	0.05 mg
28.	Vitamin B12	1.02 µg	1.16 µg
29.	Biotin	19.83 µg	19.71 µg

and sold as food products for human consumption [2]. They are consumed globally, and their production represents an important segment of the world food industry. In USA, eggs are considered to be staple food in most American households [19]. In 2005, six developing countries (China, India, Mexico, Brazil, Indonesia, and Turkey) contributed more than two-thirds of global egg production [20]. In 2009, the worldwide egg production was 67.4 million metric tons, of which China produced about 41 %, followed by USA (8 %), India (5 %), Japan (4 %), and Mexico (4 %) [21]. The US table egg production had a value of approximately 4.24 billion dollars in 2009. Coronary heart diseases (CHD) are the leading cause of death in most of the developed and developing countries [22]. A 1 % reduction in plasma cholesterol can lead to 2 % of reduction in CHD risk [23, 24]. In a typical Western diet, approximately 30 % of the cholesterol intake comes from table eggs [25] and the table eggs were found to be high in cholesterol. Therefore, in 1979, American Heart Association (AHA) recommended that people should limit their daily egg intake in order to reduce the risk of CHD [26]. Although Howell et al. [27] found no significant correlation between dietary cholesterol intake level and plasma cholesterol level, the negative public perception about egg cholesterol levels cannot be changed easily, and has adversely affected egg consumption. Brown et al. [28] indicated that this information about cholesterol is a major reason for the continuing decline in USA egg consumption. In USA, omega-3 eggs helped to revive the egg industry, as omega-3 eggs are designed to help to lower cholesterol, and provide a healthier option for

health-conscious egg lovers. Omega-3 enrichment makes egg healthier. Hence, enrichment of eggs with n-3 PUFA is considered one of the best alternatives sought to augment omega-3 consumptions in humans. Before discussing more about omega-3 eggs, briefing about omega-3 fatty acids and their health benefits is essential.

Omega-3 Fatty Acids and Health Benefits

Dietary essential PUFA include two families: omega-3 and omega-6 (designated for the position of the double bond nearest to the methyl end of the molecule). They are essential to humans because the human body cannot synthesize them. n-3 PUFA have got to be supplied in daily diet. Alpha-linolenic acid (ALA, C18:3) is the representative of omega-3 family, and linoleic acid (LA, C18:2n-6) is the representative of omega-6 family. Longer chain omega-3 fatty acids, include eicosapentaenoic acid (EPA, C20:5n-3), docosahexaenoic acid (DHA, C22:6n-3), and long-chain omega-6 fatty acids include arachidonic acid (C20:4n-6) (AA).

The modern diet is deficient in omega-3 fatty acid and excess of omega-6 fatty acid. Therefore, there is a very severe imbalance of omega-3 to omega-6 fatty acid ratio, which is said to be the root cause of our health problems. The relative excess of omega-6 fatty acid not only inhibits the formation of EPA and DHA, but also enhances the synthesis of eicosanoids which are involved in inflammatory, cardiovascular, and immunological disorders [29]. In addition, n-3 PUFA have been reported to play a key role in prevention and treatment of many other chronic diseases, such as neurological disorders, cancer, inflammatory diseases, obesity, and diabetes mellitus [30, 31]. Lewis et al. [32] noticed that consumption of n-3 PUFA is critical and these vital fatty acids can check cancerous growth, reduce threat of CVD, and decrease/loss of immunity. Several studies have shown positive roles for n-3 PUFA in infant development, and in combating cancer, coronary heart diseases (CHD), hypertension, obesity, type II diabetes, and more recently, various mental illnesses, including depression, attention-deficit hyperactivity disorder (ADHD), and dementia [33, 34].There is also emerging evidence that adequate intakes of omega-3 fatty acids are important during pregnancy to safeguard cognitive, retinal, and immune development in the infant [35, 36]. There is also a high requirement for DHA in the last trimester of pregnancy and in the first 3 months of life. DHA is important in the developing nervous system of newborns and influences structural and functional parameters during rapid brain growth [37]. Some clinical trials indicate benefit for infants in terms of immune function, visual acuity, and cognitive function [38]. In view of the importance of DHA for the development of brain, retinal, and neural tissues in fetuses and young children, adequate DHA intake in pregnant and nursing woman is very much essential [39]. In humans, ALA is converted into EPA and DHA; however, this conversion is hampered due to the excess of omega-6, as the same set of enzymes are involved in metabolism of n-3 and n-6 PUFAs [40]. The conversion efficiency from ALA to EPA and DHA in humans is reported to be less than 10 % [41]. Elderly people, hypertensive individuals, and some diabetics have a more limited capacity to synthesize EPA and DHA from ALA. Fish is the richest dietary source of EPA and DHA, and supplementation trials with fish oils during pregnancy have reported benefits for infants, such as better cognitive function [42] and a lower risk of atopy [36]. A large part of the population do not consume fish [43]. The presence of pollutants such as heavy metals and polychlorinated biphenols (PCBs) in fish [44, 45], raises the safety concern in fish consumption. Therefore, other dietary sources for EPA and DHA are being sought. Omega-3 enrichment of eggs is probably the best long-term solution to boost intake of long-chain n-3 PUFA [46] in humans as a way of promoting health and reducing the risk of diseases.

Omega-3 Eggs

Enriching eggs with n-3 PUFA is considered the most efficient way to incorporate these acids into an animal product as the linear relationship between deposition of 3 PUFA and dietary n-3 PUFA has been long established [32]. The inclusion of n-3 PUFA in eggs is achievable by feeding n-3 PUFA-rich diets to layer birds. The laying hen is a good biological model, which converts ALA to EPA and DHA, in the liver and deposits this n-3 PUFA into eggs. The omega-3 eggs taste the same as regular eggs and therefore offer an easy way of increasing omega-3 in the diet, without changing the diet or taking omega-3 supplements. The development of omega-3 eggs began at the University of Nebraska. Researchers found that adding a percentage of flax, canola oil, or other omega-3-rich products to chicken feed would produce eggs that have an increased level of omega-3. The University of Nebraska holds the trademark for omega-3 eggs and the patent for the feeding system, and the name Omega-3 Eggs is licensed to regional poultry farms. In this review article, different approaches toward enrichment of eggs with n-3 PUFA are discussed.

Advantages of Omega-3 Eggs Over Ordinary Table Eggs

Omega-3 eggs have all goodness of ordinary table eggs. The increase in yolk omega-3 is accompanied by a substantial decrease in saturated fatty acids, creating a healthier fat profile. In the Omega-3 egg, the ratio of omega-6:omega-3 has been improved to 2.6:1. The Omega-3 egg provides 250–350 mg n-3 PUFA, of which 100 mg is DHA. Due to a special feeding of omega-3-enriched poultry feed, the cholesterol content of Omega-3 eggs has also been significantly reduced to 180 mg/egg, compared to the standard egg cholesterol value of 210 mg/egg [47]. This makes omega-3 eggs "healthy eggs." The number of calories, the amount of protein, and the total fat remain almost the same to that of regular eggs. Lewis et al. [48] suggested most consumers can eat twelve omega-3-enriched eggs per week without an increase in total or low-density lipoprotein (LDL) cholesterol level. Van Elswyk et al. [49] stated that eating four omega-3-enriched eggs per week resulted in a significant decrease in blood platelet aggregation, which is a risk factor for CHD. On consumption of DHA-enriched eggs over ordinary eggs in case of mildly hypertriglyceridemic men and women, significant reduction in serum triglyceride concentration and greater increase in HDL cholesterol concentration was found [50]. Omega-3 eggs were successful in reducing blood pressures. Some children are known to have food allergy—especially for raw table eggs. Egg white is one of the leading causes of IgE-mediated food allergy in childhood [51], affecting approximately 1.6 % of children [52, 53]; the prevalence is considerably higher in children with atopic dermatitis or other food allergies [54, 55]. Previously, it had been thought that the majority of allergic children become egg tolerant by school age. However, a recent study has suggested that egg allergy persists well into the adolescent years for many children with egg allergy [56]. The food supplementation with n-3 PUFA may be promising to treat food-associated allergic disorders. De Matos et al. [57] have shown that diet supplementation with n-3 PUFA from fish oil led to a reduction of gut inflammatory response against food antigen ovalbumin, which suggests that n-3 PUFA may modulate the allergic immune response. Hence, we can expect omega-3 eggs to have beneficial effects in controlling food-associated allergic disorders. In study conducted in Australia on a large cohort of children showed that supplementation of pregnant women with high-dose n-3 PUFAs did not reduce IgE-associated food allergy in the first year, while incidence of eczema and egg allergy was reduced [58]. In addition, several studies have reported that n-3 PUFAs are able to manipulate many immune functions in favor of controlling diseases caused by excessive inflammatory response.

Health Benefits of Omega-3 Eggs

Depending upon source of omega-3 for egg enrichment, these eggs are also termed as ALA-enriched eggs or DHA-enriched eggs. The majority of the positive effects is related to presence of DHA in the eggs, rather than ALA [59–61]. As a guideline, two omega-3 eggs can offer almost same amount of omega-3 present in 100 grams of fish. Omega-3 eggs extend all the possible health benefits of DHA which include the accelerated development and improved functioning of brain, the decreased hazard of heart attack, and some support in rheumatoid arthritis, inflammatory disorders, and other diseases [37, 62–65]. Oh et al. [61] reported the positive consequences of consuming four omega-3 eggs per day on blood pressure, blood lipid profile, and lipoproteins fractions. Decrease in blood triglycerides, reduction in platelet aggregation, effect on LDL particle size, and mediating ecosanoids metabolism are some of the contributions of omega-3 eggs. So far, no harmful consequence from intake of omega-3 eggs is reported. Additionally, Cherian and Sim [37] showed that feeding ALA-enriched eggs to nursing women resulted in enrichment of breast milk with ALA and DHA. Research has shown that the regular inclusion of omega-3 eggs in the diets of breast-feeding mothers can significantly improve the omega-3 status of the infants. An analogous study by feeding of egg yolks of DHA-enriched egg to infants demonstrated increased maturation of visual sharpness [66]. Ferrier et al. [59] studied serum and platelet lipid profiles of 5 healthy volunteers who weekly consumed ALA-enriched eggs. Within a week, serum triglyceride levels (a significant predictor of coronary heart disease) declined by 35 %. Daily consumption of ALA-enriched eggs by healthy volunteers resulted in the enrichment of platelet phospholipids in DHA, which may reduce platelet aggregation. The above-described effects are expected to have an overall favorable influence on the risk of CVD.

Fish Oil Versus Omega-3 Eggs

Fish oil omega-3 is in triglycerides form, while omega-3 from omega-3 eggs is in phospholipid form. Fish EPA/DHA need to be broken down by the liver to release free fatty acids, while omega-3 EPA/DHA of omega-3 egg can be used by the cells directly and have higher bioavailability. The recent popularity of krill oil supplements is due to the presence of omega-3 in phospholipid form and claims of superior bioavailability of omega-3 relative to fish oils' omega-3. The levels of omega-3 phospholipid in krill oil capsules were compared with that in omega-3 phospholipids in omega-3 eggs and found that omega-3 eggs have more DHA phospholipid than popular krill

Table 5.2 Omega-3 in egg yolk using different sources of 3 PUFA

S. no.	Omega-3 in mg per egg yolk			Diet enriched with various sources of omega-3 PUFA	Ref. no.
	ALA	EPA	DHA		
1.	13	25	28	Control	[81]
	18	15	83	1.5 % menhaden oil (MO)	
	90	3.3	68	5 % whole flax	
	110	5.3	68	5 % ground flax	
	163	7.6	73	15 % whole flax	
	212	20	90	15 % ground flax	
2.	38.5	–	53.3	Control	[68]
	306.3	–	83.7	10 % flaxseed	
3.	15.6	0.6	30.6	Control	[150]
	173.9	16.9	108.4	Fish oil (FO) + ground flaxseed	
4.	26	0	26	Control	[94]
	26	13	85	2 % deodorized MO	
	38	31	123	4 % deodorized MO	
	58	61	196	6 % deodorized MO	
	23	16	87	2 % regular MO	
	30	30	114	4 % regular MO	
	37	45	114	6 % regular MO	
5.	70	1	62	Control	[117]
	65	12	96	2 % microencapsulated FO	
	73	24	129	4 % microencapsulated FO	
	70	40	162	6 % microencapsulated FO	
6.	17	–	35	Control	[162]
	277	–	114	Flaxseed	
7.	0	0	20	Control	[50]
	0	0	146.6	HMA	

oil capsules [67]. In view of cost of krill oil capsules, the omega-3 eggs are cheap and deliver higher amount of omega-3 DHA phospholipid.

Feed for Omega-3 Enrichment

The fatty acid composition of eggs usually reflects the composition of bird's diet, and it can be modified by changes in the hens' diet [68, 69]. PUFA content and profile in the egg can be modified through dietary supplementation; however, the amount of saturated or monounsaturated fatty acids is hardly influenced by the lipids in the feed [70–73].Omega-3 eggs can be obtained within 1–2 weeks after feeding layer birds with omega-3-rich feed [74]. Many studies have confirmed this fact over the decades, with early studies usually comprising oilseeds [75–77] and later studies incorporating fish oils, newer oilseeds such as canola, and novel marine algae [73, 78, 79]. Different omega-3s in egg yolk using different sources of n-3 PUFA are summarized in Table 5.2.

Resources for Omega-3 Enrichment

There are 3 major categories of omega-3-rich resources.

(i) Plants
(ii) Fish
(iii) Marine algae

(i) **Plants**

ALA is produced by various plant seeds, such as canola, soybean, walnuts, and flaxseed; the latter is one of the most concentrated sources of ALA [78]. In general, the two most important plant seeds that are used in layers' diets for the

purpose of incorporation of omega-3s in the eggs are flax seeds and canola seeds.

(a) **Flaxseeds**

Flaxseed is a rich source of protein (22.4 %) and lipids (37.4 %) for poultry [78]. Over the past 20 years, the influence of dietary supplementation with flaxseed or flaxseed oil on hens' performance and egg characteristics has been studied extensively. Flaxseed or flaxseed oil is widely used in poultry egg and meat enrichment due to its high (50–60 %) ALA content [80]. It is generally accepted that the amount of ALA in yolk increases linearly with the diet level of flaxseed up to 10 %. However, the conversion of ALA to EPA and DHA does not increase proportionally with increase in ALA [81]. The use of higher levels of flaxseed in the diet is limited due to the presence of some anti-nutritional factors such as phytic acid [80] cynogen glycosides, and antivitamin B6 [82]. Linatine is a trypsin inhibitor [82] found in mucilage, which decreases digestion of feed particles. According to Kennedy et al. [83] and Aymond et al. [84], flaxseed contains certain diphenolic compounds—lignans, which are phytoestrogens. They compete with estrogen, thus decreasing its level in the blood and potentially interfering with productivity of laying hens. According to Scheideler et al. [85], other factors must be considered, such as the level of flaxseed inclusion, physical condition of the flaxseed (whole or ground), storage temperature of diet, as well as the amount of antioxidants added in the feed.

(b) **Canola**

Canola (the low glucosinolate, low erucic acid form of rapeseed) is a trademarked cultivar of the rapeseed plant. The word "canola" is derived from "Canadian oil, low acid." Either the oil or the full-fat seed may be fed to poultry birds. Full-fat canola seed contains 41–43 % oil, of which about 12 % is ALA and 20–25 % protein [86], and therefore is a valuable source of energy and protein [87–89]. Canola has a rather high level of ALA, typically 9–10 % of total fatty acids, and a low level(less than 10 %) of saturated fatty acids [90]. Compared with flax, canola gives smaller increases in ALA, but similar increases in long-chain n-3 PUFA. Brettschneider et al. [91] reported that total n-3 PUFA in eggs were 127 mg and 159 mg supplemented by 15 and 30 % canola seeds, respectively. The transfer efficiency of the ALA from the diet to the eggs was lower in canola seed diet compared with that of flaxseed diet.

(ii) **Fish**

Compared with flaxseed, eggs enriched with fish meal or fish oil (FO) contain more DHA and EPA, which are thought to have a higher bioavailability in humans [90, 92]. The increase in n-3 PUFA upon fish oil supplementation is accompanied by a decrease in AA and total n-6 PUFA [71, 73, 93], while yolk cholesterol content remains unaffected [92, 93]. Menhaden oil (MO) is the most popular fish oil as a source to enrich eggs with EPA and DHA. Generally, a linear response of DHA content to feeding levels of menhaden oil is observed [73]. However, menhaden oil supplementation can more easily cause off-flavor in the eggs [94]. Hargis et al. [95] observed that adding 3 % of menhaden oil in diet could slightly increase EPA to approximately 30 mg/yolk compared to DHA at 180 mg/yolk.

(iii) **Marine algae**

Marine algae are an efficient dietary alternative to current long-chain n-3 PUFA sources [96]. Herber and Van Elswyk [97] found that marine algae contained about 11.2 % of long-chain n-3 PUFA on a dry matter basis. It was also found that the presence of carotenoids in marine algae may enhance the oxidative stability of omega-3 eggs [98]. Technology has been developed to produce marine microalgae with an extremely high DHA content (±18 % of dry mass) via a fermentation process [98]. Eggs from hens fed heterotrophic microalgae (HMA), which are rich in DHA with little or no EPA, typically show similar PUFA profiles as eggs from hens fed fish oil [71, 99]. Due to high carotenoid content in HMA, eggs from hens fed HMA showed significantly high carotenoid content in egg yolk [100], which influenced yolk color dramatically [79, 100, 101]. Similarly, upon addition of *Porphyridium* sp. (Rodophyta) to hens' diet, Ginzberg et al. [102] noted a shift in yolk color to darker yellow, which was caused by an increase in yolk carotenoid content. Several HMA products are commercially available, in the form of whole biomass as well as extracted oil which is being used to produce omega-3 eggs. Today, such eggs are available on the market in several countries, and their share is expected to grow as the market for functional foods is still expanding [102, 103].

Production Parameters and Egg Quality Characteristics

Most authors reported that in hens, the ALA to EPA/DHA conversion is rather limited, similar as in humans [68–71, 104]. This is caused by the low activity of desaturase enzymes involved in conversion of ALA to EPA/DHA. However, the conversion efficiency is affected by several factors such as the following:

1. The presence of high amounts of n-6 PUFA in the diet which increases the competition for the desaturase

enzymes, causing a decrease in ALA conversion efficiency [104]. As a consequence, the omega-6/omega-3 ratio of the diet is one of the major influencing factors.
2. Addition of antioxidants such as vitamin E also seems to modulate the elongation—desaturation pathway in a favorable way [105–108].
3. It was noticed that hens' age and strain also have an effect on the efficiency of ALA elongation and desaturation [83]. It has been postulated that older hens have a larger liver, allowing a more effective conversion of ALA into DHA. The increase in amounts of n-3 PUFA in the egg yolk was paralleled by a decrease in 6 PUFA, especially AA [69, 109, 110].

A recent study by Hayat et al. [111] concluded that feeding layer hens a diet including 10 % whole flaxseed with different levels of antioxidants increased ALA content in eggs up to 20 times that of the control diet. Contents of EPA and DHA were also increased, while the content of saturated fats was lowered. The DHA content of a fortified egg is 150 mg/egg approximately. Flaxseeds in a high dose (more than 10 %) may cause a decrease in egg quality such as decreased eggshell thickness, decreased yolk weight, and increased albumen percentages [69]. In a study in 2005, da Silva Filardi and co-workers [112] found that inclusion of canola oil to the feed of commercial layers does not cause any significant effect on performance parameters. Addition of canola oil to the feed decreases the concentration of LA and at the same time increases ALA and DHA in the yolk [113, 114]. These reports confirm that canola results in acceptable omega-6/omega-3 ratio in eggs. Moreover, Nwokolo and Sim [115] fed full-fat canola seed to layers at 10 % of diet, and increased egg ALA by 50 % and DHA by 26 %, compared with control. Cherian and Sim [109] fed 16 % canola seed to laying hens, and egg ALA increased from 0.6 to 2.4 % of total fatty acids and long-chain n-3 PUFA increased from 1 to 1.7 %. In all cases, increases in n-3 PUFA % were accompanied by corresponding decreases in saturated fatty acid content. There has been interest in feeding canola to laying hens in industrial scale, due to favorable changes in fatty acid profiles of the eggs. The levels of erucic acid and glucosinolate in canola are low enough to be of little or no concern for poultry [114].

Although fish oil contains EPA as well as DHA, eggs from hens fed fish oil are mostly enriched with DHA, while EPA contents increase to a much lesser extent [73, 116], suggesting that DHA might be preferentially incorporated into membranes in comparison with EPA [73]. Addition of EPA-rich fish oil with DHA-rich fish oil in diet is compared for PUFA content in eggs; for both treatments, the major n-3 PUFA in eggs was DHA and the proportion of ingested long-chain n-3 PUFA (EPA+DHA) that was deposited in yolk fat (in the form of DHA) did not differ significantly between both diets. These results indicate that dietary EPA is largely converted to DHA. The efficiency of this conversion is only slightly lower than that of direct deposition of dietary DHA in yolk. Several authors reported impaired production parameters, especially a decrease in egg and/or yolk weight upon feeding fish oil [94, 117, 118]. It has been suggested that long-chain n-3 PUFA consumption causes a decrease in serum triglycerides in the hens and hence a decrease in amount of lipids available for yolk formation [95]. With increasing levels of fish oil addition, the increase in yolk DHA content was not proportional indicating a lower deposition efficiency at higher inclusion levels [73, 95, 103, 117, 118]. It has been shown that EPA and DHA are preferentially incorporated in phospholipids [103, 113]. Addition of flaxseed or fish oil, feed supplementation with HMA, does not affect the yolk cholesterol content [79]. n-3 PUFA levels especially DHA (196 mg) were quite high, in the eggs resulting from 4.8 % HMA addition [100]. Parpinello et al. [119] reported that eggs produced through feed supplementation with 2 % HMA were of good sensorial quality. HMA contain several carotenoids, of which β-carotene and canthaxanthin are the most abundant [101]. A shift in yolk color toward red was observed in eggs from hens fed HMA [103], reaching a plateau within 2 weeks. This suggests that carotenoids have been transferred to egg yolks. Carotenoids offer several advantages, since they act as antioxidants, providing health benefits and increasing the oxidative stability of yolk lipids. The consumer acceptance of a reddish yolk color varies between geographical regions. In Europe, for example, consumers in northern countries prefer pale yolks, while in the south, dark-yellow, reddish yolks are preferred.

The inclusion of fish oil more than 1.5 %, produced omega-3 eggs which are generally unacceptable to Western consumers due to fishy smell [117–119]. This fishy taste paralleled the amount of 3 PUFA in the eggs [73].

Benefits of Omega-3 Enrichment to Birds

Omega-3-enriched feed supplementation not only produces healthy omega-3 eggs, but also imparts health benefits to birds. Bhalerao et al. [120] evaluated various health parameters such as morbidity, mortality, immunity, and cardiac and mental health and concluded that omega-3 fed birds had much less mortality than control birds (0.3 vs. 3 %). Birds were less disease prone and had higher immunity. The omega-3-fed birds had shown lower heart beats

signifying better cardiac health. These birds could also withstand the brain trauma than control birds. The lower mortality and morbidity add to the profitability of poultry farm owners.

Means to Improve Oxidative Stability and Quality of Omega-3 Eggs

EPA and DHA contain more double bonds than ALA. As a result, they are even more susceptible to oxidative breakdown. Hence, increasing the level of these sensitive fatty acids in egg yolk may bring about a higher lipid oxidation, which could impair sensorial quality, i.e., the development of off-flavors. The stability of omega-3 eggs is important for maintaining egg quality. Therefore, antioxidants are added to reduce oxidation process. Vitamin E and selenium are key components of the antioxidant defence system to reduce lipid oxidation. Vitamin E is an essential nutrient because it cannot be synthesized in the human body and must be contained in diet [121]. Major vitamin E sources are vegetable oils and some other plant-derived foods. While the body can absorb both natural and synthetic forms of vitamin E (alpha-tocopherol), natural forms have higher bioavailability than synthetic ones. The addition of antioxidants such as vitamin E and/or vitamin C significantly improved laying hens' performance, egg production, vitelline membrane strength, yolk and albumen height, and foam stability [122–124]. It is recommended that poultry feed should contain 100 IU vitamin E per kg or commercial omega-3 egg production. Vitamin E in the hen diet increases the content of vitamin E in the egg yolk in a dose-dependent manner [125], and transfer efficiency decreases with increasing levels of dietary vitamin E in the diet. Vitamin E along with selenium had shown improved oxidative stability. Rutz et al. [126] found that the supplementation of organic selenium up to 0.5 ppm to layer diets significantly improved egg production, egg weight, feed conversion ratio, albumen height, and specific gravity. In addition, eggshell weight and shell thickness were increased by the combination of organic selenium, organic zinc, and organic manganese. The tolerance for organic selenium is much greater than that for inorganic selenium (sodium selenite and sodium selenate). Also inorganic selenium has lower transfer efficiency to eggs than that of organic selenium (selenomethionine) [127]. Compared to the inorganic selenium (sodium selenite), organic selenium (Se-enriched yeast) contributed to a higher selenium content in eggs and increased egg weight [128]. The combination of vitamin E and organic selenium could therefore be an effective antioxidant for omega-3 eggs production and improves stability during storage. Use of butylated hydroxytoulene (BHT synthetic antioxidant) 50 mg along with vitamin E lowers levels of saturated fat when compared with hens fed the other diets [124]. Such eggs have increased ALA, EPA, and DHA levels and a decreased AA as well as total omega-6:omega-3 ratio when compared with control eggs. The antioxidant supplementation had no effect on egg cholesterol content. Yuan Ren [129] examined stability of n-3 PUFA-enriched eggs fortified with antioxidants (vitamin E or organic selenium [Sel-Plex] or both) following storage and cooking. It is possible to make the omega-3 eggs more stable with dietary antioxidants such as vitamin E or organic selenium [Sel-Plex] or both. The advantages of omega-3 eggs with antioxidants are decrease in susceptibility to lipid oxidation and prevention of fishy taste as stated by Surai [130].

Commercial Poultry Feed for Omega-3 Eggs

Using various omega-3-rich substances, proprietary poultry feeds are being produced by many poultry feed manufacturers. Some omega-3 egg manufacturers have patented poultry feed that produces eggs with super high levels of omega-3. Some companies add fish oil to the poultry feed to increase the DHA content of egg yolks. Antioxidants are generally added to the hens' diet to minimize lipid oxidation and to enhance customer's satisfaction. In poultry feed industry, adding vitamin E to the hen's diet is a common practice for omega-3 egg production [107, 108]. Different companies have begun to produce extruded flaxseed feed ingredients to address the market demand [131]. One such company is Valorex® located in La Messayais, France [132]. O&T Farms is another company located in Regina, SK, Canada, which markets its extruded flaxseed product under the name LinPRO®, which is a 50:50 combination of full-fat flaxseed and field peas [133]. On their Web site, the company states that the process used to produce LinPRO® is a dry-extrusion process under controlled temperature and pressure conditions [133]. This company's product is largely marketed to the poultry and swine industries [134]. The company claims that LinPRO® is used in the production of more than 65 % of omega-3 eggs in Canada.

Commercial Aspects of Omega-3 Eggs

Omega-3 eggs have become available on the market in many countries since the late nineties [103]. Even though omega-3 eggs usually are sold at a premium price compared to the table eggs, the number of omega-3 eggs purchased by consumers has increased significantly all over the world, as a result of the growing knowledge of its

health benefits [135]. These products and their variants are taking up more store shelf space in recent years, which is driven by increasing consumer demands of omega-3 eggs as well as eggs with multiple enrichments, such as vitamins, selenium, and lutein. [136]. In USA, the demand for omega-3 eggs is climbing steadily and is now believed to constitute as much as 5 % of the egg market [137]. Since the major benefits of omega-3 fatty acids relate to cognitive development and heart conditions, they have the greatest impact on health at the beginning of the lifecycle and at older ages. Therefore, marketers of omega-3 eggs are targeting two very different consumer groups—very young children and aging adults.

Major Findings and Conclusions of Consumer Surveys [47, 49, 135–141]:

According to Mintel's consumer survey [137], omega-3 eggs are the most popular products among the omega-3-fortified products. In a study on consumer acceptability by Marshall et al. [134], they found that 71 % of consumers would be willing to pay a premium price for omega-3 eggs when their omega-3 levels are comparable to those in fish. Other findings are as follows:

(i) As consumers have become increasingly concerned about their health, publishing in the popular media can be an effective communication approach for changing their dietary behaviors and to improve awareness about health benefit of omega-3 eggs. The consumers obtain new scientific nutritional knowledge through popular media, and consumers' purchase choices reflect their knowledge of new nutritional information and its connection with food. New nutritional information provided by popular media substantially affects consumers' food choices by reducing uncertainty about the health attributes of those foods.

(ii) The number of consumers, who are aware of omega-3 eggs, is increased over time. It is assumed that they get more knowledge on omega-3 eggs health benefits and adjust their egg purchase behavior according to that. It seems to be fair to conclude that such information has value on consumers.

(iii) Time plays a role on consumers' choice because their knowledge on nutritional information accumulates over time. The consumer's knowledge on the nutritional benefits of the omega-3 eggs in 1998 and that in 2007 is different. The current information would not have the same impact in the absence of prior exposure to similar information. The consumers' choice is not only affected by time but also by the credibility of media.

(iv) Consumers are quite sensitive to the prices of regular eggs. However, while purchasing omega-3 eggs, its health benefits seem to have a stronger impact than price. If the regular egg's price goes up, the price difference between regular eggs and omega-3 eggs shrinks, which induces consumers to choose omega-3 eggs. If the price of regular eggs decreases, the price difference between regular eggs and omega-3 eggs widens and discourages consumers from choosing omega-3 eggs.

(v) People living in urban areas may have easier access to the information on omega-3 eggs and to the new products available on the market, compared to the people living in rural areas. If households are living in an urban area, the probability of buying omega-3 eggs increases.

(vi) Elderly people tend to pay more attention to their health, and higher education may indicate higher information literacy. Higher household income, age of head, and education has positive relationships with the purchasing of omega-3 eggs. Higher income might diminish their hesitation against higher prices of omega-3 eggs. Household size had a negative relationship with omega-3 eggs' purchase, probably because larger households have to allocate their money for food over more household members, so the price becomes a more important factor for them in choosing from available alternatives.

(vii) The developmental benefit of omega-3 eggs in particular applies to babies, children, and pregnant women, so it is expected that young households or households with children would be more sensitive to the information on the developmental benefits of omega-3. Although developmental benefits are expected to be more attractive over health benefits to the households with children, there is no convincing evidence for such a tendency. Hence, the information about omega-3's developmental benefits and health benefits produced similar results.

(viii) If omega-3 were in the recommended daily food guide, then consumption of omega-3 eggs would most likely be increased. Recognition of omega-3 eggs by the FDA would also have positive effect.

(ix) Increased lobbying efforts by the omega-3 egg producers could speed up the process of FDA recognition of omega-3 eggs. Effective lobbying for omega-3 requires the development of a nationwide coalition of omega-3 egg producers, which would enable them effective promotion on a larger scale at a lower cost.

Global Omega-3 Eggs Market

Omega-3 eggs are not only available in USA but also in other countries such as Canada, Great Britain, Japan, Australia, Pakistan, and India, where greater acceptance of omega-3 eggs has occurred. Commercial omega-3 eggs were first introduced to the public in 1997. The per capita consumption of eggs in 2001 was 252.3 eggs or more than 72 billion eggs per year in USA and designer eggs account for five percent of eggs consumed, which equates to three billion eggs annually [137]. After eleven years, omega-3 eggs represent 12 % of eggs marketed in Canada [139].

Major Market Players

The idea of egg enrichment with n-3 PUFA with antioxidants and other vitamins has been used to produce VITA Eggs by Freshlay Foods (Devon, UK). They state that their eggs were enriched with n-3 PUFA, Se, vitamins D, E, B12, and folic acid. Another player is Belgium-based Belero, which launched omega-3 eggs in the USA as Christopher Eggs [140]. Each Christopher Egg has 660 mg of omega-3. They claim to have the maximum omega-3 per egg. Eggs enriched in omega-3 and vitamin E produced by Belovo under the trade name of Columbus first appeared in Belgium in 1997, and since then, they have been sold in the UK (from 1998), Netherlands (from 1999), India, Japan, and South Africa (from 2000). Currently, production of Columbus egg exceeds 50 millions/year in Europe. Belovo feeds its hens omega-3's derived from flaxseed. Another active player in US market is Martek Biosciences, whose marine algae are fed to chickens in order to increase the DHA level of its eggs, which are currently sold under the Gold Circle Farms brand. The DHA amount found in four Gold Circle Farms Cage-Free eggs is equal to one fish serving. Each Gold Circle Farms egg also provides six times more vitamin E than a regular supermarket egg (20 % of RDA). Similar eggs are produced by Pilgrim's Pride Company and OmegaTech in the USA [140]. Few US-based omega-3 eggs manufacturers are Egg Innovations, Eggland's Best Inc., Giving Nature Foods, Michael Foods Egg Products Co., and Sparboe Companies, Chino Valley Ranchers. Omega-3-rich eggs were introduced to the UK market first by Columbus and more recently by Stonegate Farms under the brand name "Intelligent Eating Healthy Eggs." The latter comes from another joint venture with Nu-Mega, which involves chicken feed containing Nu-Mega's DHA-rich tuna oil. Land O Lakes Omega-3 All-Natural Eggs claim to contain 350 mg of omega-3s per egg, but the types and amounts of omega-3 are not specified on the carton. Ditto for Organic Valley Omega-3 Extra Large Eggs, which boast 225 mg of the fats per egg (types are not specified).Smart Balance, by contrast, reveals that its Omega-3 Grade A Natural Large Eggs each contain 160 mg of ALA and 32 mg of DHA. Gold Egg Omega Choice provides 130 mg of DHA per egg.

Designer Egg—Value Addition Concept

Designer egg term was first mentioned by Dr. Jeong Sim and his colleagues in 1990s and referred to omega-3-enriched eggs [141]. The demand for such products is ever growing all over the world [142]. Other available designer eggs include those enriched with vitamins [143], lutein [144], or selenium, conjugated linoleic acid (CLA)-enriched [145], low cholesterol [146], as well as various combinations of these enrichment ingredients.

Lutein-enriched Eggs

Lutein is a type of carotenoid pigment, present in deep orange-colored marigold petals and certain type of red capsicum. It is an antioxidant which limits the oxidative stress in tissues that result from metabolism. Lesson and Caston [144] reported that it is possible to increase egg yolk lutein 5–8 times above regular concentrations, representing an additional 1.5–2 mg contribution to lutein daily intake. Although general egg quality was not affected, the egg yolk of these eggs will have attractive deep orange color. The conversion efficiency of lutein from feed to eggs is approximately 10 % with 125 ppm in the diet, declining to 2–3 % once the hen supplement level reaches 500 ppm. This transfer efficiency was decreased when flaxseed was added to the diet. Lutein-enriched eggs can be produced by incorporating 0.5 % orange marigold petal meal in poultry feed. These eggs are popular due to its beneficial effects to improve eye vision especially night blindness and controls retinal diseases such as retinitis pigmentosa, macular degeneration, and diabetic retinopathy [147].

CLA-enriched Eggs

Conjugated linoleic acid (CLA) has been shown to have antiadipogenic, anticarcinogenic, antiatherogenic, antidiabetogenic, and anti-inflammatory properties [148, 149]. Furthermore, individual isomers of CLA have distinct effects on tumorigenesis and lipid metabolism. Previous studies have shown that concentration of CLA in the yolk lipids linearly increases as dietary CLA increases [71]. Maximum CLA concentrations in the yolk lipids of hens fed 0.5, 2.5, or 5.0 % CLA occurred 11 days after the start of the experiment and were 0.82, 5.82, and 11.20 % of the total

fatty acids, respectively [146]. Feeding 5.0 % CLA decreased feed intake but did not affect rate of egg production, weight of eggs, albumen, or yolk.

Low Cholesterol and Cholesterol Lowering Eggs

These eggs will have 10–15 % less cholesterol than regular eggs and in addition will contain several cholesterol lowering compounds such as quercitin, euginol, allicin, phytosterols, n-3 PUFA, lycopene, and nicotinic acid. Due to such enrichment, these eggs not only contain low cholesterol in egg yolk but also lower serum cholesterol on its consumption [150, 151].

Herbal-enriched Egg

These eggs can be produced by feeding hens with functional feeds containing herbal ingredients, omega-3 PUFA, antioxidants such as vitamin E, selenium, and carotenoids. The commonly used herbs are holy basal leaves (tulsi), white basal (naitulasi), curry leaves, garlic, fenugreek seeds, spirulina, cumin seeds, turmeric, coriander, mint, tomato pulp, grape pulp, etc. [146, 152]. The usual concentration of these herbs is around 0.3–1.0 %in poultry feed. These herbs have active principles such as alkaloids, allicin, betaine, curcumin, flavonoids, lycopene, oryzanol, and phytosterols. These herbal ingredients impart anticarcinogenic, antioxidant, antidiabetic, antimicrobial, immunomodulating, cholesterol lowering, and other health-promoting properties to the enriched eggs. Hence, consumption of such herbal-enriched eggs will significantly improve well-being and health of the consumers [146].

Immunoglobulin-enriched Eggs

These eggs are produced by hyperimmunized hens [153]. These eggs will have more than double quantity of immunoglobulin Ig Y along with other immunomodulators. They are also enriched with vitamin E, selenium, which will boost the immunity especially in children and elderly people. These eggs can also be used for immunoglobulin production on commercial scale.

In addition, tailor-made specialty eggs can also be produced like folic acid- and vitamin D-enriched eggs, iron-enriched eggs, memory eggs, antidiabetic eggs, heart-friendly eggs, and pediatric/geriatric eggs by feeding hens with various active ingredients from herbal or other sources. Research on different aspects of designer egg production is of continued interest for the last two decades. Several experiments, mostly in western countries, have been conducted on the effectiveness and potential impact of enrichment on production parameters of layers [154, 155], egg quality [156–158], sensory evaluation [159], and health benefits [160–162].

Conclusion

In conclusion, the egg is the best vehicle through which various health boosting constituents can be brought in the daily food as discussed above in designer eggs. Designer eggs production may give poultry farmers an opportunity to be part of an emerging industry which can increase marketability and economic returns by offering consumers an alternate way of obtaining these health-promoting nutrients through their diet. These research findings can be translated into practical applications and scaled up by poultry nutritionists. However, there is a dire need for the dissemination of information and outreach regarding the nutritional quality and usefulness of designer eggs, especially in developing countries. Further research is also required to incorporate other health-promoting substances and medicines in eggs for onward entry into human food chain. Thus, the role of designer eggs in human health and nutrition is overwhelming. The efforts are needed to take these eggs to common mass especially underprivileged population. Designer eggs provide unique solution with "win-win" situation to all stake holders like farmers (the oil seed growers), poultry industry, and finally health-conscious consumers, provided the products' health benefits effectively reach to consumers and the product has consumer acceptance. Now that, we know that omega-3 eggs are healthier eggs with lesser amount of cholesterol and can lower both cholesterol and triglycerides; further, it can also be beneficial for the bird's health. Therefore it is prudent to suggest that all eggs in the market should be omega-3 eggs only.

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Omega-3 Polyunsaturated Fatty Acids and Hyperlipidaemias

6

J.J.A. Ferguson, C.B. Dias, and M.L. Garg

Abbreviations

AA	Arachidonic acid
ALA	α-linolenic acid
apoB	Apolipoprotein B
apoC-III	Apolipoprotein C-III
apoE	Apolipoprotein E
CETP	Cholesterol ester transfer protein
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
FFA	Free fatty acids
FXR	Farnesol X receptor
HDL	High-density lipoprotein
HNF-4α	Hepatocyte nuclear factor-4α
HSL	Hormone-sensitive lipase
LA	Linoleic acid
IDL	Intermediate-density lipoproteins
LDL	Low-density lipoprotein
LpL	Lipoprotein lipase
LXRα	Liver X receptor-alpha
NEFA	Non-esterified free fatty acids
n-3PUFA	Omega-3 polyunsaturated fatty acids
n-6PUFA	Omega-6 polyunsaturated fatty acids
PPAR	Peroxisome proliferator-activated receptors
RCT	Randomized control trials
RXRα	Retinoid X receptor-alpha
SREBP	Sterol regulatory element-binding proteins
TG	Triglycerides
VLDL	Very low-density lipoprotein

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Hyperlipidaemia

Hyperlipidaemia is a major risk factor for cardiovascular disease. It is a multifaceted condition involving multiple aetiologies and is often a consequence of lifestyle and dietary patterns and/or metabolic effects in the body [1]. Hyperlipidaemia is characterized by a vast array of

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abnormalities in blood lipid concentrations such as increased flux of free fatty acids (FFA), elevated triglycerides (TG), low-density lipoprotein (LDL) cholesterol and apolipoprotein B (apoB) levels, and decreased concentration of high-density lipoprotein (HDL) cholesterol [1].

The primary lipid abnormality is elevated circulating concentrations of non-esterified free fatty acids (NEFA) originating from adipose tissue. This is caused by insufficient FFA metabolism and esterification, as well as down-regulation of signalling pathways. As a result, retention time of fatty acids by adipose tissue is decreased, leading to increased flux of FFA returning to the liver. Additionally, this stimulates TG synthesis in the liver as well as the synthesis of apoB and the assembly and secretion of very low-density lipoprotein (VLDL). In turn, this process raises plasma TG, which promotes the formation of TG-rich HDL particles that are highly catabolic, therefore causing a reduction in HDL cholesterol in the presence of raised FFA [1]. Raised plasma TG could also represent a reduced clearance rate of TG, which is commonly demonstrated in individuals who are obese, insulin resistant, and/or possess genetic lipid abnormalities [2].

Hyperlipidaemia also involves raised LDL, which undergo lipolysis and therefore fail to efficiently bind to LDL receptors, while cholesterol esters are exchanged with TG to form TG-rich lipoproteins that are highly atherogenic, such as small, dense LDL cholesterol particles [1]. Common treatments for the management of hyperlipidaemia include pharmacological therapies such as statins, fibrates, niacin, bile acid sequestrant resins, and other inhibitors of cholesterol absorption [1]. Potential complementary alternatives for hyperlipidaemic management examined in controlled trials include a wide range of functional foods such as flaxseed, policosanols, fibre, macadamia nuts, almonds, red yeast rice, guggulipid, garlic, and soya proteins have demonstrated efficacious reductions in total cholesterol and LDL, however, no effect on TG or HDL [1].

Omega-3 Polyunsaturated Fatty Acids (n-3PUFA)

Structure

Polyunsaturated fatty acids consist of two key parent metabolically active fatty acids: linoleic acid (LA) and α-linolenic acid (ALA) [1]. In animal cells, LA and ALA are elongated and desaturated, forming the metabolically active longer chain omega-6 (arachidonic acid, AA) and omega-3 (eicosapentaenoic acid, EPA; docosapentaenoic acid, DPA; and docosahexaenoic acid, DHA) polyunsaturated fatty acids and their derivatives (2- and 3-series eicosanoids, respectively) [1]. Omega-3 (n-3) and omega-6 (n-6) PUFA are long-chain polyunsaturated fatty acids and are defined based on the location of the first double bond of the fatty acid molecule when counting from the methyl end [3]. Omega-3 and n-6 are essential PUFA because humans lack the Δ12- and Δ15-desaturase enzymes required to create a double bond at the n-3 or n-6 position of a fatty acid carbon chain. Small amounts of longer chain n-3PUFA (EPA and DHA) can be synthesized from ALA. However, LA metabolism uses the same enzymes involved in this process, therefore limiting n-3PUFA synthesis; hence, they must be obtained from the diet [3].

Dietary Sources and Metabolism

ALA is derived from plant sources and is a major component in chloroplasts of green leafy vegetables (e.g. broccoli, spinach, and cabbage), nuts (e.g. walnuts), seeds (flaxseed, rape, and chia), nut/seed oil products, and cereal products. EPA, DPA, and DHA are primarily found in oily seafood such as mackerel, tuna, salmon, herring, trout, and sardines [3]. ALA is converted into EPA, DPA, and DHA in the human body, albeit to a limited extent, and vegetarians or vegans have often been reported to have low levels of circulating long-chain n-3PUFA [4].

Omega-3PUFA and Blood Lipids

It is well established that dietary supplementation with n-3PUFA alter serum, plasma, and tissue lipid levels in a dose-dependent manner [1, 2, 5–7].

Triglycerides

Dietary supplementation with n-3PUFA at a pharmaceutical dose of approximately 3–4 g/day for 4 weeks reduces plasma TG by about 25–50 % [2, 8–10]. The hypotriglyceridaemic effect of n-3PUFA appears to be heightened with higher baseline TG levels [8, 11–14]. It has been shown that baseline TG levels >500 mg/dL are associated with greater reductions in overall TG levels after n-3PUFA supplementation [10, 12–15]. In direct comparison studies, it has been repeatedly shown that DHA induces greater reductions in TG levels, up to 6 mg/dL more compared to EPA [6, 12, 16]. It is thought this could be due to greater activation of lipoprotein lipase (LpL), a key enzyme involved in TG clearance and conversion of VLDL to LDL, by DHA compared to EPA [6]. Dietary ALA supplementation has only shown minimal TG-lowering effects, to a lesser extent than EPA and/or DHA [13, 17].

Mechanisms by Which n-3PUFA Modulate Triglycerides

Very Low-Density Lipoprotein Production and NEFAs

Plasma lipoprotein concentrations represent a balance between rate of production and/or the rate of clearance from the plasma. Therefore, elevated plasma TG are indicative of VLDL-TG overproduction that has overcome peripheral clearance, or a reduction in the rate of plasma TG clearance that is unaccompanied by a compensatory reduced hepatic production [2].

Overproduction of TG is a result of increased availability of fatty acids in the liver which is either due to diet (delivered via chylomicron remnants), lipogenesis, and/or circulating levels of NEFA [2]. Independent of metabolic state, NEFA are the key source of fatty acids for the production of VLDL-TG; therefore, a reduction in NEFA flux to the liver is required to decrease the production of VLDL-TG [2]. The consequences of n-3PUFA supplementation include reduced hepatic production of VLDL-TG and increased clearance of VLDL, achieved via a reduction in circulating plasma NEFA levels [2, 18]. Studies involving both normotriglyceridaemic and hypertriglyceridaemic individuals, including tracer studies [19, 20], have repeatedly demonstrated reductions in NEFA levels with concurrent reductions in plasma TG after n-3PUFA supplementation [2, 21–25]. Overall, these changes in NEFA flux reduce the rate of fatty acid incorporation into VLDL particles and thus appear to be the main contributor to n-3PUFA induced decline in VLDL-TG production [2]. Plasma NEFA levels are also largely attributed to the adipocyte lipolysis which is induced by hormone-sensitive lipase (HSL). N-3PUFA counteract HSL by suppressing adipose tissue inflammation and enhancing uptake of fatty acids in adipose tissue, skeletal, and heart muscle, thus minimizing 'spillover' of fatty acids into the NEFA pool [2]. Reduced production of VLDL-TG in the liver has been demonstrated as the cause for reduced plasma TG by n-3PUFA, irrespective of study differences such as cause of hypertriglyceridaemia, sample size, methodology, and tracers used.

TG Clearance and Lipoprotein Lipase (LPL) Activity

Tracer-labelled studies have revealed that n-3PUFA improve TG clearance as well as increased plasma LpL activity in the plasma of humans, with enhanced effects in the postprandial state [15, 21, 26, 27]. Improvements in postprandial TG clearance were associated with increases in LpL mRNA and post-heparin lipase in the adipose tissue of subjects with pro-atherogenic lipid status [28]. This suggests that n-3PUFA supplementation may shift the delivery of fatty acids from liver towards the adipose tissue and/or heart and skeletal muscle [2, 15, 29]. The high NEFA levels characterizing hypertriglyceridaemia increase apolipoprotein C-III (apoC-III) expression in VLDL particles which in turn inhibits LpL-mediated lipolysis. N-3PUFA promote LpL-lipolysis by blocking the accumulation of apoC-III in VLDL particles [30, 31]. Although the contribution of increased TG clearance induced by n-3PUFA may be small compared to overall reduction in TG levels, it still serves as a beneficial hypotriglyceridaemic mechanism that promotes a favourable shift in fatty acid trafficking.

Transcription Factors

Alterations in the transcription of nuclear receptors mediate the TG-lowering effects of n-3PUFA. Peroxisome proliferator-activated receptors (PPAR), sterol regulatory element-binding proteins (SREBP), liver X receptor-alpha (LXRα), retinoid X receptor-alpha (RXRα), hepatocyte nuclear factor-4α (HNF-4α), and farnesol X receptor (FXR) are the key players in lipid metabolism [1, 2, 29]. The most consistent TG-lowering mechanisms driven by n-3PUFA is the activation and regulation of PPAR. PPAR regulate the expression of proteins and enzymes involved in lipid and energy homoeostasis pathways such as increased hepatic hydrolysis of TG-rich lipoproteins; storage of fatty acids as TG in adipose tissue; reductions in free fatty acids, VLDL, and TG production; and the promotion of β-oxidation [2, 29]. EPA and DHA are endogenous ligands which activate PPAR and eicosanoid metabolites of 3-series produced from n-3PUFA, and they are more potent activators of PPAR than those of 2-series formed from n-6PUFA [2]. Tissues rich in n-3PUFA, therefore, experience enhanced PPAR activation resulting in increased fatty acid catabolism. Fish oil n-3PUFA lower TG levels via increased expression of PPAR-α which reduces the expression of apoC-III, a key inhibitor of LpL, a major enzyme involved in TG catabolism [11, 31–33]. Thus, n-3PUFA promotes low levels of apoC-III, which enhances the hydrolysis of TG and is protective against postprandial hypertriglyceridaemia [34, 35].

SREBP are transcription factors that regulate the expression of enzymes involved in fatty acid and TG synthesis, the primary activator of hepatic lipogenesis being SREBP-1c [2, 36]. The expression of SREBP-1c relies on the binding of LXRα to RXRα for activation; however, EPA and DHA inhibit this binding process and therefore prevent SREBP-1c expression and protein maturation [2, 33], leading to an overall decline in hepatic lipogenesis. DHA is a known activator of FXR, and therefore, some effects of EPA and DHA could be modulated via this receptor; however, other PUFA are more potent activators [2].

FXR regulates lipoprotein metabolism by stimulating the expression of apoC-II, an activator of LpL. Since DHA is a

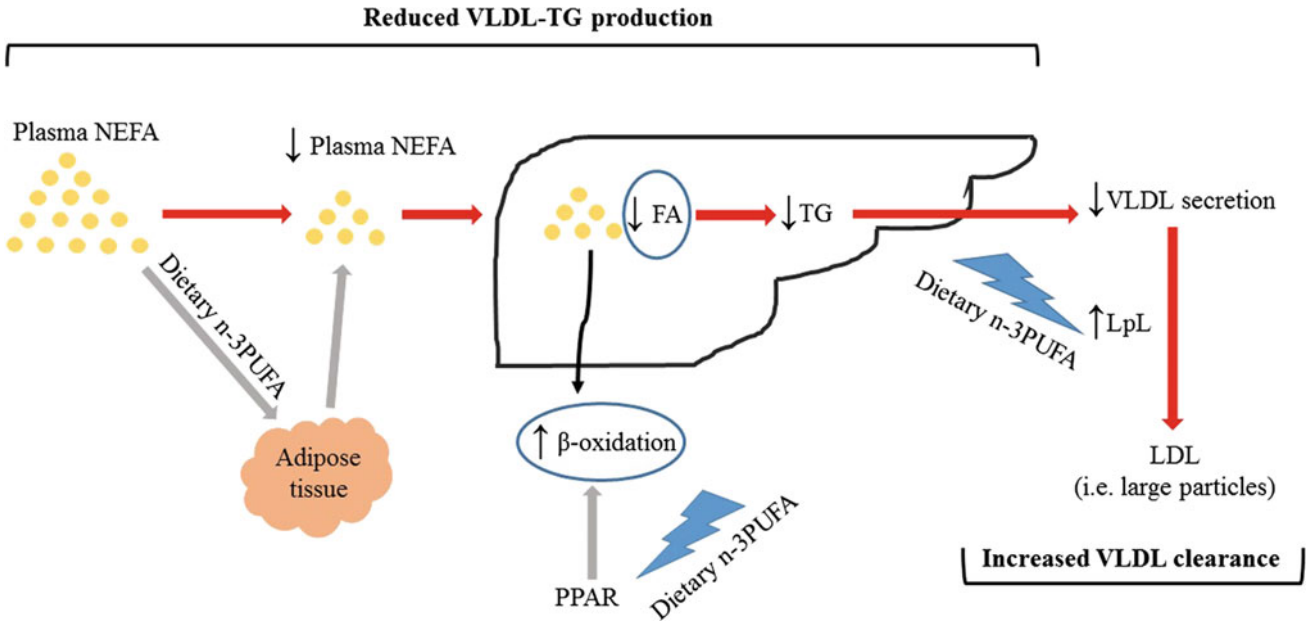

Fig. 6.1 Mechanisms by which n-3PUFA modulate triglyceride (TG) levels. Circulating non-esterified fatty acid (NEFA) levels are reduced with dietary n-3PUFA supplementation, via increased uptake of NEFA into tissues such as adipose, skeletal, and muscle; this causes a reduction in NEFA flux entering the liver. This combined with n-3PUFA induced an increase in β-oxidation via activation of peroxisome proliferator-activated receptors (PPAR) in turn reduces hepatic very low-density lipoprotein (VLDL)-TG production. Hepatic VLDL secretion is reduced, and plasma lipolytic activity is increased via stimulation of lipoprotein lipase (LpL), causing a reduction in VLDL clearance and conversion of VLDL to larger low-density lipoproteins (LDL) particles. FA: Fatty acids, ↑: increased, ↓: decreased

key activator of FXR, this could be an indirect pathway that lowers TG levels [2].

Trafficking of Fatty Acids Between Tissues

Studies on cultured hepatocytes have shown that n-3PUFA inhibit the assembly and secretion of VLDL-TG via stimulating apolipoprotein B-100 degradation [37, 38] and reduce the fatty acid pool via up-regulation of hepatocyte β-oxidation [37, 39].

Despite stimulating fatty acid uptake, dietary n-3PUFA decrease adiposity in animals on high-fat diets as well as up-regulate postprandial LpL expression in human adipose tissue [2]. Additionally, n-3PUFA promote LpL activity in heart and skeletal muscle, which are the major tissues for fatty acid utilization and where LpL-lipolysis is most efficient [2]. Thus, n-3PUFA induce increased uptake of fatty acids in adipose tissue, heart, and skeletal muscle which reduces the availability of fatty acids for hepatic lipogenesis and VLDL production (Fig. 6.1).

Total Cholesterol

The scientific literature regarding the influence of n-3PUFA on total cholesterol levels is inconclusive. Both hyper- and hypocholesterolaemic effects have been reported in animal and human studies after n-3PUFA supplementation [8, 29, 33, 38, 40]. Several reports including systematic reviews and intervention studies have concluded that n-3PUFA treatment has no significant effect on total cholesterol levels [8, 12, 13, 18, 41–43]; however, one systematic review found that fish oil supplementation increases blood cholesterol levels [12]. The lack of significant definitive findings in the literature regarding n-3PUFA and total cholesterol is indicative of the modulatory effects n-3PUFA have on total cholesterol components: LDL cholesterol and HDL cholesterol.

LDL Cholesterol

LDL Concentration

Several studies have demonstrated that TG-lowering effects of n-3PUFA are accompanied by a parallel increase in circulating LDL cholesterol [11, 27, 31, 40, 44, 45]. Intervention studies have shown fish oil supplementation can raise LDL cholesterol by 5–21 % [7, 18, 41, 42, 44]. The explanation for this is related to the increased conversion of VLDL particles to intermediate-density lipoproteins (IDL) and LDL particles, thus decreasing the fractional catabolic rate of LDL particles [27, 41]. Some studies have shown that the degree of LDL elevation induced by marine n-3PUFA treatment is dependent on the baseline TG levels, since

patients with higher baseline TG experience the greatest rise in LDL cholesterol [7, 13, 27, 31]. DHA raises LDL cholesterol levels significantly more than EPA [6, 43]. The observed increase in LDL cholesterol after n-3PUFA treatment in hypertriglyceridaemic subjects is commonly seen as unfavourable; however, it is often overlooked as indicating a potential improvement in less atherogenic, larger LDL particle size.

Particle Size

The link between LDL particle size and atherogenicity has become more widely understood in recent years. Hypertriglyceridaemia and a diet high in saturated fats is often associated with and may contribute to the formation of small, dense LDL particles (subclass pattern B) [1, 27, 45, 46]. In hypertriglyceridaemic adults, dietary supplementation with marine n-3PUFA has resulted in elevated LDL cholesterol concentration which was attributed to a shift in particle size to larger, less atherogenic LDL (Fig. 6.2). Small, dense LDL particles are considered pro-atherogenic as they are the most readily oxidized and fail to bind to LDL receptors which leads to prolonged retention time in circulation with increased likelihood of initiating or promoting atherogenic processes [46]. Larger, buoyant LDL particles (subclass pattern A) are favourable as they do not reside in the body for prolonged periods and are less likely to adhere to the arterial wall [43, 44, 46].

Minimal information is known about the effects of marine n-3PUFA on LDL particle size as only a few studies to date have examined the effects of marine n-3PUFA supplementation on LDL particle size, yielding inconsistent findings. Some studies have reported a shift in LDL particle size from subclass pattern B to A [27, 41] in hyperlipidaemic subjects. One study reported a 26 % decrease in the percentage of LDL subclass pattern A particles after dietary supplementation with 6 g/day fish oil for 6 weeks [18]. Conversely, a review found n-3PUFA supplementation did not affect LDL particle size distribution despite inducing elevated LDL cholesterol; however, the mean fish oil dosage across studies was 2.4 g/day which could be insufficient to induce such an effect [47]. In studies reporting a shift in particle size, reported pre-treatment or baseline TG levels appear to be indicative of LDL particle size shift in normo- and hyperlipidaemia individuals supplemented with marine n-3PUFA [13, 27]. The mechanism by which this occurs is unknown, and several hypotheses have been postulated. It is thought that the rate of LDL synthesis is heightened, with incorporation of n-3PUFA into the surface phospholipids of LDL subclass pattern B particles, thus changing their structure to possess antioxidant characteristics that are cardio-protective [1]. It has been reported that the key driver for shifting LDL particle size from subclass pattern B to A is the TG concentration achieved upon n-3PUFA supplementation rather than the percentage reduction in TG levels [11]. Post-dietary n-3PUFA intervention TG levels of <150 mg/dL are associated with the largest degree of conversion from LDL subclass pattern B to A [11]. Individuals with type 2 diabetes and dyslipidaemia have demonstrated a greater increase in LDL particle size after DHA supplementation compared to EPA [48], and this has also been demonstrated in mildly hyperlipidaemic men [43] and healthy subjects [48].

Further research into the clinical relevance of dietary n-3PUFA supplementation is required to ascertain the influence of n-3PUFA on the shift in distribution of LDL particle size and therefore atherogenicity of LDL.

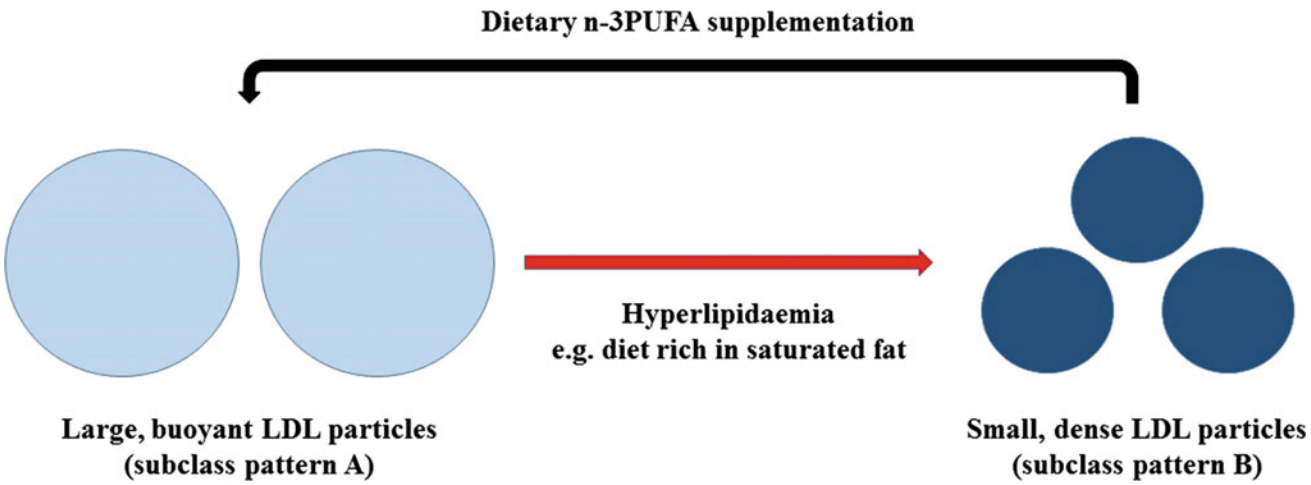

Fig. 6.2 Low-density lipoprotein (LDL) particle size distribution. Hyperlipidaemia is characterized by elevated LDL cholesterol and the formation of small, dense LDL particles (subclass pattern B) which are pro-atherogenic. Dietary supplementation with n-3PUFA has shown to redistribute LDL particle size to form large, buoyant LDL particles (subclass pattern A) which are anti-atherogenic

HDL Cholesterol

Low HDL cholesterol level is a common characteristic of hyperlipidaemia [1]. A systematic review of 47 randomized control trials (RCT) including 16,000 hypertriglyceridaemia subjects concluded that dietary n-3PUFA treatment induces only a small rise in HDL cholesterol; this is supported by other studies [8, 11, 12, 33, 43]. A recent systematic review concluded that approximately 4 g/day of EPA and/or DHA led to an average increase in HDL cholesterol of 10 % [9]; however, another review article reported a significant 13 % increase in HDL at the same dosage [14]. The key effect of marine n-3PUFA on HDL cholesterol metabolism is facilitated by a decrease in activity of cholesterol ester transfer protein (CETP) [49–51]. CETP is responsible for transferring cholesterol esters from HDL to VLDL and LDL particles in exchange for VLDL triacylglycerols, and it is up-regulated in individuals with hypertriglyceridaemia [6]. Since triacylglycerol levels are also lowered, the exchange is further lessened, promoting the formation of large HDL particles that are rich in cholesterol, rather than triacylglycerol-enriched HDL particles that are more vulnerable to degradation [49, 50]. HDL cholesterol encompass two major subfractions: HDL2, which is light, large, lipid rich and may be most protective against coronary heart disease, and HDL3, which is dense, small, and protein rich [52]. Studies have shown that approximately 4.5 g/day of fish oil supplementation has been shown to redistribute HDL cholesterol particle size from HDL3 particles to HDL2 particles in normotrigylceridaemic individuals [53] and healthy males [54].

Studies comparing the effects of EPA versus DHA on HDL cholesterol in hyperlipidaemia and normolipidaemic individuals reported greater increase in HDL after DHA supplementation [6, 13, 16, 43, 51]. After DHA supplementation, one study reported a significant 29 % increase in HDL2 cholesterol [43], and another reported a significant 50 % increase in the HDL2/HDL3 cholesterol ratio [54], and this indicates DHA may preferentially target HDL2 cholesterol and thus result in the overall net effect on HDL cholesterol levels post-treatment. In vitro studies have shown that DHA significantly reduce CETP activity compared to EPA, and it is thought this could be the explanation for the greater rise in HDL observed with DHA supplementation [55]. Although the effect on HDL cholesterol is small, dietary intervention with n-3PUFA is still beneficial as it not only induces slight elevations in HDL cholesterol levels, but potentially a shift in particle size providing protection against coronary heart disease. DHA-induced rise in HDL2 cholesterol could have noticeable clinical implications for the treatment of cardiovascular disease since HDL2 is considered to be most cardio-protective [43].

N-3PUFA and Combination Therapies

The combination of n-3PUFA with other lipid-lowering therapies has also been explored. Few studies have compared the use of n-3PUFA alone and combined to natural compounds or lipid-lowering drugs on the management of hyperlipidaemia. Combination therapies can be especially useful when dealing with mixed hyperlipidaemias.

Omega-3 fatty acid supplementation can be a powerful tool for the reduction in plasma TG and increase in HDL cholesterol. Notwithstanding the importance of increasing LDL particle size, the combined therapy may also be useful in reducing LDL cholesterol [27, 31, 40, 44, 45, 56], thus inducing an overall anti-atherogenic blood lipid profile. Studies combining n-3PUFA with other lipid-lowering therapies are few and often contradictory.

Combined n-3PUFA and Lipid-lowering Drug Therapy

Statin treatment is a common tool in the prevention of hyperlipidaemia, although the use of statins may incur in serious side effects such as muscle weakness, increased risk of type 2 diabetes, erectile dysfunction, acute kidney failure, and cataract formation, among other effects [57, 58]. The co-administration of n-3PUFA and statin treatments may allow a reduction in statin doses and consequently a reduction in side effects. Studies suggest the lipemic response may be dose dependent, indicating no improvement in plasma TG with n-3PUFA supplementation at a dose of 1.9 g or lower. Hypercholesterolaemic subjects undergoing statin treatment did not see any improvement in plasma TG when supplemented with 1.9 g n-3PUFA (EPA + DHA) daily for 20 weeks [59]. Statin users receiving 1.8 g EPA daily for 48 weeks did not present any significant difference in change of TG levels when compared with subjects who did not receive n-3PUFA [60]. However, studies providing higher doses of n-3PUFA (minimum 2.4 g daily) have observed significantly lower plasma TG levels in hyperlipidaemic patients [61–63]. Statin users on a 41-month trial presented improvement in plasma TG when consuming 0.4 g EPA + DHA and 2 g linolenic acid daily, but not 0.4 g EPA + DHA alone [61]. Subjects with mixed dyslipidaemias undergoing statin treatment (simvastatin) presented significantly lower plasma TG levels when supplemented with 3.6 g n-3PUFA (EPA + DHA) daily for 6 weeks [62]. Subjects treated with atorvastatin and 3.36 g n-3PUFA had a significant reduction in plasma TG, total cholesterol, VLDL cholesterol and non-HDL cholesterol compared to subjects treated with atorvastatin only [63].

Despite the proven LDL cholesterol increasing effect of n-3PUFA, the change in total and LDL cholesterol was no different between patients on statin treatment with or without n-3PUFA supplementation [59, 60, 62–64]. This may suggest that statins may be able to neutralize the n-3PUFA potential to increase LDL cholesterol.

LDL cholesterol particle size has also been discussed. LDL particle size was shown to increase in subjects with type 2 diabetes and mixed dyslipidaemias under statin treatment, independently of n-3PUFA supplementation during an 8-week intervention, although greater increase was observed for subjects consuming 4 g n-3PUFA in association with statins [65]. Statin therapy alone has previously been shown not to increase small, dense LDL particles in patients with and without coronary artery disease [66]. Hence, n-3PUFA combined with stating therapy would increase LDL particle size while maintaining low levels of LDL concentration.

Combined n-3PUFA and Other Natural Therapies

Natural therapies, such as plant sterols and fibre, have also been explored for the management of hyperlipidaemias. Their combination with n-3PUFA may offer a more extensive approach when dealing with heart health, not only improving blood lipid profile, but also providing additional health benefits without side effects.

Plant sterols or phytosterols are substances found in all plant-based foods and present a similar structure to cholesterol. Plant sterols effectively reduce intestinal absorption of dietary and biliary cholesterol, consequently reducing plasma cholesterol levels [1]. Therapies combining 1.7 g plant sterols and 5.4 g n-3PUFA daily have reported improved fasting plasma TG, compared to plant sterols and n-3PUFA alone [1]. Those subjects also presented a greater decrease in total cholesterol-to-HDL cholesterol ratio compared to subjects consuming fish oil alone. However, Micallef and Garg [1] and Khandelwal et al. [67] indicated no further improvement in plasma TG or HDL cholesterol in subjects consuming an n-3PUFA and plant sterol mixture compared to fish oil alone. Furthermore, Khandelwal indicated that n-3PUFA counteracted the non-HDL cholesterol-lowering effects of plant sterols [68] and did not affect LDL cholesterol [67, 68]. In contrast, Micallef and Garg [1] demonstrated that the combination of plant sterols and n-3PUFA decreased total and LDL cholesterol when compared to n-3PUFA alone.

Plant sterols and n-3PUFA were also combined with B vitamins, causing a significant decrease in total, LDL, and VLDL cholesterol, and no significant change in HDL cholesterol or TG from baseline to 16 weeks of intervention [69]. However, the authors did not compare their results with a fish oil- or plant sterol-only control group.

Dietary fibre is another natural tool in the management of blood lipids. It has shown to reduce total and LDL cholesterol in hypercholesterolaemic subjects [70, 71] and reduce LDL cholesterol in healthy subjects [72] with no effect on HDL cholesterol or triglycerides [70–72]. β-glucan, a soluble fibre, has been shown to decrease total and LDL cholesterol, while increasing HDL cholesterol [73]. Therefore, the addition of fibre to a diet rich in n-3PUFA would contribute to further improve blood lipid profile. However, studies considering this combination are lacking. In animal studies, consumption of soluble fibre (as psyllium) has improved total cholesterol, especially VLDL cholesterol, in animals consuming a high cholesterol and n-3PUFA diet, although total cholesterol remained higher in animals consuming a control diet rich in n-6PUFA and cholesterol [74]. The findings of these studies are promising and warrant further investigation using the combination of dietary fibre and n-3PUFA therapy for an effective management of hyperlipidaemias.

Research on combination of n-3PUFA with other lipid-lowering therapies indicated further improvement in TG, total cholesterol levels, and LDL particle size with no significant change in LDL cholesterol levels. Thus, indicating that combining n-3PUFA with other lipid-lowering therapies may be important for optimizing blood lipid profile in hyperlipidaemia patients.

The effect of combination therapy on blood lipid profile seems to be dependent on the dose of both n-3PUFA and the complementary strategies.

Factors Affecting n-3PUFA Efficacy in Hyperlipidaemia

Apolipoprotein E Polymorphisms

Apolipoprotein E (apoE) is a structural and functional entity of lipoprotein complexes such as chylomicrons, VLDL, LDL, and HDL [18]. ApoE plays an essential role in lipid metabolism, particularly in the removal of atherogenic remnants from TG-rich particles, reverse cholesterol transport, cellular cholesterol efflux [75], and the clearance and metabolism of lipoproteins by the liver [18]. ApoE has also been shown to be positively associated with hyperlipidaemias [76]. ApoE exists in three common isoforms: apoE2, apoE3, and apoE4, and the majority of people possess the E3 allele [18, 77]. Polymorphisms in the genes that code for ApoE cause some forms of familial hypercholesterolaemia [78]. ApoE4 increases blood LDL cholesterol levels via preferential binding to VLDL and remnants, which

accelerates their clearance causing a down-regulation of LDL receptors [78]. Postprandial studies have reported higher levels of total cholesterol, LDL cholesterol, small dense LDL particles, and lower levels of HDL cholesterol in apoE4 carriers [18, 75, 76, 78], and intermediate and low levels in E3 and E2 carriers, respectively [18] with E2 carriers tending to have higher TG levels [76, 77]. Findings are inconsistent with respect to apoE genotypic variants and TG response to fish oil supplementation. One study reported no difference in the hypotriglyceridaemic effect between genotypic subgroups [79], and another reported minimal impact of apoE genotype on the fasting TG fraction except E2 carriers experiencing a greater reduction in postprandial response [18]. A meta-analysis revealed E2 carriers experienced greater reductions in total cholesterol and LDL cholesterol after statin therapy, followed by E3, and E4 carriers [77]. Conversely, DHA oil supplementation for 4 weeks in healthy normolipidaemic males induced a greater rise in LDL cholesterol levels in E4 carriers compared to E3 carriers [79]. Another study reported a significantly greater reduction in the percentage of small, dense LDL particles in E4 carriers after fish oil supplementation, followed by E2 and E3 carriers [18]. Biokinetic studies have reported that differing LDL cholesterol response among apoE genotypic variants to fish oil is attributed to a greater conversion of VLDL to LDL in E4 carriers compared to other apoE allele counterparts [79–81]. Recent evidence suggests apoE genotypes induce different effects on lipid metabolism according to age [18]. A study reported E4 carriers, aged >50 years, had 29 % higher fasting TG levels compared to E3/E3 individuals and the reason for this is unknown [75].

Studies conducted to date suggest that apoE4 carriers have a 40-50 % increased risk of developing cardiovascular disease; however, large, standardized intervention trials according to apoE genotype are warranted to establish the impact of diet (i.e. saturated fat, total fat, fish oil) and lifestyle factors (i.e. smoking, weight) on genotype–lipidemic associations. The differences in lipid response to n-3PUFA supplementation across apoE genetic variants suggest not only an impedance on the efficacy of n-3PUFA treatment and management for hyperlipidaemias, but also a key determinant for the risk of developing cardiovascular disease [77, 79].

Gender Differences

It has been widely reported that females have higher circulating DHA concentrations compared to males, independent of dietary intake [82–86]. Fatty acid stable isotope studies have confirmed that endogenous conversion of ALA to DHA in females is higher [82, 83, 85, 87] and appears to be associated with oestrogen levels [82]. There is substantial evidence to suggest that oestrogen has indirect effects on lipid metabolism such as increased PPARα activity and subsequently enhanced DHA biosynthesis [82]. Oestrogen interacts with PPARα gene transcription which leads to increased PPAR activity [82], and oestrogen up-regulates the conversion of ALA to DHA in: post-menopausal women on hormone replacement therapy or women taking the oral contraceptive pill or even male-to-female transsexuals receiving oral forms of oestrogen [85, 88]. In turn, this heightened activity of PPARα is thought to cause increased transcription of enzymes involved in DHA biosynthesis [82]. The evidence is varied with respect to EPA composition, as some human and animal studies have reported higher EPA levels in males compared to females [84, 85, 89, 90], whereas some studies report no difference [88, 91–93]. Interestingly, a systematic review of 51 publications and another study reported lower levels of DPA (the intermediary between EPA and DHA) in total plasma lipids of females compared to males [83, 86], and this could indicate a greater conversion rate of DPA to DHA.

In support of these findings, a recent study conducted by Ferguson et al. [94] demonstrated that females aged 65 years and over had significantly higher n-3PUFA status (percentage of erythrocyte EPA and DHA [95]) when compared to male counterparts. After controlling for confounding factors (e.g. age, BMI, dietary intake), only females demonstrated a negative association with erythrocyte n-3PUFA status and TG levels.

As discussed in previous sections of this chapter, strong evidence suggests that DHA induces a greater hypolipidaemic affect than EPA, and given females have greater circulating concentrations of DHA compared to males, gender could be a potential influencing factor on the efficacy of n-3PUFA on the treatment and management of hyperlipidaemia.

Further understanding of the interactions involved is vital in order to comprehend the potential health benefits of dietary n-3PUFA and to help determine appropriate dietary and pharmacological recommendations for ALA, EPA, DPA, and DHA for not only hyperlipidaemic management but to maximize overall n-3PUFA intake.

Conclusion

Hyperlipidaemia is a multifaceted condition and continues to be a major risk factor for cardiovascular disease. N-3PUFA are diet-derived fatty acids that play a pivotal therapeutic role in the management, treatment, and prevention of hyperlipidaemias. N-3PUFA modulate key pathways involved with lipids such as the synthesis and clearance of TG, fatty acid trafficking between tissues, LDL cholesterol

concentration and particle size, and HDL cholesterol concentration. The interaction between n-3PUFA and lipidemic pathways in the body is influenced by gender and genetic polymorphisms, and further investigation into these factors is warranted to determine their impact on the efficacy of n-3PUFA as a therapeutic agent for hyperlipidaemias.

Summary

Hyperlipidaemia is a multifaceted risk factor for cardiovascular disease, involving multiple aetiologies such as diet, lifestyle, and/or metabolic effects within the body. Hyperlipidaemia is characterized by a vast array of lipid abnormalities which promote the development of pro-atherogenic blood lipid profile and in turn, cardiovascular disease. Common treatments for hyperlipidaemia involve pharmacological therapies which can induce severe side effects. Dietary n-3PUFA offer a natural, safe, and effective alternative to the prevention and treatment of hyperlipidaemias.

N-3PUFA are well known for their modulatory effects on blood TG levels. TG are dose dependently reduced after n-3PUFA supplementation, with greatest reductions seen with higher baseline TG levels. Moreover, contrary to the general belief, it has been hypothesized that co-administration of n-3PUFA with saturated fats may offer better outcomes as saturated fats may allow greater incorporation of n-3PUFA into blood/tissue lipids [96]. This is achieved by an overall decrease in plasma NEFA levels via reduced hepatic production of VLDL-TG and increased clearance of VLDL; improved TG clearance; increased plasma LpL activity; activation and regulation of transcription factors which regulate lipid metabolism; and increased fatty acid uptake into tissues. Total cholesterol remains unaffected by dietary n-3PUFA intervention; however, this is likely indicative of the modulatory effects n-3PUFA have on total cholesterol components such as LDL cholesterol and HDL cholesterol.

Several studies have demonstrated that TG-lowering effects of n-3PUFA are accompanied by a parallel increase in circulating LDL cholesterol, which is attributed to an increase in particle size to that which is anti-atherogenic. The link between dietary n-3PUFA and LDL particle size requires further research as few studies have examined this link and some present inconsistent findings. Studies that have reported a shift in particle size from small, dense LDL to large, buoyant LDL particles suggest baseline TG levels are indicative of n-3PUFA induced LDL particle size shift; however, further investigation is warranted in order to confirm the mechanism behind this observation.

Dietary n-3PUFA induce only a small rise in HDL cholesterol, and the degree of elevation among studies varies; however, DHA has been shown to have a greater effect on raising HDL levels compared to EPA.

Studies combining n-3PUFA with other lipid-lowering therapies are few and contradictory. Combined n-3PUFA and lipid-lowering drug therapies may allow a reduction in drug dosage and therefore potentially minimize side effects. Combined n-3PUFA and natural therapies such as plant sterols have shown noticeable beneficial effects in fasting plasma TG compared to administration of either substance alone. Both combined therapies are dependent on the dose of n-3PUFA and the complementary strategies.

ApoE polymorphisms and gender differences are key factors affecting the efficacy of n-3PUFA in hyperlipidaemias and influence an individual's susceptibility towards hyperlipidaemic blood lipid profile and the beneficial health effects of n-3PUFA. Further research and intervention trials examining these key factors are necessary to ascertain appropriate and individualized dietary strategies and recommendations for the therapeutic use of dietary n-3PUFA.

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World Market of Omega-3 Fatty Acids

7

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List of Abbreviations

ALA	Alpha-linolenic acid
EPA	Ecosapentanoic acid
DHA	Docosahexanoic acid
SDA	Stearidonic acid
AA	Arachidonic acid
GOED	Global Organization for EPA & DHA
PUFA	Polyunsaturated fatty acid
CAGR	Compound annual growth rate
USFDA	United States of America Food and Drug Administration
RDI	Recommended daily intake
FAO	Food and Agriculture
WHO	World Health Organization
IBD	Irritable bowel syndrome
OTC	Over the counter
US	USA
EU	European Union
TCI	Technology Crops International
GLA	Gamma-linolenic acid
EC	European Commission

Introduction

When Hippocrates, the father of medicine, was asked how could a person remain healthy, he answered: "Let food be your medicine." He reminds us today that we are what we eat and therefore must make careful choices in order to maintain a healthy and disease-free life.

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Since last few decades, consumers more and more believe that foods contribute directly to their health [2, 3]. It was mainly the advances in understanding the relationship between nutrition and health that resulted in the development of the concept of functional foods, which means a practical and new approach to achieve optimal health by promoting the state of well-being and reducing the risk of disease. A nutraceutical is a food or a part of a food with demonstrated safety and health benefits that go beyond the basic dietary needs and is presented in "a non-food matrix or non-conventional food format." This definition clearly differentiates nutraceuticals from functional foods; the functional foods are the products in a conventional food format with added substances, which promote a healthy state in an

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individual; and a nutraceutical needs to be presented in a non-food matrix or non-conventional food formats [4]. In European law, nutraceuticals are labeled as food supplements. The most important regulations on European level that control nutraceuticals are Directive 2002/46/EC, which establishes harmonized rules for the labeling of food supplements and introduces specific rules on vitamins and minerals in food supplements [5], Regulation (EC) 1924/2006 on nutrition and health claims made on foods [6] and Regulation (EC) 258/97 on novel foods [7].

Coinciding with the emergence of nutraceuticals, consumer demands have changed considerably toward food products that contribute directly to their health, with correspondingly high expectations of consumers and the food industry [2, 8–10]. The global nutraceuticals market was estimated to be US $176.6 billion in 2013 [11, 12]. In the last twenty years, the emerging nutraceuticals technology has created a global market with impressive growth rates estimated between 15 and 20 % annually [13, 14], with Japan, USA, and the European Union (EU) as major markets (global market share 39, 31, and 28 %, respectively). The development and commerce of these products is rather complex, expensive, and risky, as special requirements should be answered. Besides potential technological obstacles, legislative aspects, as well as consumer demands, need to be taken into consideration when developing functional food. In particular, consumer acceptance has been recognized as a key factor to successfully encash market opportunities.

Types and Sources of Omega-3 Fatty Acids

Omega-3s belong to a broader group of fats called polyunsaturated fats, found in a wide variety of foods, most famously in fish. They are classified as essential fatty acids because our body cannot synthesize them but they are necessary for the regular metabolic activities. These are found in plants and marine life. Common available source of omega-3 fatty acids include algae, flax, and fish. Omega-3 fatty acids are associated with numerous health benefits. One of the biggest proven health benefits is its ability to lower the level of bad cholesterol-LDL cholesterol thereby leading to lower risk of coronary heart diseases and blood pressure. Because of recent research suggesting potential cardiovascular prevention and other health benefits, omega-3 fatty acids are currently a hot topic in nutrition research. Omega-3 contains at least three double bonds that make them more unstable, delicate, and susceptible to damage.

There are 3 major types of omega-3 fatty acids:

1. Alpha-linolenic acid (ALA) C18:3
2. Eicosapentaenoic acid (EPA) C20:5
3. Docosahexaenoic acid (DHA) C22:6.

1. **Alpha-linolenic acid (ALA)** is the most common omega-3 fatty acid in the vegetarian diet. It comes from plants and is found in vegetable oils—primarily flaxseed, walnut, canola, and soybean oils. ALA is a strictly essential fatty acid and is used in our body to produce EPA and DHA. Flax seed oil, which contains more than 50 % ALA, is the most commonly used in supplements. Other good sources include chia seed oil, perilla oil, lingonberry seed oil, and kiwifruit seed oil. Purslane, a vegetable used in soups and salads along the Mediterranean basin and in the Middle East, is unique because it is the richest source of ALA and one of the few plants known to contain EPA. Under the right circumstances, our bodies can transform ALA into EPA and DHA. Our body's ability to make EPA and DHA from ALA partly depends on the other types of fat that we eat. One of those other fat types is omega-6 fat. Omega-6 fats are more plentiful in foods than omega-3 fats; therefore, we often consume much more of them. The high consumption of omega-6 fats can directly reduce the amount of ALA that our body converts into EPA and DHA. Our body cannot do an effective job of converting ALA into EPA and DHA without a satisfactory supply of certain nutrients such as vitamin B3, vitamin B6, vitamin C, and the minerals such as zinc and magnesium [15]. If we are deficient in one or more of these nutrients, our bodies may not be able to provide optimal amounts of EPA and DHA, even when our ALA intake is sufficient. The studies showed that the conversion rate of ALA to EPA/DHA can be as high as 21 % in young women [16]. There have also been some recent publications that show in non-fish-eating diets that the conversion rate is very high as well [17].
2. **EPA** and 3. **DHA** are known as the "long-chain" or marine omega-3 fatty acids since they are mainly found in fish and fish oils. Fish oil is the most widely used commercial source of omega-3 due to its high content of EPA/DHA. Further EPA and DHA are claimed to have the most potent health benefits of the omega-3 fatty acids. EPA plays a role in cardiovascular health and is recommended by the American Heart Association for its cardiovascular benefits. DHA is the most abundant in the brain and retina and is an important structural component of the nerve cells in the brain and eyes and a key component of the heart. The fish do not make EPA and DHA themselves; rather, they consume them in the form of algae and phytoplankton. Hence, algae, and phytoplankton, themselves can be used as an omega-3 source. These include Crypthecodinium cohnii, which is used industrially to produce DHA by fermentation, and Phaeophyceae brown algae, which contains EPA. As EPA and DHA are naturally contained in algae, the focus on obtaining EPA and DHA from algae was

prompted to seek "vegetarian," and sustainable sources of EPA and DHA which are more acceptable than fish.

Three types of systems are currently used for algae cultivation:

1. Open-pond cultivation (autotrophic)
2. Photobioreactors (closed system)
3. Fermentation (heterotrophic).

Each system has its own benefits. In an open-pond system, algae are grown in open bodies of water (either shallow ponds or open containers or tanks) in seawater and natural exposure to sunlight. It is a simple system that requires land for growing but thereafter relies on natural "inputs" instead of expensive equipment. Open-pond cultivation method allows to grow algae on arid, barren land, in seawater without use of expensive equipment and enclosures, hence can be scaled up to thousands of acres, benefiting from significant economies of scale. A variation on an open-pond system and a photobioreactor system also employs natural sunlight but in a closed system which may provide more control over conditions such as sunlight exposure, temperature, and protection against contamination. The third type of system, fermentation, is a closed system that has no exposure to sunlight but relies on sugar to ferment and grow algae in fermentation vessels. DSM and Lonza use this system. Both companies stress that this process ensures end products are free of sea-borne contaminants like heavy metals often associated with omega-3s from fish sources. Also like different types of fish, algae strains differ in the ratio of EPA:DHA. In general, heterotrophic algae naturally contain a higher DHA % while autotrophic algae tend to contain a higher EPA % [18].

Krill

Presently, the krill oil market may be a smaller fraction of the omega-3 supply market compared to other major sources. But it is growing more quickly and will soon achieve premium position and value as the consumers like the benefits of krill. In the past few years, Antarctic krill has started to be used as a source of omega-3 oils. According to Frost & Sullivan/GOED (Global Organization for EPA & DHA), the krill ingredients market saw an astounding 41.5 % growth last year. Krill's EPA and DHA fatty acids are bound primarily to phospholipids, as compared to the DHA/EPA fatty acids in fish and algae that are primarily bound to triglycerides. Fish EPA/DHA which are bound to triglycerides need to be broken down by the liver to release the oils, while krill EPA/DHA which are present in the form of phospholipids can be used by the cells directly therefore have higher bioavailability. Hence, a greater effect is seen from a smaller dose of krill oil. Further it also contains the antioxidant astaxanthin, which aids in preventing the oxidation of the omega-3s, thus helping to stop them from going rancid [17].

The Omega-3 Market

The omega-3 market is essentially "**Innovative market**" driven by years incredible research studies that have underscored the significance of omega-3 fatty acids in maintaining overall health (including heart, brain) in addition to safeguarding against cancer and birth defects and offsetting symptoms of diabetes, arthritis, cognitive decline, depression, and several other conditions [19]. Scientific research continues to be the backbone of this phenomenal global growth and drives the demand for higher quality and quantity of omega-3 products. No other type of fat than omega-3s has been getting more publicity, due to its' incredible health benefits. However, much of the omega-3 publicity has been focused on dietary supplements rather than food. In particular, more consumers are increasingly aware of the health benefits of consuming omega-3 products and are increasing their own per capita consumption. Also, omega-3s' proven health benefits have increased consumers willingness to pay for omega-3s and have convinced of their long-term health and wellness benefits. Where omega-3s are available in a conventional food format, they are considered as functional foods, for example omega enriched food products—bread, eggs, and milk while omega-3s in a non-food matrix or non-conventional food formats, they are considered as a nutraceuticals example softgel capsules, omega-3 concentrates. Here, while discussing omega-3 World market, we have clubbed together functional food—omega-3s and nutraceutical omega 3s.

Worldwide, consumption of omega-3 polyunsaturated fatty acids (PUFAs) was estimated at 123.8 thousand metric tons worth US$2.3 billion in 2013, and 134.7 thousand metric tons valued at US$2.5 billion in 2014. By 2020, it is projected that demand for omega-3 PUFAs globally would reach 241 thousand metric tons with a value of US$4.96 billion, thereby posting a volume CAGR of almost 10 % and a value CAGR of 11.6 % between 2013 and 2020 [20].

A new market report published by Transparency Market Research suggests the global omega-3 (EPA/DHA) ingredients market could be worth $4 billion in 2018, growing at a CAGR of more than 15 % from 2013 to 2018. Global demand for omega-3 ingredients was estimated to be worth $1.56 billion in 2010 and is expected to cross $4 billion in 2018. In the overall global market, for omega-3 products North America is the largest market (38.5 %), followed by Europe (21.9 %), China (13.6 %), rest Asia (12.4 %),

rest world (9.9 %), and Japan (3.7 %). Asia–Pacific is expected to be the most promising market in the near future, according to the report, "Global Omega-3 (EPA/DHA) Ingredients" [21].

The Omega-3 World Market for Ingredients

The world leader on the production and exports of flax seed is Canada; in 2012, the country produced 518.2 thousand tons. At the same time, Canada slightly decreased exports: 391 thousand tons as opposed to 404 thousand tons earlier. According to the data of Oil World in 2011/12 flax seed world production totaled 2.1 million tons and essentially exceeded the last year production (1.82 million tons) [22]. The largest planted areas of flax seed are in Canada, China, USA, Russia, Kazakhistan, and Ukraine. The major buyers and consumers of flaxseed are the EU countries. The large-scale importers are also China and the USA.

Fish and fish oil being rich source of EPA and DHA, continue to dominate the omega-3 ingredient supply. Fish oil is the chief raw material used for extraction of omega-3 ingredients. However, the reduced or static production levels of fish oil act as a major inhibitor for the growth of the market. This situation is expected to escalate owing to the uneven frequency of the El Nino, which further reduces the overall fishing volumes. The fish oil supply has remained around the 1000 tons mark for the past few years, and FAO estimates suggest that the supply is not expected to increase significantly in near future. This global situation of fish oil market has opened vast opportunities for the development of alternate sources such as flax seeds and algae for resourcing omega-3 fatty acids. The global omega-3 fatty acid demand was 21.9 kilo tons in 2012 and is expected to increase to over 60 kilo tons by 2020, at a CAGR of 13.7 % from 2014 to 2020. Anchovies and sardines lead, accounting for close to 80 % of supply, according to Global Organization of EPA and DHA (GOED)/Frost & Sullivan. They are followed, to a much smaller extent, by cod and tuna, which each account for less than 10 % of the market. Part of the appeal of anchovies and sardines is their high EPA/DHA content per fish, said Ellen Schutt, GOED's communications director. Oily fish such as herrings, mackerel, and sardines contain particularly high levels of omega-3s, with the levels of each oil varying between fish. Frost & Sullivan estimated 2011 global revenue of $1.86 billion for DHA and EPA ingredients market. More significantly, it predicts a 13.6 % CAGR for 2012–2016. China is now the third largest market for EPA and DHA oils which consumes around 14,009 metric tons next to North America (39,806 metric tons) and Europe (22,574 metric tons). China will be larger than Europe shortly. EPA and DHA oil consumption figures for some of the other part of the world are—Japan 3809 metric tons, Rest of Asia 12,792 metric tons, and Rest of the World 10,259 metric tons [17, 18].

Increased consumer demand and technological advancements are the key factors driving omega-3 fatty acid toward the mainstream food products. Omega-3 fatty acids are one of the most extensively researched and scientifically proven functional ingredients in the market place. The proven benefits of supplementation with omega-3 fatty acid and the consequent increase in consumer demand have put them at the forefront of the functional ingredients sector. Positive media coverage on the proven health benefits of omega-3 fatty acid and the consequent increase in consumer confidence has also given them an edge in the functional ingredients market. Nutrition Business Journal valued the US market at more than $600 million in current figures—and growing at around 30 % per year. New Nutrition Business magazine puts a similar figure on the European market and predicted both would soon reach $1 billion mark. Frost and Sullivan's European analysis revealed omega-3 fatty acid market growing at 24.3 % annually and projected to be $1.6 billion by 2014. This figure includes marine, algal, and flaxseed sourced omega-3s. A study from the Institute of Entrepreneurship and Innovation from Vienna University of Economics and Business estimate a worldwide growth up to 2 billion USD till 2019 in the consolidated omega-3 market.

On the downside, the market is ridden with legislative challenges. One such challenge is the proposed rule of US Food and Drug Administration (FDA) that restricts the nutrient content claims of omega-3 fatty acids. Due to this all the claims that characterize the level of EPA and DHA in foods categorized as "high," "more," or "good source" will be banned," says the analyst. At a time when the market for omega-3 fatty acid is witnessing robust growth due to escalating interest from consumers and health professionals, a ban on the nutrient content claim poses a major challenge to the growth of the omega-3 fatty acid market. However, regulatory uncertainties did not deter the surging US Omega-3 fatty acid market. GOED is actively involved in lobbying with the regulatory authorities worldwide and is working toward establishing a recommended daily intake (RDI) that would enable the use of nutrient content claims in the USA," notes the analyst. There are clear signals that emerging economies such as India and China are also interested in omega-3 fatty acid products.

The global omega-3 ingredients industry is marking major milestones. The omega-3 ingredients market is witnessing continued growth in established sectors such as supplements and functional foods. Omega-3 ingredients market includes EPA and DHA omega-3s from marine oils such as fish oils, krill oils, squid oils, and algal oils. The end use includes dietary supplements, food and beverages, pet nutrition, infant nutrition, pharmaceuticals, and clinical nutrition. There is also renewed interest in the rapidly growing innovative

markets as big pharmaceutical companies have launched "Prescription Omega-3S" in market. One of the primary reasons for the continued development and growth of the omega-3 ingredients market is the fact that the application areas of omega-3 are increasing while existing applications are finding new markets. For example, infant formula is an application segment of omega-3 that has found incredible attention in the recent past. The global market revenue for marine and algae EPA and DHA ingredients has surpassed $2 billion in industry revenue in 2012. [23]. The market is expected to grow at a double-digit rate from now to 2016, due to China and the rest of the Asia–Pacific region quickly gaining market share in terms of unit demand; thus, these regions will offset demand growth in the European market.

The Omega-3 World Market for Application Segment

The following are the major application segments [21]:

1. Supplements and functional foods
2. Pharmaceuticals
3. Infant formulas
4. Pet and animal feed
5. Others such as clinical nutrition and beverages.

Supplements and functional foods was the largest application segment with a share of over 55 % and consuming close to 12.8 kilo tons of omega-3 ingredients in 2012. Pharmaceutical and infant formulas were the other large application segments together accounting for 33 % of the global revenue. Consumption of omega-3 ingredients in infant formula is expected to be the fastest growing segment over the forecast period with an estimated CAGR of 15.3 % from 2014 to 2020. The infant formula market is the world's fastest growing packaged food category, primarily due to the development of markets in Asia (particularly China) and Eastern Europe and to a lesser extent in the Middle East and Latin America.

Global Consumption of EPA and DHA Omega-3 Products in 2011 shows that dietary supplements (62,569 metric tons) dominate consumption followed by pet foods (21,623 metric tons) then Fortified Food and Beverage (12,950 metric tons) Infant Formula (3457 metric tons) and Pharma (1922 metric tons) [18, 24].

1. Supplements and Functional Foods

Today, the dietary supplement segment remains the largest segment of the omega-3 market, but growth in this area is expected to decrease over time due to market saturation. In turn, the pharma segment is expected to see a significant share gain, due to traditional supplement consumers selecting higher quality supplement products and doctor-prescribed alternatives. Consequently, the concentrates are expected to see significant growth in market share relative to standard fish oils. In 2012, supplements and functional foods was the largest application segment with a share of over 55 % and pharmaceutical and infant formulas were the other large application segments together accounting for 33 % of the global revenue.

Global production of omega-3 products is estimated at 2.49 million metric tons, worth $4.5 billion in 2013 and expected to grow a mammoth 32.8 % annually in volume (15.1 % in value) by 2018, by which time 3.3 million metric tons worth $9.1 billion will be produced. "According to Amadee Bender, founder, CEO of Amadee & Company, Inc. plant omega-3 production value is expected to grow twice as fast as marine during the next five years; plant omega-3s will account for 52 % of production value compared to 48 % for marine. Plant omega-3s will benefit from consumers' desire to get away from animal-based products The EPA/DHA-fortified foods and beverages will reach $10.2 billion, EPA/DHA nutritional supplements will reach $4.6 billion, EPA/ DHA pharmaceuticals will reach $3.1 billion, and clinical nutrition products will reach $1.7 billion.

Supplement Formats:

The other ways in which consumers take in omega-3s are fortified foods. Globally increasing numbers of fortified food packs are being flooded on the supermarket shelves highlighting their omega-3 content. The fish oil supplements such as cod liver oil in past was given from a bottle by the spoonful form, but today, this is not preferred delivery form due to unpleasant taste. In addition as soon as it comes into contact with the air, it starts to oxidize and begins to get fishy taste and smell due to rancidity. Refrigeration can help, but this has led to the development and rise in popularity of alternative dosage forms like softgel capsules [25].

- **SOFTGELS** are the perfect delivery form for omega-3s; hence, nowadays the supplement market is dominated by softgel capsules. The consumers find softgels are easy and convenient to take, and importantly, the gelatin shell provides protection from oxidation, preserving the quality of the oil [26].
- **CHEWABLE SOFT GELS**: This form can improve palatability and consumer acceptance. The softgel is made chewable by mixing the gelatin with a higher proportion of potato or corn starch than in a regular softgel. Inside the capsule, the omega oil will be mixed with flavoring agents, usually in the form of oils, and carriers such as silica to give the contents the desired

texture. On chewing, the gelatin capsule will start to dissolve and the oil released. They need to be handled and packaged with special care because of the reduced strength that the chewable nature of the softgel imparts [27].

- Another format is **chewable tablets** are developed and aimed at children that usually involve dispersing coated oil in microencapsulated form into an excipient powder. These were specifically designed to meet the growing market demand for omega-3 products for children, in the light of research reports that suggested that omega-3 fatty acids do give behavioral improvements and improve brain development in the young. These solid dose forms are generally pleasant and easy for children to take, but the levels of omega-3 fatty acid are quite low and are more expensive. This results in need of taking more doses per day to meet recommended daily intake levels. Another problem is that the high compression force required for tableting can crack the coating on the oil, allowing air to come into contact with the oil, causing its oxidation. This may result in the tablets getting unpleasant fishy taste. Despite of all these facts, their popularity is growing. A better flavor can be achieved with gummies, because of the high levels of sweeteners they contain. However, they can probably be better considered confectionery products with a slightly healthier "edge" than a true dietary supplement [28].
- Omega-3 fatty acids can also be spray-dried into powders by mixing with other ingredients, such as starches or proteins, which protect them against oxidation. Omega-3 powders are preferable in products such as baked goods or functional beverages, where the ingredient has greater dispersibility and enhanced stability, as well as better flavor masking. In liquid applications, omega-3 fatty acids often need to be mixed with emulsifiers to make it water miscible and prevent oil separation [25].

2. **Pharmaceuticals**

Pharmaceuticals are about treating disease conditions and the dosages required to do so exceed the amount of EPA and DHA one can put into supplements; hence, pharmaceutical companies have started incorporating higher concentrations of omega-3s. The EPA/DHA **pharmaceuticals** market is projected at $3.1 billion in 2016, with a five-year CAGR of 10 %. Moreover, due to entry of generic category in omega-3 pharmaceutical market, it will surpass the said projections. Anticipated dollar growth in Asia–Pacific region is 12.5 %, followed by North America at 10 %.

Omega-3 Therapeutic Potential:

Omega-3 drugs had the potential to offer safe alternatives in certain health areas with a better safety profile. Early trials on omega-3s found significant reductions in mortality in patients with cardiovascular diseases. Three omega-3 drugs-Lovaza (GlaxoSmithKline), Vascepa (Amarin Pharma), and Epanova are the major brand in the heart health market and all targeting at hypertriglyceridemia (triglyceride levels ≥500 mg/dL). Lovaza/Omacor is the most prominent in a pharma market estimated at about €2 billion. This product is commercialized in 57 countries and sold under different brand names—Lovaza in US Omacor and Zodin in Europe and Asia, and Eskim, Esapent, and Seacor in Italy. One more product is produced by Mochida Pharmaceuticals (Japan) and sold under the trade name of Epadel. As per Global Organization for EPA and DHA Omega-3 (GOED), there are many companies such as Acasti Pharma, Amarin Corp, Catabasis Pharmaceuticals, Epax AS, Endeavor Therapeutics, Hanmi Pharmaceuticals which are developing Omega-3 Pharmaceutical products such as Capre, Vascepa, AMR101, CAT 1002, CAT 1000, AKR 963, Endeav101, HCP1007 which are in different stages of clinical trials.

These products will be helpful in preventing cardiac event, inflammatory diseases, type 2 diabetes with inflammation, speech disorders in autism spectrum, hyperlipidemia with high triglycerides, etc. Apart from above-mentioned companies, there are many other generic drug manufacturers such as Apotex Inc., Chiesi Farmaceutical, Endo Pharmaceuticals, Institut Biochemique, Nissui Group, Sandoz (Novartis), Teva Pharmaceuticals, Par Pharmaceuticals who are likely to launch omega-3 fatty acid [29].

Harry Rice, PhD, vice president of scientific and regulatory affairs for the GOED says that the supplements industry stands to benefit from the consumer attention and research that drug companies will bring to omega-3s. As pharma firms continue to invest more in research to unveil the benefits of omega-3s for other health conditions, the information generated will be of much useful to supplement companies and will result in a more educated consumer, not only about cardiovascular health, but also for other health indications.

The cardiovascular/metabolic market is the largest pharmaceutical drug segment, and the market opportunities are increasing due to westernization of lifestyles, aging populations, and increased access to health care. Due to the long timeframe for drug approval, the omega-3 drug market will grow more slowly than expected. Executive director Adam Ismail of GOED said there remained a place in the market

for high- and low-dose omega-3 products, which would undoubtedly lead to more pharma products for different conditions [30].

3. Infant Formula

Today, approximately 87 % of infant formula is EPA/DHA-fortified, creating a $10.2 billion market, or 40.0 % of the total infant formula market. Packaged facts projected the consumer market for DHA-fortified infant formula will reach $14.2 billion in 2016, reflecting a CAGR of 6.8 % for the 2011–2016, during same period Asia–Pacific will post the highest regional five-year growth rate, at 14 %. The infant formula market is the world's fastest growing packaged food category, primarily due to the development of markets in Asia (particularly China) and Eastern Europe and to a lesser extent in the Middle East and Latin America. Market development is linked with the economic growth of those countries and the growing number of working women who want premium products and the convenience of infant formula [31]. The need for infant formula enriched with DHA and ARA(Arachidonic Acid) for non-breastfed infants has been recognized by various official bodies including the United Nations FAO/WHO (Food and Agriculture Organization/World Health Organization), which recommends that all infant formula should contain DHA and ARA. WHO recommends minimal consumption of 40 mg/kg/day for ARA and 20 mg/kg/day for DHA for infants. An estimated 87 % of infant formula sold in 2011 was fortified with DHA and ARA. Algae oils have a high DHA content and dominate in DHA infant formula fortification. Most algal DHA, i.e., 75 % is going in infant formulas and provides less than 0.2 % of the worlds omega-3 nutrition needs today [18]. With roughly 25 million babies born each year, Asia has the fastest growing infant nutrition market in the world. India's birth rate exceeds China's by over 10 live births per 1000 in population. At an estimated $400 million, India's baby food market is just a fraction of the Chinese market, but the Indian market is expected to grow to $700 million by 2015 [24, 32].

4. Pet and Animal Feed

Growth in the **pet food and supplements** category will be driven by the recent "humanization" trend in companion animal products and services. Market size for the omega-3-fortified pet food and supplements is projected at $1.05 billion in 2016, reflecting a five-year CAGR of 3.4 %. The global expansion of pet superstores will create more opportunities for EPA/DHA-fortified pet foods, especially in developing market such as Asia–Pacific, Latin America, and Eastern Europe. In more developed markets, additionally, private label pet food is becoming more premium, including those fortified with fish oils. In the developing world, as the middle class emerges and expands (with their increased disposable income and development of a Western middle-class mindset about companion animals), there is growing demand for omega-3 pet food. The pet owners consider their pets as family members and want to provide the best to their pets and hence feed them omega-3-fortified food products, to extend health benefits of omega-3 to their pets. The nutritional profile of pet foods, treats, and supplements becomes more of a priority as pets move from outdoor to indoor animals and regarded as part of the family. As one reflection of this trend, specialized pet food formulas have been introduced in rapidly growing markets such as Brazil and China. Key products include many puppy and kitten foods, select premium dog and cat food formulas, and dog treats and dietary supplements marketed as solutions for improving skin and fur coat [32].

5. Clinical Nutrition

At present, **c**linical nutrition is a small segment of omega-3 market but it has got a lot of potential and certainly a lot of benefits for people who are recovering from a stroke or temporary unconsciousness. Clinical nutrition includes enteral and parenteral nutrition products and to be delivered under a doctor's supervision. Enteral nutrition is ingested both orally and through tubes delivered directly into the digestive system while parenteral nutrition is injected intravenously. Enteral and parenteral products of the clinical nutrition category benefit from the addition of omega-3 fatty acids because of their anti-inflammatory properties and are beneficial to trauma patients and those with chronic wounds. Clinical nutrition is also provided to cancer patients and patients with general malnutrition. Factors driving growth include aging populations and increased use of enteral products in homecare settings. Elemental enteral formulas are prescribed for patients with impaired digestive and absorptive capacity and feature partially or fully hydrolyzed proteins. The clinical nutrition market is conservative in pace of development as new product introductions, and formula changes require clinical trials and must go through a rigorous process of regulatory approval. In spite of these hurdles, enteral formulas with the addition of omega-3 fatty acids are growing at good pace. The value of the clinical nutrition market for products with EPA/DHA fortification is projected to reach $1.7 billion in 2016, reflecting a five-year CAGR of 4.3 %. Asia–Pacific should post the highest regional growth rate at 5 %, and will be followed closely by the other global regions. Example of EPA/DHA enriched enteral formulas is Optimental of Abbott Laboratories, which is used for acute conditions such as trauma, surgery, burns, pancreatitis, and

multiple fractures and for chronic conditions such as irritable bowel syndrome (IBD), radiation enteritis, and malnutrition. The specialized enteral formulas are designed to meet the nutrient needs of patients with specific illnesses. Specialized enteral formulas that have EPA/DHA include Abbott Laboratories OXEPA and Nestlé's Impact formulations, which help to support the body's immune defense system for a faster recovery, and are usually given to surgical and trauma patients. In Europe, parenteral preparations of omega-3s are becoming more popular and soon may be of interest in rest of the world [32].

New Emerging Market for Novel Omega-3 Products

Fish oil will continue to drive the market, but more of the market is trending toward high-concentrate products, as consumers want to get more EPA/DHA per capsule or softgel especially in North America. Higher concentrations give consumers the benefit of getting the same health-promoting levels of EPA and DHA, but in smaller or fewer softgels. Frost & Sullivan says the high-concentrate omega-3 consumer market is worth around $1 billion and growing at 20 % yearly. It seems that single-strength (30–50 % concentrate) products are the entry point into and once consumers become more "omega-3 savvy," they trade up to higher concentrates —double and triple strength for greater benefits. Through the media, doctors, health care practitioners, people understand the value of higher potencies to support the body's natural anti-inflammatory response, which benefits heart health, brain health, mood, and a variety of other conditions. Also, doctors are recommending higher potencies, so the market for concentrated EPA and DHA is likely to increase significantly. Claus Kjaersgaard, vice president of consumer health care told that "the high-concentrate omega-3 market is definitely growing fast and overall the omega-3 market is forecast to see double-digit annual growth rates for each of the next five years including the *single-strength (30 %) segment." *Single, double, and triple strength is the terminology originated from the USA [33].

The logic is as follows

1. *Single strength will be 300 mg/g (180 mg EPA and 120 mg DHA).
2. Double strength refers to a concentration of 600 mg/g (360 mg EPA and 240 mg DHA).
3. Triple strength will be 900 mg/g (540 mg EPA and 360 mg DHA).

As concentrations increase, the number and size of capsules/tablet decrease and therefore become more convenient to consume; hence, consumers are interested in higher doses.

The omega-3 drug market is further expected to diversify due to the global entry of generic omega-3 drugs from companies such as Teva Pharmaceutical, Apotex, Par Pharmaceutical, Institut Biochemique, Chiesi Farmaceutici, Nissui Group, Endo, and Sandoz (Information courtesy of Frost & Sullivan.). According to Frost & Sullivan/GOED demand for EPA/DHA for drug purposes in 2011 stood at a mere 2 % compared to 61 % omega-3 fatty acid demand for supplements, however, the dollar value of the drug market even at its small portion of the market was already at 19 % compared to 48 % for supplements. "The benefit of growing drug demand is that it will boost credibility for the omega-3 category overall. It can be a tremendous opportunity for both the Rx and OTC businesses." He continues, "In the case of omega-3, there is very little risk of conflict between supplements and drugs. Supplements are targeted at the 'prevention' and 'maintenance' segments, and drug is targeted at the 'treatment' and 'preemptive' segments. As such, these segments are largely complementary and that there is certainly room for both segments to grow".

The pharmaceutical-branded fish oils are generally of higher potency, the extra costs involved in manufacturing these types of potencies, along with the extra costs for being a pharmaceutical fish oil, really make them difficult to compete for the same market share. Frost & Sullivan/GOED predicts the high concentrates consumer market to double in the next five years. There is tremendous opportunity in driving the high-concentrate premium segment further. With the DHA algal market fairly established, a newer development for the algae market has been finding algae sources that produce higher amounts of EPA. For supplement marketers, the ability to offer customers a vegetarian ingredient that not only directly provides DHA but also now EPA is exciting, allowing companies to capture the health benefits of both the fatty acids.

DSM/Martek was the first to market an EPA algae ingredient last year. To produce product high in both EPA and DHA, the company grows two different strains of marine algae separately, extracts the EPA and DHA separately, and then blends them in one product. Eventually, the company will produce a custom blend EPA: DHA ratio to maximize desired health benefits. Similar to the fish oil industry, algae oil suppliers will increasingly focus on higher concentration of omega-3 fatty acid. Each species of algae is unique in that it provides different ratios of EPA and DHA,

and there will be room for multiple species of algae with different omega-3 profiles [17].

Other Omegas and Sources

Other emerging sources of omega fatty acids, both plant and marine, will continue to expand this ever-broadening market. Below are a few omega-3 fatty acid sources gaining the interest and attention:

Ahiflower oil is a plant-based branded ingredient from supplier Nature's Crops Specialty Oils/Technology Crops International (TCI). Ahiflower oil is a good source of Stearidonic Acid (SDA), an omega-3 fatty acid found in soybeans, hemp, and blackcurrant oil. Ahiflower's oil is derived from Buglossoides arvensis seed. "This is the first time Buglossoides arvensis oil will be available for commercial use," says Steve Howatt, vice president of agronomy and business development TCI. "TCI began with the wild plant species and spent many years improving its oil profile using natural plant breeding techniques. Now the crop contains high levels of oil, which also contains increased concentrations of SDA" Howatt says, Ahiflower oil contains up to 20 % SDA, as well as over 35 % ALA, total omega-3 fatty acids concentration of over 55 %. It also contains 5 % GLA. "Ahiflower will provide the highest concentration source of natural, plant-derived SDA available from a non-GMO source," he states. Howatt states that SDA, compared to plant-derived omega-3 fatty acid ALA, is more efficiently converted in the body, especially when it comes to EPA. He explains that the poor conversion of ALA to EPA and DHA is generally attributed to a key enzyme called delta-6-desaturase (D6d). "The conversion of ALA potentially can be blocked at the D6d stage, as this enzyme can be inactivated or down-regulated by a number of factors," he says, such as high intakes of saturated fat, trans fatty acids, carbohydrates, or alcohol, zinc deficiency, disease conditions, or aging. By contrast, says Howatt, "SDA is an intermediate between ALA and EPA/DHA which does not require the D6d enzyme for metabolism into EPA. As a result, SDA is converted to EPA much more efficiently than ALA; thus, lower quantities are required in the diet." He estimates that 1 tsp (5 ml) of Ahiflower oil contains approximately 840 mg SDA and 1800 mg ALA—which converts to approximately 300 mg EPA based on 30 % conversion for SDA to EPA and 3 % ALA to EPA. By comparison, 1 tsp of flaxseed oil contains 2600 mg ALA, which converts to 80 mg EPA. "Thus," he says, "Ahiflower oil would conservatively provide approximately four times as much 'EPA equivalent' as a similar amount of flaxseed oil."

On the marine front, a newer source of omega-3 fatty acids—DHA in particular—is squid. "Squid, or calamari, is an excellent and abundant source of omega-3 fatty acids, delivering 30 % omega-3 in non-concentrated natural oil," says Todd Parker, president of Pharma Marine USA LLC (Sovik, Norway), "The fatty acid profile is similar to tuna and salmon in that the DHA levels are higher than the EPA levels." Parker says Calamari oil is on par or better than many other marine sources of omega-3s and has a naturally higher ratio of DHA: EPA (2:1) than other marine sources and meets or exceeds standards set by GOED [17].

Room for Everyone

The omega fatty acids are a great business, and all categories are growing well. There is a lot of positive activity backed with lot of scientific backup. There is plenty of room for a variety of sources, applications, and concentrations [17]; this will ultimately benefit the consumer by providing choices of source, profile, and concentration to meet individual preferences and health needs. The omega-3 category is nowhere near reaching its potential. But in order to unlock that potential, we need to adopt consumer-based innovation approach. So, in conclusion "*the omega-3 market is a great business with win–win situation to both producers as well as consumers*".

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Fortification of Food with Omega-3 Fatty Acids

8

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List of Abbreviations

EFAs	Essential fatty acids
PUFAs	Polyunsaturated fatty acids
MNM	Micronutrient malnutrition
NRVs	Normal recommended values
ALA	Alpha-linolenic acid
LA	Linoleic acid
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
CFR	Code of Federal Regulations
US FDA	United States of America Food and Drug administration
GRAS	Generally recognized as safe
RDI	Recommended daily intake
CRN	Council for Responsible Nutrition
EDTA	Ethylenediamine tetra acetic acid
UHT	Ultra-high temperature
UK	United Kingdom
FA	Fatty acids
LO	Linseed oil
FO	Fish oil
LC-PUFA	Long-chain polyunsaturated fatty acids
SDA	Stearidonic acid
AA	Arachidonic acid
EC	European Commission
NHCR	Nutrition and Health Claims Regulation
DSHEA	Dietary Supplement Health and Education Act

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Introduction

Fortification is the practice of deliberately increasing the content of essential micronutrients, i.e., vitamins, minerals, and polyunsaturated fatty acids (PUFAs) in a food, so as to improve the nutritional quality of the food and provide a health benefit with minimal risk to health [1].

When and where existing food supplies fail to provide adequate levels of the required micronutrients in the diet, then the fortification of food is an easy way to fulfill that requirement. The food fortification reinforces and supports ongoing nutrition improvement programs and should be regarded as part of a broader, integrated approach to prevent micronutrient malnutrition (MNM). While fortified foods contain increased amounts of selected micronutrients, they are not a substitute for a requirement of good quality diet [2]. Although the topic of present review is "Fortification of food with omega-3 fatty acids," it is very much essential to understand fortification concept in general and its advantages and industry-driven fortification with legal frame work, before proceeding to fortification of food with omega-3 fatty acids.

Advantages of Food Fortification as a Strategy to Combat MNM [3, 4]

- Fortified foods are better at lowering the risk of the multiple deficiencies that may be due to a poor quality diet. This is an important advantage to growing children and to women of fertile age.
- Fortification of widely distributed and widely consumed food has the potential to improve the nutritional status of a large proportion of the population, both poor and wealthy.
- Fortification requires changes in neither existing food patterns nor individual compliance. On a regular consumption of fortified foods, body stores of nutrients will be maintained more efficiently and effectively.
- It is feasible to fortify foods with several micronutrients simultaneously without adding substantially in total cost, to combat multiple micronutrient deficiencies.
- When properly regulated, fortification carries no risk of chronic toxicity.

Although food fortification has an enormous positive impact on public health, there are some **limitations** as given below:

- Fortified food often fails to reach to poor people who are at the greatest risk of micronutrient deficiency due to their low purchasing power and higher price of fortified products.
- Nutritional benefits of fortified food are not of higher priority over price, taste, packaging, easy accessibility, and convenience to consumers.
- The need for micronutrients is often unrecognized by consumers due to lack of awareness.
- Fortified foods offer a preventive rather than a therapeutic benefit; hence, no immediate advantages are noticed by consumers.
- More affluent market segments often feel that they do not need additional micronutrients.
- Technological issues relating to food fortification have yet to be fully resolved, especially with regard to appropriate levels of nutrients, stability of fortificants, nutrient interactions, physical properties, as well as acceptability by consumers including cooking properties and taste.

Most early developments of functional foods were those fortified with vitamins and/or minerals such as vitamin C, vitamin E, folic acid, zinc, iron, and calcium [5]. Subsequently, the focus shifted to foods fortified with various micronutrients such as omega-3 fatty acids, phytosterol, and soluble fiber to promote good health or to prevent diseases such as cancers [6]. More recently, food companies have taken further steps to develop food products that offer multiple health benefits in a single food [7]. Today, the consumers and industry are at so ease with the concept of fortified foods that they no longer require the "public health campaign" to accept it. These products have been mainly launched in the dairy, confectionery, soft drinks, bakery, and baby food market.

The prominent types of functional foods are as follow:

(1) Fortified product is a food fortified with additional nutrients, e.g. infant formula, health drinks.
(2) Enriched product are the food with added new nutrients or components not normally found in a particular food, e.g., breads enriched with omega-3s, fruit juices fortified with omega-3s.
(3) Enhanced products are the food products in which one of the components has been naturally enhanced through special growing conditions, new feed composition, genetic manipulation, e.g., eggs with increased omega-3 content achieved by feeding omega-3- enriched poultry feed. [8]
(4) Altered and enhanced product are the food from which a deleterious component has been removed, reduced, or replaced with another substance with beneficial effect, e.g., de-fating of milk for saturated/bad fat and re-fating with healthy omega-3 fatty acids.

It is a different world today than it was 30 years ago. Hence, there is a great need for the Fortification Policy (1980) to be reevaluated. In the 1920s, food fortification began as a cooperative response to a public health need which today has escalated into an industry-driven fortification with no longer need for a public health campaign. Today in an era of complex diseases, with increased health awareness the consumers are seeking the food with health benefits. Although food fortification has been very effective in eliminating widespread nutritional deficiencies, it should not be left to the food and beverage industry to decide. Food regulatory authorities need to be vigilant and meticulous while evaluating health claims made by food and beverage industry [9, 10].

Industry-Driven Fortification

Food manufacturers add micronutrients to their products not just to increase their nutritional value but also to increase their appeal to the health-conscious consumers. This business-oriented initiative can play a positive role in public health by improving the supply of essential nutrients. A perception of value must be created which compensates for the price increase to develop an active consumer preference. In order to provide a competitive edge for fortified products over non-fortified products, aggressive marketing techniques are being used [11, 12].

Fortification Policy

Although fortification has proven effective in treating nutritional deficiencies in the past, food fortification should support dietary improvement strategies and not be seen as an alternative strategy [13]. Government has a key role to play in ensuring that food fortification is effective and safe. Food laws together with a broader food control system are the primary tools for establishing an appropriate level of control over food fortification practices. The main aim of regulating the level of fortificants in processed foods is preserving the nutritional balance and safety of the diet for the population at large; for this, it is also desirable to regulate which food can be fortified [14]. To this end, minimum levels are set to ensure that reasonable amounts of micronutrients are added to food products and must be stated on the product label. For market-driven fortification of processed foods, setting safe maximum limits is important, as the fortified processed foods are usually consumed by all family members, rather than any specific age groups. As the same serving size of the fortified food is common to all family members, setting maximum limits that are based on the normal recommended values of adult males will deliver unnecessarily large amounts of micronutrients to children. Therefore, in view of safety concern, some form of risk assessment appraisal is required while establishing maximum limits [15].

National Food Law and Fortification

Food law is the mechanism commonly used by governments to set technical provisions for fortified foods, the most important of which relate to their composition, labeling, and claims. The fortification provisions in food law ensure that all compositional parameters applicable to both fortificants and food vehicles deliver safe and appropriately efficacious public health outcomes and the labeling claims and advertising of fortified foods is factual and not misleading; the food fortification requirements may be established either in food and health act or in technical food regulations. One advantage of setting fortification provisions in regulation, rather than in legislation act, is that amendments can be made more quickly and easily [1].

Guidelines on Food Fortification

Regulators need to balance between required/permitted label information and advertising of fortified food products. In case of mandatory fortification, the manufacturer is legally obliged to add one or more micronutrients to that food. Mandatory fortification is written into food law, usually in the form of regulation which specifies a legal minimum and maximum (where appropriate) levels for each micronutrient in the identified food or category of foods. The legal minimum levels are established on the basis of efficacy in terms of health benefit, whereas maximum levels are determined on the basis of safety. Both the legal minimum and the maximum levels serve to protect human health. There is no concrete evidence of any negative effect of fortified foods on the overall population. Regulations also stipulate the identity and purity requirements of the permitted compounds, or make reference to pharmacopoeias and other technical publications that set out such requirements. The amount of food that is used as a reference for these levels (i.e., mg per kg or per serving) is defined. Minimum micronutrient levels should be that amount that would be expected to contribute a benefit on consumption of normally expected quantities of diet. A risk-based approach for setting maximum fortification limits is becoming more commonly accepted, with the development of reference upper safe values for intakes. The range of foods to which micronutrients may be added should be prioritized or restricted on the basis of their nature and importance in the diet of the general population. The per serving basis has the attraction of being more relevant for consumers, especially if the nutrient declaration on label is

made on the same basis. However, for international trade, due to variation in serving size among different countries there is likely to have more problems. Provision of information about nutrient contents including the micronutrient(s) with which the product is fortified is normally required on the label under the rules on nutrition labeling [1].

The governments have established regulations about how much qualitative or quantitative nutritional information is needed to include on the label and advertising. Nutrition and health-related claims focus on the nutritional properties or its nutritional and health benefits for consumers. Health-related claims include nutrient function and reduction in disease risk claims. The claims regarding the nutritional properties or its nutritional and/or health benefits for consumers are frequently made by manufacturers as a means of promoting their products. An appropriate regulation of nutrition and health-related claims ensures that the information manufacturers convey to consumers about their products is truthful and not misleading. The Codex Guidelines for use of nutritional claims provides guidance on the conditions for nutrition/health-related claims and establishes the general principle that these claims should be consistent with the national nutrition policy. The use of disease reduction claims of fortified products needs to be carefully evaluated or such claims may be controlled by setting qualifying and disqualifying criteria [16]. In 1994, the Dietary Supplement Health and Education Act (DSHEA) came into force (FDA.gov 2012). Under the DSHEA, nutraceuticals were considered dietary supplements. The Act states that the dietary supplement or dietary ingredient manufacturer is responsible for ensuring that a dietary supplement or ingredient is safe before it is marketed. The DSHEA provided opportunities for the emerging nutraceuticals industry in the USA by allowing health claims on nutraceutical products. It became possible to make structure/function claims on a nutraceutical (these claims describe the role of a nutrient or dietary ingredient intended to affect the structure or function of the body). The manufacturer is responsible for ensuring the accuracy and truthfulness of these claims; they are not approved by FDA. Therefore, if a nutraceutical includes such a claim, it must state in a "disclaimer" that FDA has not evaluated this claim. Stipulating best before date provides the shelf life of the product.

Omega-3 Fortification

The modern diet is deficient of omega-3 fatty acid and the health benefits and the consumer demand for the omega-3-fortified products are well recognized. Therefore, a lot of work is going onto bring back omega-3 in food chain through omega-3 fortification of commonly consumed food products. Examples of foods fortified with omega-3 include milk-based products, meat, eggs, juices, table spreads [17], salad dressings, sauces, breakfast cereals, baked goods, infant formulas, baby foods [18, 19]. Also new food and beverage products with added omega-3 have emerged in the market because of the mounting evidence of the overall health benefits associated with omega-3 fatty acids [18–21]. Although many foods in the market are fortified with omega-3 fatty acids, before opting one should seek answers to the following important questions:

(1) What kind of omega-3 fatty acids have been added to the food and how much?
Alpha-linolenic acid (ALA) or eicosapentaenoic Acid (EPA) or docosahexanoic acid (DHA) or combination of fatty acids and quantity of each.
(2) Source of omega-3 fatty acids.
Plants, fish, or algae.
(3) Quantity obtained per serving.

Biotechnological techniques are used for increasing the omega-3 content of some animal-derived foods by adding omega-3 fatty acids to animal feed so that their tissues become enriched. Some examples of such foods include omega-3 eggs and omega-3 chicken meats.

Safety

In order to meet the safe and lawful requirement for health claims under 21 CFR 101.14, the use of EPA and DHA, when used in conventional food or as a dietary supplement at levels necessary to justify the claim, must be demonstrated, to Food and Drug Administration's (FDA) satisfaction. FDA evaluates whether the substance is "safe and lawful" under the applicable food safety provisions of the Act [22]. For conventional foods, this evaluation involves considering whether the ingredient that is the source of the substance is generally recognized as safe (GRAS) approved as a food additive, or authorized by a prior sanction issued by US FDA. The FDA has allowed fish and algal oils to be GRAS for food fortification. Algal oil is useful for those who do not eat fish [23].

FDA concludes that the use of EPA and DHA as dietary supplements and as an ingredient in conventional foods is safe and lawful under 21 CFR 101.14, provided that the daily intakes of EPA and DHA do not exceed 3 grams per person per day from conventional foods and dietary supplement sources. Further FDA guide the manufacturers to declare the amount of EPA and DHA per serving in the claim label of the conventional foods and dietary supplements that bear an omega-3 fatty acid qualified health claim [23].

In European law, nutraceuticals are labeled as food supplements. The most important regulations on European level that control nutraceuticals are Directive 2002/46/EC, which establishes harmonized rules for the labeling of food supplements and introduces specific rules on vitamins and minerals in food supplements [24], Regulation (EC) 1924/2006 on nutrition and health claims made on foods [25] and Regulation (EC) 258/97 on novel foods [26]. The European Nutrition and Health Claims Regulation EC 1924/2006 (NHCR), which came into force on July 1, 2007, controls any nutrition, health, and disease reduction claims made about a food. One of the requirements of the NHCR for making a nutrition claim is that the food contains significant amounts of the named nutrient to be a benefit. The significant amount is the amount that can be expected to produce the desired effect taking into account what quantities will be eaten.

Q. What kinds of claims can be made for a food product?

The claims should not be misleading and generally there are three types of claims:

- A food claim that expresses the composition, quality, quantity, or origin of a food product.
- A nutrient content claim that characterizes the energy value of the food, or the amount of a nutrient contained in a food. It provides a quick and easy way to identify foods with specific nutritional features of interest such as "high in omega-3". Omega-3 content claims are likely to be nutrition claims.
- A health claim is any representation in labeling or advertising that states, suggests, or implies that a relationship exists between consumption of a food or an ingredient in the food and a person's health.

Market of Omega-3-Fortified Products

At present, more and more manufacturers introduced new omega-3-fortified products into the market as a method of differentiation and to maximize on the functional food trend, in spite of that, omega-3-fortified food segment is far from saturation. Market growth in the omega-3-fortified foods segment is expected to be in the region of 10 % per year for the next 3 years. This will be driven by more new product introductions. After 3 years, growth is expected to level off as the market stabilizes.

The incorporation of EPA and DHA into functional foods has exploded due to the following factors:

(1) The growing range of scientific research on the benefits of EPA and DHA.
(2) Increased awareness.
(3) Expanding fortification capabilities of food and beverage manufacturers.
(4) Health and wellness are priorities held and marketed by many of the world's largest food and drink companies.

Earlier food applications were limited to lipid friendly spreads and yoghurts because of flavor and stability considerations. However, recent technological advances have made the use of omega-3s in other food categories more common—including dairy products, beverage and juices, infant nutrition products, breads and baked goods, processed meats, cooking oils, and various prepared foods.

Nestle, as "the world's leading nutrition, health, and wellness company," with other global food and beverage leaders like Danone, Numico, is looking to the functional food category for growth opportunities. The success depends on the creation of a differentiated product benefit that fits within their brand portfolios. While there are ground- breaking smaller companies in each region that offer EPA/DHA-fortified foods, the potential for significant category expansion lies largely in hands of multinational food corporations. The rate at which they bring new products to market and the brand equity and marketing clout they devote to these efforts decide the extent of market expansion. Many of the products are branded toward children at a time when EPA and DHA are well known among consumers to be a benefit for young brain development. It is only natural for a parent who would spend extra for a DHA-fortified formula to want to choose a DHA-fortified milk or yogurt as the child grows. Any food that is made with the dairy product can easily be fortified using microencapsulation technology to encapsulate the fish oil with dairy proteins. One difficulty with omega-3 fortification is that there is as yet no FDA-approved recommended daily intake (RDI) to provide guideline quantities. Even international standards for omega-3 fortification levels vary widely across countries. For example, in Britain the RDI is 800 mg per day, and 3 grams per day in some Scandinavian countries and 1.5 grams per day in Canada. In addition, there is no formal FDA-approved health claim, although a limited claim linking fish oil supplements with a reduced risk of coronary heart disease has been permitted. With the increasing awareness of omega-3s, the labeling regulations will change and RDI will be established soon. The Council for Responsible Nutrition (CRN), Washington, D.C., omega-3 working group (created in March 2001), has developed a voluntary monograph that establishes minimum quality standards for treatment of marine products and uniform standards of measure of fat content, which should be stated in milligrams per gram (mg/g) [27].

As consumers continue to demand more nutritious products, food manufacturers are seeking many more opportunities to include omega-3 in their formulations. However, flavor is the major obstacle hampering progress on

the enrichment and fortification of food products with fish omega-3s. In addition, omega-3s are extremely sensitive to heat, light, and oxygen and go rancid very quickly due to oxidation resulting in repelling flavor, and reduction in shelf life of the product. This is also likely to cause health damage due to increased free radical formation in the body. To overcome this problem, the addition of antioxidants, such as vitamin E and ethylenediaminetetraacetic acid (EDTA) (metal chelating agent) [28], is recommended. Omega-3 fatty acids oxidation can also be controlled by pH adjustment in emulsions or by producing low-viscosity emulsions for ease of handling and incorporation into water-based foods. Some products that are emerging as the best for omega-3 fortification include frozen foods, soups, refrigerated foods, salad dressing, yogurt, spreads, juices, egg products, and cheeses. Omega-3-fortified products should not be packed in transparent packaging since light can cause them to oxidize. Another technique used to protect oils is microencapsulation, which can guard against damage incurred during processing, and serve to render fish oil products tasteless and odorless [29]. Omega-3s can also be spray-dried into powders by mixing with other ingredients, such as starches or proteins, which protect them against oxidation. In general, powders are preferable in products such as baked goods or functional beverages, where the ingredient has greater dispersability and enhanced stability, as well as better flavor masking. Powders may also be easier to incorporate into beverages [30]. In liquid applications, essential fatty acids (EFAs) often need to be mixed with emulsifiers to prevent their separation. As far as the practicalities of food fortification are concerned, each manufacturer must consider its target consumers as well as the particular health issue it wishes to address, before deciding the type of omega-3s best suited for its products. If a product is aimed at the mass market, vegetarian options may be preferable, but for more specific targets or health complaints, the greater efficacy of fish oils may offer better marketing advantages [31, 32].

The dairy and bakery companies are trying to enrich various products using omega-3s and other healthy nutrients into their products to appeal the consumers through their already known significant health benefits. Omega-3 fortification of already healthy foods, such as fruit juices, cereals, cereal bars, and dairy products, will be the boon to consumers. Other foods to look out for fortification include pasta, salad dressing, and ice cream [33].

Omega-3-Fortified Food Products Available in Market

(1) Breads and Bakery

Bread plays a central role in the Western diet, and innovative ingredients such as omega-3 are used by the bakery industry to offer products with added health benefits [34]. High-quality fortified breads are increasingly popular with consumers for addressing omega-3s deficiencies. Omega-3 bread contains microencapsulated omega-3 fish oil 100 milligrams per 100 grams [35]. They are a good vehicle for providing EPA and DHA at adequate levels. Breads and cereals are the area in which there is a high growth in omega-3 fortification. Bread is in fact one major staple that has already been extensively fortified with omega-3s. One particularly successful brand in this respect is Tip Top Up bread from George Weston in Australia [36]. This product was launched in 2002 as the result of a joint venture between Nu-Mega Ingredients and George Weston Foods, which involved adding Nu-Mega's microencapsulated HiDHA oils to the bread [9].

(2) Dairy Products

Dairy products have proven to be particularly suitable for fortifying with omega-3s, in terms of both ingredient compatibility and popularity with consumers. The addition of omega-3 to infant formulas has extended to their addition to fluid milk and other products around the world. Typical omega-3-fortified dairy products include yogurt, milk drinks, ice cream, margarines, spreads, fresh, and ultra-high temperature (UHT) milk [37]. USA and Europe are the strongest markets for dairy products, but differ in their segmentation. Europe has a much stronger yogurt market (four times larger than the USA), while the USA has a stronger milk market. In the US market, dairy products are usually sold and stored cold and have a short shelf life, which helps to maintain the stability of the omega-3s [38].

Nestle markets its Omega Plus line in South America and the Far East. The following omega-3-enriched products have also been introduced into the world market: cheese (130 milligrams EPA+DHA per 100 grams), mayonnaise, sweet-flavored nutrition bars, cream cheese. In addition, EPA/DHA can be easily added to butter and spreads. Many consumers expect margarines and spreads to be enriched

with omega-3s and plant sterol to offer special benefits for lowering cholesterol [9].

(3) **Beverages and Juices** [9, 39, 40]

In the past, beverage manufacturers found it challenging to fortify drinks with omega-3 fish oils due to the ingredient's "fishy" taste and smell. However, microencapsulation of omega-3 ingredients eliminates fishy smell and EPA/DHA with a neutral sensory profile are now available and can be added to various beverage products. In the US market, fortified fruit juices are very popular and the leaders in the fortified juice category are Minute Maid Enhanced Pom Blueberry, Tropicana Pure Premium Health heart Orange juice, and Indian rivers' fortified grapefruit juice [7]. Genesis introduced a line of omega-3-fortified orange juices which is fortified with algae oil. In Canada, A. Lassonde produces a variety of juices fortified with microencapsulated fish oil. In the UK, Big Shotz, described as a super vitamin health drink, offers over 70 % of the new European recommended daily adult level of omega-3 in one 120-ml bottle [35]. In Norway, Smartfish Recharge is a fresh juice-based drink for children and adults fortified with marine omega-3.

(4) **Baby Food and Pediatric Juices** [35, 41]

The popularity of DHA-fortified infant formulas has given rise to toddler and pediatric foods and drinks fortified with DHA. Gerber DHA and Probiotic Cereal (in two varieties, oatmeal and rice) and 2nd Foods Smart Nourish Purees (in various fruit and vegetable varieties) are fortified with tuna oil. PBM Nutritionals [42], the largest producer of store brand infant formula, produces its Bright Beginnings Pediatric Drink with DHA algal oil. DrSEARS Cool Fuel uses refined fish oil from anchovies and sardines.

(5) **Omega-3 Eggs**

The aim of increasing the omega-3 fatty acid content of poultry products is to augment the omega-3 consumption in humans. This has opened the possibility to develop "Omega Eggs" with higher levels of omega-3 fatty acids for the health-conscious consumer at premium prices. Omega Eggs are a type of "designer egg," in which the yolk's fatty acid profile has been modified by altering the hens' diet. The fatty acid composition of poultry meat and eggs usually reflects the composition of bird's diet [43, 44] and fatty acid profile in the eggs can be modified by changes in the hens' diet [45–47]. The inclusion of omega-3 fatty acids in poultry products is achievable by feeding omega-3 fatty acid-rich diets to layer birds. A large (60 g) egg labeled as "Omega Egg" contains at least 350 mg of omega-3 fatty acids [48]. The increase in yolk omega-3 is accompanied by a substantial decrease in saturated fatty acids, creating a healthier fat profile. The laying hen is a good biological model, which converts ALA to EPA and DHA, and deposits both important omega-3 fatty acids into egg yolk. In the Omega Egg, the ratio of omega-6: omega-3 fatty acids has been improved to 2.6:1. The Omega Egg will provide 250–350 mg omega-3 fatty acids, of which 100 mg is DHA (C22:6). Due to a special feeding of omega-3-enriched poultry feed, the cholesterol content of Omega Eggs has also been consistently reduced to 180 mg/egg, compared to the standard egg cholesterol value of 210 mg/egg. Omega-3-rich eggs were introduced into the UK market first by Columbus and more recently by Stonegate Farms under the brand name "Intelligent Eating Healthy Eggs." The latter comes from another joint venture with Nu-Mega, which involves chicken feed containing Nu-Mega's DHA-rich tuna oil. Columbus on the other hand feeds its hens omega-3s derived from flaxseed.

In the USA, the demand for omega-3-enriched eggs is climbing steadily. In fact, omega-3-enriched eggs are now believed to constitute as much as 5 % of the US egg market. Active in this market is Martek Biosciences, whose marine algae are fed to chickens in order to increase the DHA level of its eggs, which are currently sold under the Gold Circle Farms brand. Designer eggs comprise about 4 % of the total egg production in Canada. Another player is Belgium-based Belero, which launched omega-3 eggs in the USA as Christopher Eggs [49].

(6) **Meats and Meat Products Enriched with Omega-3**

Similar to omega-3 eggs, chicken meat has great potential to become omega-3 functional food for humans. This represents another upcoming growth area. Consumption of poultry meat has increased by 73 % in the last 30 years worldwide and is also increasing in India. Chicken meat is the fastest growing component of global meet demand. In India, the consumption of chicken meat is higher compared to other meats, as it is preferred over other meat for its health appeal because of its higher protein content and lower cholesterol and fat. Compared to other meats, chicken meat has lower saturated fat. However, commercial chicken meat is known to be deficient in omega-3 FA and rich in omega-6 FA. Therefore, it is a viable means to increase the availability of omega-3s and safeguard the health of consumers. It is possible to modify the lipid profile of commercial chickens by manipulating the broiler diet. Enriching chicken meat with omega-3s can be a rewarding enterprise to realize added value to the final product and provide health benefits to the consumer through omega-3-enriched functional foods [50].

The cholesterol content of omega-3 eggs is lower than that of regular eggs; however, a 100 g portion of chicken

meat contains, at the most, 100 mg of cholesterol. Therefore, chicken meat enriched with omega-3 s has the potential to become a sought after omega-3 functional food [51]. Several attempts have been made to enrich chicken meat with omega-3 FA using different sources of omega-3 FA. Linseed has been used by several workers for the enrichment of chicken meat [52]. The advantage of linseed is that it naturally contains three times more omega-3 FA than omega-6 FAs, which is a useful factor as the final aim is to decrease the omega-6/omega-3 FA ratio in chicken meat. Further, linseed oil (LO) has 55–60 % omega-3 FA unlike fish oil (FO) which may have approximately 30 % omega-3 FA. Non-omega-3 FA in linseed is low and so better manipulation of omega-3 FA in chicken meat is possible with linseed and LO, when used in moderate amounts. While making a choice between the two major sources, linseed or fish, linseed, and more particularly linseed cake in moderate amounts seem to be a better alternative for omega-3 FA enrichment of broiler diets [51]. A level of 300 mg of omega-3 FA per 100 g of meat is required to qualify as omega-3 functional food according to Canadian standards; however, it has not been found to be suitable for commercialization of chicken meat due to many reasons [53]. Firstly, it increases the cost of production, secondly, the shelf life of the product may be affected, and thirdly, and most importantly, higher omega-3 FA adversely affects organoleptic properties and consumer acceptability.

Although fish or fish oil (FO) can directly enrich chicken meat with EPA and DHA, the flavor of meat is drastically affected [54, 55]. Therefore, the use of fish or FO for omega-3 FA enrichment has to be at tolerable levels resulting in regulated incorporation in chicken meat [56]. Additionally, omega-3 FA enrichment using sources such as fish or linseed could lead to alterations in shelf life of chicken meat due to the presence of long-chain polyunsaturated fatty acids (LC-PUFA), which are more susceptible to oxidation and may lead to undesirable changes in quality parameters, such as loss of water holding capacity, texture, and flavor due to rancidity [57, 58].

(7) Prepared Products

Modern, fast-paced lifestyles have resulted in the more frequent consumption of prepared, processed meals. Food manufacturers continue to capitalize on this trend by developing more appealing and healthier products. Processed foods are often fortified to restore nutrients lost in their production, while others are fortified to increase a product's added nutritional value or to create a differentiated new product. Infants and young children are one of the groups at risk of dietary deficiencies. They need a high level of nutrients, but their food choices are limited and the amount they consume is relatively low. At the same time, prepared products such as chicken nuggets or fish fingers are very popular with children. In the UK, both of the leading frozen fish finger brands, Birds Eye and Young's, include an omega-3 fish fingers product in their ranges, and the Birds Eye version is now the number-three frozen fish brand line in the UK. Similar products are offered throughout Europe, including Captain Iglo Omega-3 Fish Fingers in the Netherlands [49].

(8) Confectionary and Chocolates

The global confectionery market is expected to cross US $ 200 billion and nearly 25 million tonnes by 2016. Since the flavor of omega-3 oils in the forms of algal or fish oils is usually quite distasteful to young children, manufacturers have opted microencapsulation strategies to incorporate fish or algal oils to sweet, fruit-flavored pastilles, gums, or jellies. As an alternative to fish and algal sources of omega-3 fatty acids, which are costly and pose significant technical challenges, stearidonic acid (SDA)-enriched soybean oil plays a beneficial role in the incorporation of nutritionally meaningful levels of omega-3 fatty acids in conventional diets while enabling the successful formulation of stable foods, including confectionery products. The development of SDA-enriched soybean oil makes exploitation of the market for omega-3-enriched confectionery products so much easier to realize than the alternative offerings currently available, on several primary counts: (a) SDA is oxidatively more stable compared to EPA and DHA. The comparative shelf life testing has demonstrated that off-flavors and odors are not produced in SDA-fortified confectionery products in the same way as those fortified with fish or algal oils, (b) SDA readily converts to EPA, whereas ALA does not convert to that significant extent, and (c) SDA is derived from a land-based renewable source, thus easing the burden on the world's fish stocks [49].

Omega Cookie is the wholesome combination of omega-3 fish oil and gluten-free oat fiber and helps to keep cholesterol and triglyceride levels in check. It contains calcium, vitamin D, and 2000 mg of EPA/DHA, making the Omega Cookie truly a Super food Champion. Each Omega Cookie contains more than two teaspoons of fresh fish oil (2000 mg EPA/DHA), which is the daily dose recommended for maximum potency. An Omega Cookie has less sugar than an apple, and the sugar comes from the cookie's natural ingredients—honey and fruit or dark chocolate chips (which have plenty of antioxidants). The Omega Cookie's fat content comes entirely from fish oil and extra virgin olive oil which are heart healthy fats. The Omega Cookie contains five grams of dietary fiber, half of which is soluble—that is more than the soluble fiber content of a bowl of oatmeal. The

Omega Cookie also contains 35 % of the daily recommended calcium intake in the form of calcium citrate, which is considered to be the best kind for bioavailability. When combined with vitamin D, calcium has been known to help to fight obesity and improve heart health, in addition to strengthening the bones. The Omega Cookie's all-natural and FDA GRAS formula makes it well suited for everyone. In fact, children can reach a solid daily dosage of omega-3 by consuming just half a cookie. The Omega Cookie tastes like regular cookies, no fishy smell. The FDA's recommended daily intake is 25 grams; eating an Omega Cookie every day is a big step toward reaching daily omega-3s requirement.

The chocolate is a particularly good food to enrich with omega-3 because it contains compounds that aid in its absorption and dark chocolate is a natural source of antioxidants which help to reduce cell damage. Omega-3 chocolate is the rich, dark chocolate formulated with 70 % cocoa for maximum antioxidant benefit. Here, purified omega-3 is microencapsulated, making it odorless and tasteless. The microencapsulated omega-3 does not oxidize or lose potency. This is a very exciting product as it provides a sweet alternative to other omega-3 foods and supplements and can provide many healthy benefits for the body [41].

(9) Infant Formula

Human infants should ideally be nursed on mother's milk, which constitutes nature's best food. Breast milk is considered the gold standard of infant nutrition. Even though doctors and hospitals are placing greater emphasis on breastfeeding, infant formula often becomes a more convenient option when mothers return to the workplace following maternity leave. Increasing urbanization and a rapidly expanding middle class support lead to further growth in sales of infant formula. There has been an ever increasing reliance of infant formula feeding practices in both developed and developing countries. Women in the developing world are increasingly returning to work after childbirth, fostering greater reliance on infant formula. In the event of lactation failure, insufficient milk secretion, and where mothers are suffering from transmittable diseases, human milk substitutes serve as savers of precious life during vulnerable stages of infancy. It should be noted that there are certain advantages of infant formulae over breast milk, most significantly the fact that formulae contain no contaminants (i.e., medications and their metabolites) from the maternal diet. The commercially available infant formulae designed to mimic its unique combination of carbohydrates, fat, protein, vitamins, and minerals to that of breast milk to provide infants with the same nutritional value as of breast milk. However, breast milk contains numerous components that cannot be manufactured synthetically, including maternally derived antibodies which protect against disease and infection [59].

Infant formulae have traditionally only contained the precursor essential fatty acids alpha-linolenic acid (ALA) and linoleic acid (LA) from which the fed infants must synthesize their own DHA and arachidonic acid (ARA), respectively [60]. While there is evidence that infants can effectively metabolize ALA and LA [61], there also is evidence that they do not synthesize these substances at an adequate rate. The current hypothesis is that infant formulae containing only the precursors LA and ALA may not be effective in meeting the full nutritional requirements of infants. Therefore, the infant formula enriched with DHA and ARA for non-breast-fed infants has been recognized by various official bodies including the United Nations Food and Agriculture Organization/World Health Organization (FAO/WHO), which recommends that all infant formula should contain DHA and ARA. An estimated 87 % of infant formula sold in 2011 was fortified with DHA and ARA.

To date, infant formula has been a major segment of focus with respect to omega-3 fortification. Such fortification has taken place around the world for several years; however, currently the US permits manufacturers to fortify infant formula with vegetarian marine algae. In May 2001, the FDA issued a GRAS notification regarding the use of Martek's DHA oil, in infant formula. This favorable review opened the door for US infant formula manufacturers to add microalgae-derived DHA to domestic infant formula. Algae oils have a high DHA content and dominate in DHA infant formula fortification. In February of 2002, the FDA approved the use of DHA and ARA as additives to infant formulae in the USA. Expert panels from the Life Sciences Research Organization assessed nutrient requirements for both term and preterm infant formulas and recommended neither a minimum nor maximum content of either ARA or DHA for term infant formulae. For preterm infant formulae, they recommended maximum levels of 0.35 and 0.6 % of total fatty acid intake for DHA and ARA, respectively [62].

Now let us understand why DHA is recommended in infant feed formula.

DHA is important for the structure, growth, and development of the fetal central nervous system and retina. DHA comprises roughly 30 % of the fetal brain's weight, DHA is a major part of fetal neural tissue, and it maintains good neurotransmitter function. DHA is also a major fatty acid in the retina. DHA naturally occurs in human milk and omega-3 supplementation during pregnancy and lactation increases levels of DHA in breast milk. Since FDA approval in 2001, infant formula is supplemented with DHA to support optimal brain and eye development. The significance of DHA for brain and eye development is widely recognized,

and evidence has emerged to demonstrate that DHA supplementation during pregnancy, lactation, or infancy improves mental and visual development in infants. Emerging research suggests that DHA supplementation reduces the risk for early premature birth [41].

Infant formula is currently a commodity market, with all products being almost identical and marketers competing intensely to differentiate their product from competitors as a marketing tool and to allow companies to promote their formula as "closest to human milk" [63]. The infant formula business is lucrative because the profits per unit are high, and also huge growth is anticipated, especially in Asia. The major infant formula producers operate globally, and they are planning a massive push into Asia and other parts of the world. Another report says China's baby food and drink market is expected to be worth more than $15 billion by 2015, with perhaps 80 % of this comprised of infant formula [64]. With the infant formula industry having enormous global reach, the model of regulation under separate national jurisdictions is outdated. All the nations of the world should act together to strengthen quality control for infant formula and other foods intended for children. There is a need for a global perspective in regulation, which is based on appreciation of differences in local circumstances [16].

The technological advancement for the production of infant formula has come a long way with the manufacture of a variety of infant formulae for dietary management of normal babies, preterm infants, and infants born with a variety of nutritional as well as physiological disorders. Formulas for infants with particular needs could be defined as pharmaceuticals, and distributed on a prescription basis, rather than through commercial marketing. Most of the infant formulae utilize bovine milk because of easy availability. There has been much growth in the dried milk industry for manufacture of the infant formulae. Today nearly 185,000 metric tons of infant formula is manufactured in India alone, representing approximately 3.8 % of India's total milk production [65].

In order to understand future of omega-3-fortified food products market, we need to know market drivers, market restraints, market beneficiaries [34], which are summarized below before concluding this subject.

Market Drivers for Omega-3-Fortified Products

- Increased consumer awareness.
- Inadequate omega-3 intake levels in diet.
- Proven relevance of omega-3s in fighting against major diseases.
- Fortification trend helps to increase acceptance of omega-3-fortified foods.
- Manufacturers' initiative increases product availability.
- Increased activity in the dietary supplement category raises omega-3 profile.
- Technological advances widen the scope for fortification.

Market Restraints for Omega-3-Fortified Products

- Poor consumer understanding of omega-3s.
- Premium price of Omega-3 fortified products.
- Regulatory restrictions and the absence of globally accepted RDI.

Who Will Benefit from Omega-3 Fortification of Food Products?

Existing Companies in the Omega-3 Market and Potential New Entrants.

- Bread manufacturers.
- Dairy product manufacturers.
- Egg suppliers.
- Infant formula manufacturers.
- Confectionary and small goods manufacturers.
- Spreads/Margarine manufacturers.
- Omega-3 ingredient suppliers.

And allied …
Investment Community.
Industry Information Services.
Industry Associations.
Distributors and Retailers.

Conclusion

New food and beverage products with added omega-3 fatty acids have emerged in the marketplace because of the mounting evidence of the overall health benefits associated with omega-3 fatty acids [18–21]. Future opportunities also revolve around targeting pregnant and breastfeeding women in order to assist them in replenishing the nutrients, which they provide to their infant, both inside and outside the womb.In conclusion, the future of omega-3-fortified foods is very bright and food manufacturers need to craft innovative marketing strategies that harness the strengths of the omega-3 fortification. They would also need to sustain momentum on research and development efforts toward improved product performance.

Only then they will reap the rewards of omega-3 fortification concept whose time has come now.

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Importance of Polyunsaturated Fatty Acids from Marine Algae

9

Rafael Zárate, Nabil el Jaber-Vazdekis, and Raquel Ramírez-Moreno

Introduction

Marine algae, appeared on Earth sometime 3–4 billion years ago, these include marine cyanobacteria, marine eukaryotic microalgae, and seaweeds which appear widely spread in the oceans, going from the polar regions to tropical areas, covering many ranges of environments, such as nutrient-rich coastal seas or oligotrophic open oceans [1]. They are photoautotroph, and unlike "plants," they do not possess roots, leaves, and other organs that characterize higher plants, but they do possess chlorophyll. Marine algae range in size from microscopic individual cells of microalgae to huge seaweeds that are greater than 30–50 m long and are called macroalgae *Macrocystis*, the genus that includes kelp. Marine algae are responsible for approximately 40–50 % of the photosynthesis that occurs on Earth annually [2]. These species have been used by humans to obtain abundant valuable compounds, with interest for human health and nutrition, or cosmetics, oils (e.g., triglycerides), polysaccharides (e.g., algin, agar), pigments (e.g., phycobili proteins, carotenoids), including also biodiesel, for the chemical industry, and often the discovery of potent new pharmaceuticals [3–6].

This vast group of organisms is defined as the primary producers in the marine environment, and their presence is essential for the maintenance of the food chain and life. Regarding that, it should be highlighted their capacity to produce high valuable omega-3 polyunsaturated fatty acids (PUFAs), which have been demonstrated to possess great importance in human health, since the consumption of EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) has been reported to participate in the optimal functioning of the cell membrane, and thus in literally all possible physiological and biochemical events within the cells and bodies, participating also in reproduction, many other mechanisms, and to prevent cardiovascular, obesity, nervous systems, inflammatory conditions, and numerous pathological disorders [7, 8]. Accordingly, the global demand for omega-3 fatty acids has significantly increased over the years, but on the other hand, there is also a growing concern about how to manage and achieve a sustainable exploitation of their major source, i.e., wild fish (fish oils), which are being seriously depleted [9].

Therefore, a different scenario emerges a few decades ago, in which algae appear to be the natural takeovers due to their ability, not only to produce high amounts of PUFAs, particularly omega-3, but also the ability to be cultured and grown in controlled and optimal conditions. Numerous autotrophically and heterotrophically grown microalgae species have been studied for their high-EPA and/or high-DHA contents, such as *Nannochloropsis*, *Phaeodactylum*, *Schizochytrium,* and *Thraustochytrium* [10]. In the last few years, biotechnology approaches have also been applied to improve the quality of PUFA profiles, as well as boosting the amounts of these desirable compounds, together with the optimization of culture techniques and strategies [11]. Many advances have been attained, and progress is being achieved at a regular pace, with many interesting outcomes being reported. All these considerations make algae, particularly microalgae, a very attractive group to be exploited and in the near future to likely become one of the main sources of omega-3 PUFAs, and more importantly to offer a continuous and sustainable source and supply of these important metabolites to thus satisfy the world demand.

In this chapter, references to the marine environment and the food chain in which these organisms participate have been addressed, making also some comparisons with terrestrial plants and their fatty acids; furthermore, the

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appearance and profiles of fatty acids from both microalgae and seaweeds are also presented, together with updated information on the biotechnological approaches conducted for boosting fatty acid yields with microalgae. For the optimal exploitation and consumption of fatty acids from these natural sources, comments on the regulatory processes and their constrains were also treated. Finally, under concluding remarks, references are made to the expected impacts of new or more comprehensive approaches, the expected results for boosting production and thus establish algae as an attractive and effective source of fatty acids for human health in the near future.

The Marine Environment and Algae

The sea, home to the marine environment, covers around 71 % of the Earth's surface and represents about 97 % of its waters, corresponding to approximately 1370×10^6 km^3. Given the current state of continental drift, the seawater is not distributed evenly between the two hemispheres. In the Northern Hemisphere, the sea ratio is approximately equal to the ground (61 %), while the Southern Hemisphere is significantly oceanic (80 %). The sea is an interconnected system with five large areas called oceans (Pacific Ocean, Atlantic Ocean, Indian Ocean, Antarctic Ocean, and Arctic Ocean) and smaller sections known as seas (Mediterranean Sea, Baltic Sea, Red Sea, etc.). The Pacific Ocean is the most extensive, almost as large as the rest all together. Life in our planet is dependent upon the oceans, being the sea the main stabilizer of the world climate. In addition, the oceans are the main highway for international trade, as well as providing us water, food, energy, and sustaining the livelihoods of millions of people. Actually, marine biotechnology is being largely focused on marine biomolecules for medicine or engineering uses [12].

The presence of seawater is common in all marine habitats. The seawater is distinctly salty due to the nature of water and materials dissolved therein. Only six ions comprise 99 % of the dissolved solids. The most common are sodium and chloride, representing almost 85 %. In oceanic areas, the salinity has little variation, generally is between 33 and 37 ‰ depending mainly on the balance between precipitations and evaporation. However, in other regions, the salinity levels can change more conspicuously caused by many factors, such as the currents, the incoming of freshwater from rivers and glaciers, or the high evaporation levels in shallow areas. The sunlight penetrates only about 200 m, depending on water turbidity and angle of the sun, so at greater depths there is darkness. In addition to salinity and light, others physicochemical factors such as temperature, wind, currents, waves action, tides, acidity, dissolved gases, pressure, and substrate have influence on the functioning and diversity of the varied marine ecosystems. Also, these ecosystems depend on dissolved nutrients washed down from the land and sediments of the seabed. Rock composition, waves, tides, and currents are the principal agents responsible for erosion and deposition along coastlines and seabed. Besides, the currents are important in the transport of essential nutrients for the development of marine life, from the north to the south and vice versa and from deep to superficial areas.

Marine habitats can also be modified by their inhabitants. Some organisms such as coral, kelp, mangroves, and sea grasses are ecosystem engineers capable of reshaping the marine environment to the point where they even can create further habitats for other organisms [12].

The geological structure of the seabed is fairly similar worldwide. Marine habitats are distributed horizontally from the coasts, over the continental shelf, until the deep seabed. Alternately, the marine habitats can be vertically divided, and the entire area of the open water column is the pelagic environment; pelagic organisms live in open sea away from the seabed. The demersal environment is just above the seabed, and the benthic environment corresponds to the sea bottom, with 3.8 km of average depth. A third division is by latitude, from temperate tropical waters to polar waters. Depending on the distance from the coast, the pelagic environment is horizontally subdivided into the neritic zone on the continental shelf, which extends 68 km on average, and the oceanic zone. Progressing vertically and according to depth, the pelagic environment can be subdivided into different zones. The epipelagic, synonymous to photic or lighted region, is the zone of primary production in the ocean and with a major importance. The transition area between light and dark, called the disphotic region, is equivalent to mesopelagic. This area has enough light for vision but not enough for photosynthesis and extends down to about 700–1000 m. Next is the bathypelagic, which cover until 2000–4000 m. Overlying the plains of the major ocean basins is the abyssal pelagic, which has its lower boundary at about 6000 m. The open water of the deep oceanic trenches between 6000 and 10,000 m is called the hadalpelagic.

Corresponding to the last three pelagic zones are three bottoms or benthic zones. The bathyal is the area of seabed encompassing the continental slope and down to about 4000 m. The abyssal zone includes the broad abyssal plains of the ocean basins between 4000 and 6000 m. The hadal is the benthic zone of the trenches between 6000 and 10000 m. The benthic zone underlying the neritic pelagic zone on the continental shelf is named the sublittoral. It is illuminated and a permanently submerged area. The intertidal or mesolittoral zone includes shore areas lying between the extremes of high and low tide. It represents the transitional area from marine to terrestrial conditions. The most elevated area corresponds to supralittoral zone, called the splash.

Seawater penetrates in these areas only during storms with high tides [13].

Most marine life is found in coastal habitats, even though the shelf area occupies only 7 % of the total ocean area. Many sceneries such as sandy and rock shores, estuaries, and mangroves are colonized by great quantity of organisms constituting several different communities, such as sea grass beds, kelp forests, and coral reefs. In contrast, only about 10 % of marine species live in the open ocean.

Marine organisms have very different eating ways. The food is not only vital for their survival, but determines their trophic position and their relationships with other organisms in the ecosystem. Food chains show the flow of matter and energy, from primary producers or from traces of organic matter (called detritus chain), then to consumers until decomposer organisms. However, the reality in the ecosystems is that a species can eat different things and can be consumed by various types of organisms, so at the ecosystem level, food chains have a network aspect (food web). These networks are often complex and include further the route of microscopic decomposers and recyclers of organic matter [14].

The primary producers or autotrophs are organisms that synthesize organic matter from inorganic matter. In the marine environment, this role is played by algae and marine plants, as well as archaea and bacteria, phototrophs, and chemotrophs. Other primary producers referred to as mixotrophs can behave as both, autotrophs or heterotrophs, or combine both strategies depending on the environmental conditions, such as dinoflagellates. In addition, other autotroph organisms can establish symbiotic relationships with heterotrophs, for example, the case of the corals. Heterotrophs that feed directly from autotroph organisms are called herbivores or primary consumers. Benthic herbivores, such as sea hedgehogs, eat algae that are attached to the substrates. Others in the pelagic environment, such as copepods, feed on phytoplankton, separating them from the water through filtering structures. Secondary consumers eat primary consumers. In this group, there are carnivores, parasites, omnivores, etc. The large predators like sharks or orcas represent the upper trophic level. Ultimately, in the marine environment, there are also decomposer organisms, mostly microscopic such as bacteria and fungi, and also macroscopic such as polychaete, that feed on detritus or inert organic matter, returning to the medium some of the original molecules, which can be used by primary producers.

Food webs connect the pelagic and benthic systems, for example, the remains of organisms and organic matters, called marine snow, that fall to the seabed from the most productive surface layers provide food for numerous sessile benthic organisms that feed by filtering water and capturing food particles. Besides, while marine snow is deposited, it decomposes and enriches by decomposer microorganisms that can also serve as fresh food for benthic animals. It is known that only around 10 % of organic matter and energy pass from one trophic level to the next, and the rest is degraded for to obtain energy. Some parameters evaluate the transfer of matter and energy within an ecosystem; an example is the biomass that is the mass of all organisms that form a trophic level or ecosystem per unit area or volume. One measure of the relative importance of different marine habitats is the rate at which they produce biomass. Another parameter is the productivity, which allows to observe the rate of biomass renewal [12].

Marine ecosystems are home to a myriad of vegetable species (algae and plants) and animals, both invertebrates and vertebrates, ranging from microscopic phytoplankton and zooplankton, fungi, bacteria, and viruses, including marine bacteriophages, to large marine mammals of various sizes.

The first global Census of Marine Life (www.coml.org) has been completed recently. A consortium of around 2700 scientists from more than 80 nations has established a unique picture of the marine life diversity, distribution, and abundance. This global project has generated the most comprehensive inventory of marine life, from microorganisms to whales, from the icy poles to the warm tropics, and from shores to deep and dark seabed, as the basis for future research. In addition, this has been useful to forecast, measure, and understand changes in the global marine environment, as well as the management and conservation of the marine resources.

The number of marine species scientifically described has been around 250,000, but the census still could not reliably determine the total number of species from the sea. If the results were extrapolated, it would exist at least 1 million total marine species and tens or even hundreds of millions of kinds of marine microorganisms. In the last decade, the census found more than 6000 potentially new species and completed formal descriptions of more than 1200 of them. On the other hand, 170,000 cases of synonymy between previously known species, that is, a species described under two or more different names, have been found. Applying genetic analysis, i.e., DNA barcoding techniques, the number of species was expanded, especially the number of marine microorganism species. Moreover, on a dataset of 35,000 species from widely differing major groupings of marine life, the census identified the proximity and distance of relations among distinct species and observed organisms that had been mistakenly named separate.

Using new technologies and state-of-the-art equipments, the census found life in extreme marine habitats and was also able to register migratory routes of many species, establishing areas where they succeed and where they die. Coastal species showed maximum diversity in the tropical Western Pacific, whereas high diversity of species

frequenting the open ocean peaked across broad mid-latitudinal bands in all oceans. Regarding biomass, it is known that approximately 90 % of marine life is microbial, and the census database still has records for no more than 20 % of the sea. As an interesting data, the census has shown a global decrease of phytoplankton near the surface and the decline of large animals at the top of the food chain, but whether the total weight of life in the sea is changing remains unknown [15, 16].

Even though many types of seaweed are plant-like in appearance, in contrast plants show a very high degree of differentiation, with roots, leaves, stems, and vascular network, with their reproductive organs covered by sterile cells. Moreover, all plants have a digenetic life cycle with an alternation between a haploid gametophyte and a diploid sporophyte. Algae do not have any of these features; although some of them show differentiation of their vegetative cells, they do not form embryos; their reproductive structures consist of cells that are potentially fertile; parenchymatous development is present only in some groups and has both monogenetic and digenetic life cycles.

Historically, the major groups of algae are classified into divisions based on the types of pigments, the presence of reserve products like polysaccharides, structure of chloroplasts and cell wall, number, arrangement, and structure of flagella, reproduction cycles, and other special features. Recently, the sequence of some genes and the 5S, 18S, and 28S ribosomal RNA sequences are being used to confirm that these divisions are non-artificial, even though they were originally defined on the basis of morphology alone.

According to the most recently published classifications, the term algae refer to a highly diversified group of phototroph organisms. Prokaryotic members are grouped into the kingdom Bacteria and phylum Cyanobacteria with one class Cyanophyceae also called blue-green algae, where the members of the proposed division Prochlorophyta, considered artificial after phylogenetic analysis, are also included, while eukaryotic members are included in the kingdoms: Plantae, Chromista, and Protozoa. In general, the term algae refer to both macroalgae and a highly diversified group of microorganisms known as microalgae. The number of algal species has been estimated to be one to ten millions, and most of them are microalgae [17].

The algae are highly variable in dimensions. Among the unicellular algae, most are microscopic; nonetheless, others such as *Acetabularia acetabulum* have several centimeters (8 cm) despite being a unicellular organism. Similarly, the size of multicellular algae can range from microscopic forms (0.2–2 μm in diameter) to the giant kelp, a large brown alga that may grow up to 60–80 m in length.

They show a wide range of levels of organization and morphology, as unicellular structures with or without flagella, or colonies, aggregates of a variable number of cells with a growth by cell division of its components, without division of labor, and each cell capable of surviving on its own. Others, the filamentous algae, are the result of cell divisions in the plane perpendicular to the axis of the filament, so the cell chains are daughter cells connected to each other. Filaments can be simple and have false or true branching, other with a single layer of cells called uniseriate or with multiple layers called multiseriate. The siphonous or coenocytic algae consist of tubular filaments lacking transverse cell walls, created by repeated nuclear division without forming cell walls, and hence, they are unicellular and multinucleate. The parenchymatous and pseudoparenchymatous algae are mostly macroscopic whose overall body is called thallus. The thallus is formed by undifferentiated cells and originates from a meristematic tissue with cell divisions in three dimensions. In the parenchymatous structure, the cells of the primary filament are divided into all directions and any essential filamentous structure is lost. This structure is found, for example, in *Ulva* (Chlorophyta) and many of the brown algae. The pseudoparenchymatous thallus is made up of a filamentous construction with little or no internal cell differentiation. The branched filaments are intertwined and held together by mucilage, especially in red algae.

Methods of reproduction may be vegetative, by the division of a single cell or fragmentation of a colony, asexual by the production of motile spore, or sexual by the union of gametes. Vegetative and asexual modes provide a fast and economical means of increasing the number of individuals, but restrict genetic variability. In contrast, sexual mode involves genetic recombination and allows variation [12, 17, 18].

Algae are found almost anywhere because they can tolerate a broad range of temperatures, salinity, pH, O_2, and CO_2 concentrations. Accordingly, the algae can be planktonic, like most unicellular species, and also benthic, attached to seabed or living within sediments, limited to illuminated areas. Benthic algae can live in supralittoral zone, above the high tide level within the reach of waves and spray; in intertidal zone on shores exposed to tidal cycles or sublittoral zone in the benthic environment from the extreme low-water level to around 200 meters of deep. They can grow attached to stones (epilithic), on mud or sand (epipelic), on other algae or plants (epiphytic), on animals (epizoic), in symbiosis, as parasites, etc. It is possible to find algae in snow and ice (cryophilic) and in hot springs (thermophilic), and some of them can live on or in soil (edaphic).

The microscopic algae, the phytoplankton, are significantly involved in the accumulation of oxygen in the marine environment and also in the atmosphere. Besides, they represent carbon storage, fixing the atmospheric and seawater dissolved CO_2 through photosynthesis, helping to maintain cold the temperature of the planet and the seawater acidity at optimum levels for life. Sometimes, when the population of

these organisms is very large, due to pollution with nutrients, such as nitrogen and phosphate, the blooms can reduce the water transparency causing the death of other photosynthetic organisms. At the same time, these blooms contribute to the proliferation of other heterotrophs that consume elevated amounts of O_2, causing the depletion of this gas in the seawater so necessary for life resulting in the death of many organisms. In addition, some of them can also produce toxins able to kill other superior organisms (toxic blooms) [17].

Commonly, the prokaryote algae are called blue-green algae or cyanobacteria. These correspond exclusively to the Cyanophyta division and are non-motile Gram-negative eubacterias. They are among the first photosynthetic organisms on the planet. According to the endosymbiotic theory, the chloroplasts (photosynthetic organelles with circular DNA) found in plants and eukaryotic algae evolved from cyanobacterial ancestors via endosymbiosis. Currently, this group of algae is the most widely distributed and can tolerate wide ranges of salinity and temperature. The structure of cyanobacteria is diverse, from unicellular to colonial species appearing in planktonic and benthonic ecosystems. Cyanobacteria pigmentation includes chlorophyll-a, blue and red phycobilins (c-phycoerythrin, c-phycocyanin, allophycocyanin, and phycoerythrocyanin), and xanthophylls (myxoxanthin and zeaxanthin) that protect cells from excess sunlight and β-carotene. As reserve products, they present cyanophycin, an arginine and asparagine polymer, and cyanophycean starch. Some of them can also produce potent hepatic and neurotoxins. However, certain species have a high biotechnological potential, as well as being employed as dietary supplements [12, 17].

Within eukaryotes, algae are ordered into three major groups: red, brown, and green individuals. The red algae (Rhodophyta division) are essentially marine algae, of the 4100 species described, very few live in freshwater or terrestrial environments. This group includes different microalgae genera, but mostly consists of seaweeds. Their characteristic color is the result of chlorophyll-a masked by red or blue phycobilins (r- and b-phycoerythrin, r-phycocyanin, and allophycocyanin). Exclusive characteristics of this division are the absent of flagellate stages and the presence of hemiellipsoidal phycobilisomes and phycobilinproteins complexes anchored to thylakoid membranes into the chloroplasts. Moreover, they present α- and β-carotene, and the xanthophylls lutein. The polysaccharide floridean starch is the storage product more representative and abundant, located in the cytoplasm, unlike green algae that are located within the chloroplast. Some species of *Coralline* are directly involved in the formation and development of coral reefs. Others are recollected for different uses; for example, *Chondrus* species are useful as a gelatine substitute in food industry, and from *Gracilaria* and *Gelidium* species, agar is obtained, a gelatine-like substance, an important ingredient as solidifying agent in the preparation of culture media for bacteria and fungi. *Phorphyra* (Nori) is traditionally and widely consumed as a vegetable in Japan.

The brown algae or Phaeophyceae class (Heterokontophyta division) include approximately 1500 species, almost all of them are marine and common in cold waters along the coasts. Depending on the proportion of green pigment (chlorophyll) and brown pigment (fucoxanthin), they show a color palette from dark brown to olive green. As member of this division, the phaeophytes possess chlorophylls a and c, α-carotene, β-carotene, and ε-carotene, fucoxanthin, and vaucheriaxanthin and lack phycobilins. The polysaccharide chrysolaminarin is the principal storage product, located in special vacuoles inside the cytoplasm. They present a wide range of forms and sizes; for instance, the complex and giant kelps (*Laminaria*) are the biggest algae with a length of 80 m. Some seaweeds have a special gas-filled bladders, called pneumatocysts, which keep photosynthetic parts of the algal thallus floating on or near the surface of the water closer to sunlight. Brown algae are an important source of algin, a colloidal gel used as a stabilizer in the baking and ice cream industries, and also a major source of iodine and potash. Certain species are also used as fertilizer, and several are eaten as a vegetable such as *Laminaria* and *Undaria.*

The diatoms or basillariophytes (Heterokontophyta division) are other important and numerous groups of microalgae that contribute significantly to the total oceanic primary production [19]. About 16,000 species have been described; they are eukaryote organisms, most of them unicellular brown pigmented, with a pelagic life. As principal feature, diatoms are enclosed within a silica cell wall called frustule, usually with a bilateral symmetry. The yellowish brown chloroplasts of diatoms are typical of the Heterokontophyta division. They present chlorophylls a and c, β-carotene, and fucoxanthin as the major pigments, and chrysolaminarin as storage products and lipids. Thus, the diatoms serve as food for many animals, both directly and indirectly. Additionally, fossil diatoms, called diatomaceous earth, are used as base in dynamite, filters, insulation, abrasives, paints, and varnishes [17, 18].

Although most green algae (Chlorophyta division) are freshwater ones, there are also terrestrial and marine species. Nearly, 7000 species are described with only 10 % marine species, many of them unicellular. However, some species dominate certain marine environments with a wide salinity range. The photosynthetic pigments of chlorophytes are present at similar proportions as in higher plants, so it is believed that green algae are the ancestral form of land plant. The main pigments are chlorophylls a and b, and lutein and prasinoxanthin like xanthophylls. Chlorophylls are not masked by other pigments, so the chlorophytes show a brilliant green color. They also have carotenoids (α-, β-, and γ-carotene); phycobilins are absent in this division, and the

most important reserve polysaccharide is starch, which resides inside chloroplast as grains. Green algae vary in shape and size, including unicellular individuals as *Chlamydomonas* and macroalgae as *Caulerpa* or *Ulva.* The uses of green algae are diverse, in cosmetic industry, for human consumption (*Ulva*) and even employed for sewage purification (*Chlorella*) [12, 17, 18].

Other important members of marine phytoplankton are the dinoflagellates (Dinophyta division); approximately, half of the species are photosynthetic and significant in the food chain, but even among these, many are also predatory. They are unicellular and usually biflagellate organisms. Other morphologic characteristic is their covering with vesicles, which can be empty or filled with cellulose. Some of them are invertebrate parasites, others are endosymbionts (zooxanthellae) of tropical corals, and some can produce bioluminescence. Dinoflagellates have chlorophylls a, b, and c, β-carotene, and different xanthophylls (peridinin, fucoxanthin, diadinoxantin, dinoxanthin, and gyroxanthin); phycobilins are absent in this division. The main reserve polysaccharide is starch, located in grains in the cytoplasm, similar to green algae, but oil droplets are present in some genera. Some dinoflagellates produce toxins; consequently, under favorable conditions, dense algal blooms appear that can poison fish and other marine animals, even to humans by eating, for example, mussels that have ingested and processed large quantities of these dinoflagellates [17, 18].

Terrestrial Plants and Fatty Acids

Under this subheading, attention is drawn and some details are presented, on how the biosynthetic capability of terrestrial plants is regarding fatty acid formation, as well as showing some differences compared with the plant marine sources. Generally, a large interest exists on omega-3 fatty acids, also those taken from higher plants, mainly due to their importance in human nutrition and health, because of the relatively scarce sources of these types of compounds, and our intake of them, in comparison with the omega-6, which are much more abundant, and present in an excessive degree in our diets.

Terrestrial plants all are derived from the seas about 400–500 million years ago, in particular from green algae. This being one of the most important events in the evolution of life on Earth, affecting the development of landscapes, the appearance of new species colonizing this medium, and the atmospheric concentration of gases [20–22]. Following evolution, higher plants have developed different biochemical capabilities for fatty acid biosynthesis, and one would expect to encounter similarities between plants and marine species in relation to the synthesis and accumulation of fatty acids, but also some differences.

In general, it is known that in higher plants, the synthesis of fatty acids takes place in different cell compartments, participating in plastids and endoplasmic reticulum, involving also the release from different reservoirs and transport of precursors within the cell [23]. Predominantly, omega-3 fatty acids in higher plants display carbon chains up to 18 carbons in length, and in a few cases, the maximum number of double bonds reaches up to 4 (18:4n-3, SDA = stearidonic acid), but more commonly 3 (18:3n-3, ALA = α-linolenic acid) [24, 25]. This is where the biosynthetic capacity reaches, being unable to synthesize longer carbon chain omega-3 with larger number of double bonds, such as, for instance, EPA (eicosapentaenoic acid, 20:5n-3) and DHA (docosahexaenoic acid, 22:6n-3) of large nutritional interest [26]. Nonetheless, there are some examples, no omega-3, in which certain plants can produce longer carbon chain fatty acids, up to 22 or 24 carbons, such as erucic acid (22:1n-9) and nervonic acid (24:1n-9), but with only one double bond present at position 9 [27, 28]. Similarly, regarding the omega-6 fatty acids, plants display a reduced number of them, such as linoleic and γ-linolenic acids (18:2n-6; 18:3n-6); however, longer carbon chain ones, with a higher number of double bonds, such as dihomo-gammalinolenic and arachidonic acids (20:3n-6; 20:4n-6) are rarely encountered in higher plants, unlike other sources of fatty acids, such as fish and algae, which accumulate longer carbon chains and more unsaturated compounds, displaying almost all spectra of fatty acids. Thus, fewer plants possess the ability to accumulate Δ6-desaturated fatty acids, in particular SDA and mainly GLA. Within these fewer plants, the genus *Echium* (Boraginaceae), well represented in the Canary Islands with 23 endemic species, show attractive amounts of SDA and GLA [29–34]. Moreover, other plant families, i.e., Saxifragaceae, Scrophulariaceae, and Primulaceae, contain species accumulating these two 18 carbon fatty acids [35, 36]. However, beyond these fatty acids, higher plants do not possess the enzymatic machinery to undertake further elongation and desaturation steps to produce the more interesting omega-3, such as EPA and DHA.

Regarding the synthesis of highly unsaturated fatty acids, this can be conducted following several ways, either by anaerobic pathways (by polyketide synthase enzymes known as the polyketide pathway), which is only present in some microorganisms, or by aerobic metabolic routes, which are more universal as occurring in plants, fish, fungi, algae, etc., and consisting of successive elongation reactions (elongation of the carbon chain) and desaturation reactions (introduction of double bonds into the carbon chain). These reactions are governed by two types of enzymes, i.e., elongases and desaturases [37–40].

There exist variations in the order in which the desaturation and elongation steps occur. Thus, the "metabolic pathway 6" begins with a 6-desaturation reaction, followed

by chain elongation and desaturation of the carbon chain, leading to the formation of eicosapentaenoic acid (EPA, 20:4n-3), when the initial substrate is ALA, or to arachidonic acid (ARA, 20:4n-6), when the initial substrate is LA (linoleic acid, 18:2n-6). Next, EPA is elongated to docosapentaenoic acid (DPA, 22:5n-3), which again is desaturated at 4-position, to finally produce docosahexaenoic acid (DHA, 22:6n-3) (Fig. 9.1).

Furthermore, there is another variation called Sprecher's route, which describes the production of DHA by means of Δ6 and Δ5 successive desaturation of ALA through the formation of metabolic intermediates of C24 that are eventually shortened to DHA by one β-oxidation step taking place in the peroxisomes [41–43]. What characterizes this route is that the final product, DHA, is synthesized without the intervention of the enzyme 4-desaturase. This latter route has been characterized in mammals and fish [44–46] (Fig. 9.1).

Finally, the "alternative route 8," frequent in protists and algae, begins directly with an elongation step from C18 or C20 intermediates, followed by a desaturation at position 8, and an additional one at position 5, to eventually form dihomo-gammalinolenic acid (DHGLA, 20:3n-6) and ARA, or eicosatetraenoic acid (ETA, 20:4n-3) and EPA (Fig. 9.1) [47, 48].

Despite these apparent limitations of higher plants for producing the valuable omega-3 fatty acids, alternative approaches have been conducted employing them, such as attempts to modify the genetic makeup of higher plants, aiming at the production of these important metabolites. Furthermore, natural resources of fish oils and the omega-3 fatty acids they contain are diminishing, and are not maintained and exploited in a sustainable manner. Therefore, alternative sources of these important compounds are needed. Thus, much efforts have been applied in the genetic engineering of higher plants by introducing multiple genes of this pathway derived from other organisms, such us fungi, microalgae, bacteria, and diatoms, which have resulted in the establishment of different transgenic plants, i.e., soybean, Arabidopsis, Brassica, tobacco, and others, capable of producing DHA and EPA with attractive yields [26, 49–51].

Many obstacles appear to be solved, particularly the control of the biosynthetic intermediates which should be released from different reservoirs and transported to and

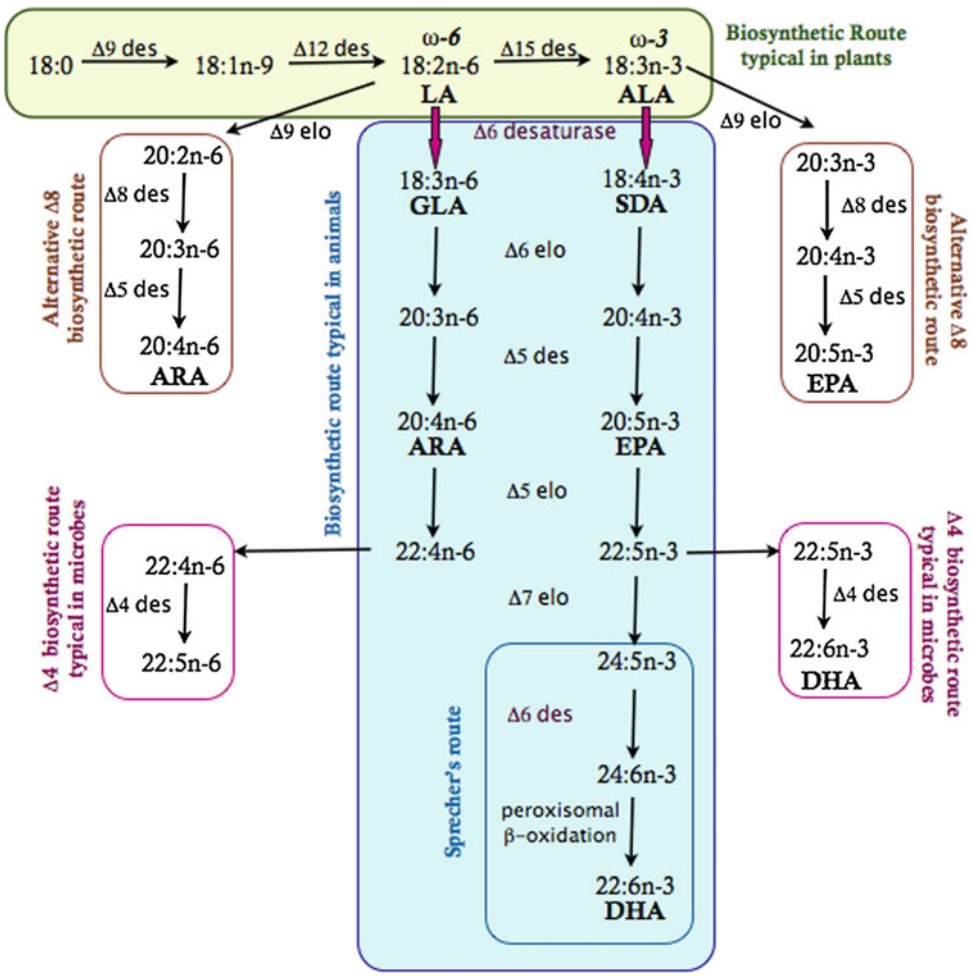

Fig. 9.1 Aerobic metabolic pathway for the formation of long-chain polyunsaturated fatty acids (PUFAs) n-3 and n-6 from short-chain essential fatty acids n-3 and n-6, respectively (*des* = desaturase, *elo* = elongase)

from different organelles; furthermore, control of the primary synthesis and redirecting the flux of substrates and biosynthetic precursors have also been accomplished, particularly being able to halt to reduce the stimulation of the omega-6 branch of the pathway after characterizing and properly utilizing acyl CoA-dependent desaturases. Thus, following the insertion of 5–7 genes in the fatty acid pathway into *Camelina sativa* resulted in the high production of EPA and DHA in seeds, accumulating 12 and 14 % respectively, levels similar to fish [52].

Analogously, other authors have also engineered this species to convert native oleic acid, LA, and ALA into mainly DHA and EPA [53]. Their strategy also maintained at bay the amount of omega-6 fatty acids produced, with only small amounts of GLA (1.2–1.4 %), and no other omega-6 PUFA accumulated. More importantly, the levels of DHA recorded exceeded 12 %, whereas total omega-3, i.e., ETA, EPA, and DPA, reached 25 %.

These results demonstrate the effectiveness of the genetic engineering of oilseed crops, and how the advances made offer a real agronomic alternative for the sustained production of long-chain omega-3 fatty acid in higher plants. This approach has all the potential to exploitation and is an alternative source of production of these important PUFAs, although many issues related to the perception and opposition to the acceptance of GM crops have to be overcome before these genetically engineered species would be implemented as an agronomic crop for fatty acid production. Nonetheless, the scientific advancements have been achieved, showing the successful generation of a terrestrial source of long-chain omega-3 fatty acids employing higher plants, pending to solve regulatory issues before the commercial production of long-chain omega-3 fatty acid through oilseed plants becomes a reality.

Codex Alimentarius and Human Nutrition

Historical documents from the earliest civilizations as Assyrian, Egyptian, Greek, or Romans provide evidence that governing authorities were already interested in the regulation of food sale to protect consumers from fraud or bad produce. However, the first overall food laws were approved in mid-nineteenth century, coinciding with the period in which the food chemistry began to be a recognized scientific discipline.

The *Codex Alimentarius,* the Food and Drug Administration from USA (FDA), and the European Food Safety Authority (EFSA) are the three major authorities regulating food safety worldwide.

Nowadays, the *Codex Alimentarius* is a collection of internationally recognized standards, codes of practice, guidelines, and other recommendations relating to foods, food production, and safety, whose aim is consumer protection [54]. This food regulation body, which is relatively young, was established in 1963 by Food and Agriculture Organization of the United Nations (FAO) and World Health Organization(WHO). It develops standards, based on independent scientific data, which are taken as a world benchmark and often serve as the basis for drafting legislation. Its main goals are to protect the health of consumers and ensure reliable and equitable practices in the international food trade; it also promotes the coordination of all food standard work undertaken by international governmental and non-governmental organizations.

The *Codex* texts, the most important global reference for consumers, food producers, government agencies, food control, and international professional associations, are developed, maintained, and updated by the *Codex Alimentarius* Commission. In addition, this organization helps developing countries to apply Codex standards and strengthen national food control systems taking advantage of international food trade opportunities. The *Codex Alimentarius* Commission includes 186 members: 185 member countries and 1 member organization (European Union), and 225 observers: 52 intergovernmental organizations, 157 nongovernmental organizations, and 16 United Nations organizations, which can attend sessions of the commission and of its subsidiary bodies, as well as the meetings [54].

The Food and Drug Administration of the USA (FDA) was founded in 1906, and it is the oldest authority for regulation, representing a reference worldwide. The FDA is a division of the Department of Health and Human Services. This organization is divided into several centers; one of them is the center for the food safety and applied nutrition. However, the FDA is responsible not only for regulating food for humans but also for animals, besides drugs (human and veterinary), cosmetics, medical (human and animal) devices, biologics, and blood products, in order to ensure the safety and quality of products consumed within the country [55].

The European Food Safety Authority (EFSA) is very recent compared to the two previous authorities and was created in 2002. The main objective was to ensure consumer protection and restore confidence in the European market [56]. EFSA is funded by the EU budget, but it is an independent European agency that operates separately from the European Commission, European Parliament, and EU Member States. EFSA provides a forum for collaboration and exchange of information of 28 European members, Iceland, and Norway and has observers in Switzerland and the European Commission. This is the European authority that provides scientific and technical support on food and feed safety, nutrition, health and welfare animal, and plant health. Additionally, it assesses environment safety and is a support to agroinnovation. At the request of the European

Commission, EFSA experts frequently participate in meetings organized by the Codex Alimentarius Commission.

These agencies require the evaluation of the different parameters before granting authorization to exploit any product derived from these organisms. In brief, this includes risk assessment based on scientific evidence and information; evaluation of the production and processing methods of the final product; food and feed safety; traceability which shall be established at all stages of production, processing, and distribution; and presentation of the product.

Table 9.1 List of microalgae and seaweeds accepted for human use by any of the three regulatory authorities ESFA, FDA, and Codex Alimentarius

Microalga	Seaweed
Chaetoceros sp.	*Ascophyllum nodosum*
Chlorella sp.	*Ahnfeltia plicata*
Crypthecodinium sp.	*Alaria esculenta*
Dunaniella salina	*Caulerpa* sp.
Haematococcus pluvialis	*Chondrus crispus*
Isochrysis sp.	*Cladosiphon okamuranus*
Nannochloropsis sp.	*Durvillaea* sp.
Nitzschia laevis	*Ecklonia* sp.
Odontella aurita	*Enteromorpha* sp.
Pavlova sp.	*Eucheuma* sp.
Phaeodactylum sp.	*Fucus* sp.
Porphyridium sp.	*Gelidium* sp.
Schizochytrium sp.	*Gigartina* sp.
Skelotenma sp.	*Gracilaria* sp.
Spirulina (*Arthospira*) sp	*Gracilaria* sp.
Tetraselmis sp.	*Himanthalia elongata*
Thalassiosira sp.	*Hizikia fusiforme*
Thraustochytrium sp.	*Hypnea musciformis*
Ulkenia sp.	*Kappaphycus alvarezii*
	Laminaria sp.
	Lessonia sp.
	Lithothamnium sp.
	Macrocystis pyrifera
	Mazzaella laminaroides
	Monostroma sp.
	Palmaria palmata
	Phymatolithon calcareum
	Porphyra sp.
	Pterocladia sp.
	Sarcothalia crispata
	Sargassum sp.
	Ulva sp.
	Undaria pinnatifida

According to the current legislations and after all the evaluations and assessments performed, the consumption of several microalgae and seaweeds, as well as many of their products, is approved for human use, as well as other industrial applications different than for human use, which has boosted the commercial exploitation of many of these species and many more to come (Table 9.1) [57].

Fatty Acids from Marine Microalgae

Microalgae in general and marine microalgae in particular are considered important organisms acting as the natural primary producers of long-chain PUFAs in the food web and similarly being major sources of intermediates for the production or accumulation of these fatty acids by other organisms, particularly fish, higher in the food chain [58]. These are also natural producers of other important compounds, such as pigments, antioxidants, sterols, vitamins, polysaccharides, and many other bioactive compounds [59–61].

Furthermore, it is known that there is high increase in the demand for fish oils rich in PUFA, particularly EPA and DHA. These are mainly obtained from extractive fishing practices, or from aquaculture, which also depend directly on extractive fishing to manufacture fish feed, affecting global fish stocks and fish sustainability. According to FAO figures [9], fish stocks are being depleted at a fast pace, and rapid actions are being implemented in order to avoid environmental tragedies; nonetheless, the tendency of stock depletion is maintained despite all these efforts, and new approaches for producing these valuable metabolites have also been devised. Likewise, microalgae offer attractive advantages over fish oils since these possess high quantities and different balances of DHA and EPA depending on the particular species. More importantly, microalgae display simpler fatty acid profiles compared to fish oils, which simplifies their adaptation or nutritional modification and becomes more attractive from a commercial viewpoint. Microalgae also produce and accumulate important antioxidants that provide stability to the PUFA, avoiding their oxidation and bad odors. In general, these also display a low n6/n3 ratio making them more nutritionally attractive. On the other hand, independent quality control analyses of microalga oils have established the absence of heavy metals and other contaminants in these organisms, which emphasizes and demonstrates a major interest and attractive features for exploring and exploiting these resources, avoiding these issues often encountered when consuming fish oils [9, 62]. This PUFA source is also suitable for the vegan and vegetarian consumers who do not manage to intake appropriate omega-3 fatty acids from plant sources.

This scenario has ignited the evaluation, research, and development of new sources of PUFA and proteins to meet

the nutritional needs of the increasing human population. Thus, in order to find and establish other sources of production of PUFA, mainly EPA and DHA, including also the sustainable and effective production of these products, microalgae research has been conducted for several decades, showing their potential commercial exploitation, which is presented under this subheading, making an effort to unite the literature in relation to the production of EPA and DHA by these organisms.

Often the term microalgae is applied to and includes also different prokaryote organisms that are not strictly speaking microalgae (eukaryote). In this chapter, and assuming the lack of rigor but for a more ordered manner for presenting the scientific results, we have decided to consider under the term microalgae, either microalgae themselves, as well as other single cell organisms that are not necessarily microalgae, such as diatoms, dinoflagellates, cyanobacteria, and protists, which are equally able to produce and accumulate these important PUFA and in many instances have been exploited or showing attractive potential for commercial exploitation.

The microalga *Nannochloropsis* (Eustigmatophyte) has long been used as a source of n-3 PUFA to mainly supply larval fish and other markets. The predominant fatty acids in *Nannochloropsis* are palmitic acid (16:0), palmitoleic acid (16:1), and EPA (20:5n3), independently of the culture conditions [63]. It has also been shown how EPA content changes with different culture conditions. EPA as a percentage of dry mass was 3.2 and 3.1 % in low and high nitrogen (N) levels, which increased by 50 and 46 % compared with 2.1 % in middle N level. The total amount of PUFAs (20:4, 20:5, 22:6) showed also an increase of 12, 23, and 41 % in low, middle, and high N levels, respectively. However, the percentage of PUFAs decreased with increasing salt concentrations and temperature [63].

Similarly, the FA profile of *Nannochloropsis oculata* showed that the main fatty acids were palmitic, palmitoleic, eicosatrienoic, and eicosapentaenoic acids, the latest being also the major constituent, with a value of 48.86 % (w/w) [64]. In fact, the genus *Nannochloropsis* is currently the source of marketed oils due to its potential to produce high-EPA lipids with very low DHA and ARA content, which is considered advantageous for the manufacture of dietary supplements [65]. Contrarily, *Nannochloropsis limnetica*, an unusual freshwater species from this genus, produced 28 mg g^{-1} DW of EPA under aerated suspension cultures at the stationary phase of growth. More interestingly, this species was able to produce 55.5 mg g^{-1} DW of EPA when cells were grown in a non-aerated culture supplemented with dipotassium phosphate, together also with linoleic and arachidonic acids (22.2 and 10.5 mg g^{-1} DW) [66].

Another species feasible of being used as a source of omega-3 FA is the diatom *Phaeodactylum tricornutum*. This microalga produces high levels of EPA, appearing as the most abundant, with yields of 23.7 % with respect to the total fatty acids and lower amounts of DHA (2.5 %) [67]. It is known that glycerol is one of the most used substrates in microalga culture because it causes strong effect on fatty acids accumulation, and is added as a carbon source to mixotrophic cultures in order to change fatty acid profile and content in microalga, especially eicosapentaenoic acid (EPA) [68]. Using glycerol as a carbon source in semi-continuous cultures, the optimal dilution rate (0.143 mol L^{-1} glycerol) produced both the highest biomass production (25.4 g L^{-1}) and the highest EPA accumulation with a 3 % increase (50 mg L^{-1} day^{-1}). Similarly, an outdoor batch culture with the strain *P. tricornutum* UTEX-640 was able to produce maximum lipid productivity. EPA content was increased up to 3 % (DW) in mixotrophic growth, giving a productivity of 56 mg L^{-1} day^{-1}, a significant increase compared to the photoautotrophic control, which yielded a maximum EPA content of 1.9 % (DW) and a productivity of 18 mg L^{-1} day^{-1} obtained in semi-continuous mode, suggesting that these culture conditions might be an excellent choice for transferring to an outdoor pilot-scale plant [69]. A linear programming approach has also been described to simulate how the growth and storage of important compounds of *P. tricornutum* could vary, based on mass and energy balances under nutrient-limiting conditions. It was predicted that both carbohydrates and lipids are synthesized simultaneously but at different rates under nutrient limitations [70].

Odontella aurita is a microalga marine diatom used for human nutrition that is known to contain high levels of EPA (26 % of total fatty acids) and several bioactive compounds, such as pigments, fibers, and phytosterols, which have beneficial effects on human health [71, 72]. In one instance, *O. aurita* was cultured under high UV radiation which did not affect the fatty acid composition of the total lipids and lipid fractions of the cells with EPA levels remaining attractively high (27–28 % of total lipids) during the 8 days of treatment. Indicating that open cultures of *O. aurita* in medium or high UV irradiation latitudes could yield high-EPA algal biomass [71]. Based on its EPA profiles, together with its other bioactive components, this species has been evaluated as an attractive dietary supplement for dyslipidemia, platelet function, and oxidative stress in high-fat fed rats. The synergistic effect of these microalgal compounds displayed a beneficial effect in reducing the risk factors for high-fat-induced metabolic syndrome, i.e., hyperlipidemia, platelet aggregation, and oxidative stress, in the treated rats [73].

Following a different approach, *O. aurita* was cultured in cylindrical glass columns and flat-plate photobioreactors, altering different conditions, such as low light and nitrogen, high light and low phosphorus, high light and low silicon, or high light and low sulfur. Under optimal conditions,

O. aurita yielded a maximum biomass production of 6.7–7.8 g L^{-1} under high light. Furthermore, the protein content decreased, while carbohydrate, mainly β-1,3-glucan, increased remarkably to about 50 % of dry weight during the entire culture period. The highest lipid content was 19.7 % of dry weight, and 80 % of fatty acid profiles were C-14, C-16, and C20. ARA and EPA accounted for 1.6–5.6 % and 9–20 % of total fatty acids, respectively [74].

Another promising species is *Trachydiscus minutes*, a yellow-green alga that produces high amounts of EPA. This microalga was cultivated in a standard medium and also in media without sulfur and nitrogen. The best productivity of EPA was in excess of 35 % of total fatty acids; the productivity was thus 88 mg L^{-1} day^{-1}. So, this organism could be considered as an alternative source for EPA production [75]. In the same fashion, *T. minutes* was cultivated under different conditions varying different parameters, such as light intensity, salinity, or nitrogen source [76]. It was shown that for producing a considerable amount of EPA, urea was a very attractive nitrogen source, behaving similar to nitrate, producing in both cases 26 mg EPA L^{-1} day^{-1}. A NaCl concentration of 0.2 % slightly stimulated EPA content, reaching 24 mg L^{-1} day^{-1}, while higher salt concentration above 0.8 % was lethal. Regarding light and temperature conditions, the microalga grew best at high light intensities and a temperature of 28 °C, reaching EPA amounts of ca. 30 mg L^{-1} day^{-1}. So, taken into account all these data, it could be suggested that for an optimal EPA production, outdoor cultivation under conditions of a temperate climatic zone in summer, using urea as a nitrogen source, could be the best combination [76].

It is well known that the dinoflagellate *Crypthecodinium cohnii* is a very attractive producer of DHA. In fact, differences in productivity among four different strains, *C. cohnii* ATCC 30 556, ATCC 50 051, UTEX L 1649, and RJH, were studied. It was shown that all the four analyzed strains produced DHA in high amounts, but with the highest production found in ATCC 30 556 strain with 159.4 mg L^{-1} grown in Porphyridium medium with 5 g L^{-1} glucose at 25 °C during 96 h. These results demonstrated that it is also feasible to grow microalgae in heterotrophic conditions, with a concomitant great potential for the production of PUFAs [77].

It is generally considered that low temperature favors the formation of polyunsaturated FA including DHA, so further experiment with this high-DHA producer strain in which temperature was shifted from 25 to 15 °C, to promote the accumulation of cellular DHA, was studied. High temperature (30 °C) favoured the growth of the microalga; in contrast, low temperature boosted the accumulation of PUFAs. The highest DHA content was obtained at 15 °C in the early stationary phase, recording 6.21 % (of dry weight), and the highest DHA productivity was of 1.47 mg L^{-1} h^{-1} [78].

Recently, a screening of approximately 300 different microorganisms belonging to the genus *Crypthecodinium* was carried out, finding that only 34 were DHA producers. All these 34 strains showed different ranges and amounts of FA, containing, for instance, ARA, EPA docosatetraenoic acid, and DHA as PUFAs. Their productivities varied from 7.87 to 502 mg L^{-1} of total fatty acids production, and particularly, the range of DHA was 8.7–66.7 % of total fatty acids. Surprisingly, the isolated strain D31 identified as a related species of *C. cohnii* possessed a unique fatty acid composition, where DHA was the only PUFA. The amount of DHA produced by this strain grown in GPY liquid medium for 7 days was over 60 % of total fatty acids, amounting 124 mg L^{-1}. DHA accumulated mainly as a polar lipid (79.4 % of total DHA), especially as phosphatidylcholine (71.4 % of polar DHA), although most oleaginous microorganisms accumulate DHA as triacylglycerol [79].

Further modifications of various culture conditions (carbon sources, nitrogen sources, salinity, and initial pH of nutrient medium) were carried out in order to evaluate the effect on DHA production. D-Glucose, D-fructose, acetic acid, ethanol, and glycerol were better sources to promote cell growth, whereas other saccharides, organic acids, and sugar alcohols did not have effect on growth. Besides, ethanol and glycerol stimulated DHA productivity, reaching amounts comparable to that obtained with glucose. DHA production was the highest with glycerol as the carbon source (103 mg L^{-1}). Different sources of nitrogen and salinities in the medium were also studied. The mixture of polypeptone and yeast extract, used as the basic GPY medium, was preferable for growth and DHA production, while the optimal saline concentration was 50 % to that of seawater. The pH was also analyzed, reporting a constant DHA production at acidic pH range (pH 3.0–6.0). Therefore, cultures under the so-called optimal conditions (glycerol as the carbon source, a mixture of yeast extract, and polypeptone as the nitrogen sources, salinity at 50 % to that of seawater, and pH 5.5) yielded 375 mg L^{-1} of DHA [79].

Pavlova lutheri is another species which produces EPA and DHA at attractive levels [80], and it is widely used in aquaculture as live feed for marine invertebrates, for example, feeding oyster larvae *Crassostrea gigas* [81]. When cultured under different irradiance levels and with different carbon sources, it was observed that lipid composition was more sensitive to light intensity variation than carbon source. The highest EPA levels were achieved predominantly in the galactolipid fraction when the cells were cultured at low light, regardless of the carbon source, accumulation that could be related to facilitate thylakoid membrane fluidity. Furthermore, the highest DHA levels were observed under high light conditions. Thus, PUFAs corresponded to approximately 45–55 % of the total fatty acids [82]. Recently, cultivation of *P. lutheri* has been carried out on

large-scale, which demonstrates that this microalga is able to grow similarly in 300 L or 250 mL cultures. This fact makes this organism, together with the optimization of culture conditions, to be considered for its potential to yield EPA and DHA at a profitable large scale [83]. As fatty acids are generally considered to be sensitive to oxidation by UV radiation (UV-R), and in order to determine the possible deleterious effect of UV radiation on fatty acid profiles of *P. lutheri*, this was cultured under high UV radiation. Exposure to UV-R treatment led to a decrease in the proportions of PUFAs, such as EPA and DHA, especially into structural lipids (glycolipids and phospholipids). It caused a reduction of 20 % in EPA levels, from 22.6 % molar of control to 18.2 %, and 16 % in DHA levels, from 13.4 % molar of control to 11.2 %, after 8 days of UV-R treatment [71].

Several species belonging to the genus *Isochrysis* are amenable for the production of DHA. For instance, *I. galbana* produced attractive amounts of DHA with 15.8 mg g^{-1} dry weight. Nonetheless, for most of the microalgae studied, the most abundant fatty acid was palmitate [84]. The effects of the addition of sodium nitrate at different intervals as a nitrogen source have a marked effect in lipid production. Cell density of *I. zhangjiangensis* was improved significantly when sodium nitrate was supplied at an interval of 24 h, as well as the lipid productivity, reaching the maximum value of 140.9 mg L^{-1} day^{-1} [85]. This algal strain can accumulate lipids under nitrogen-repletion conditions and accumulate carbohydrates under nitrogen-depletion conditions. When cultured in a high nitrate concentration, the growth of algal cells was suppressed, but the highest lipid content of 53 % was attained. Furthermore, a two-stage cultivation model was also assessed; in the first stage (0–96 h), sodium nitrate was added into the medium at an interval of 24 h. In the second stage (96–240 h), three different nitrogen treatments, i.e., nitrate-repletion condition (75 mg L^{-1}, 24 h interval addition), nitrate-depletion condition (no added nitrate), and the intermediate condition (75 mg L^{-1}, 72 h interval addition) are carried out. After these treatments, DHA remained as the major PUFA with 13 % of total fatty acids amount and EPA with 1.5 % [85].

Recently, the optimization of different *I. galbana* strains for DHA production has been studied. Although all the strains possessed almost the same FA composition, DHA content varied from 6.8 to 17.0 % of total fatty acids, being *I. galbana* #153180 the strain showing the greatest DHA content of 16 % of total fatty acids, achieving the highest DHA productivity (6.13 mg L^{-1} day^{-1}). Different parameters were varied (light, nitrogen, phosphorus, and salinity) assessing growth and DHA yields. After optimization, this strain registered the highest DHA figures, up to 17.5 % of total fatty acids (13.6 mg L^{-1} day^{-1}) or 1.7 % of cell dry weight (0.72 g L^{-1} day^{-1}) [86].

It has been recognized that *I. galbana* is a rich source of PUFAs and has been used to enhance nutritional value of different foods, such as pasta, to produce high-value nutritional PUFA sources for human consumption. The enrichment of raw fresh pastas with *I. galbana* biomass led also to a significant increase of EPA (20:5n3) and DHA (22:6n3) that were absent in raw control pastas [87].

Another species that has received large attention is *Schizochytrium* sp., mainly for the production of DHA. In particular, *S. limacinum*, a thraustochytrid closely related to heterokont algae, first isolated from a mangrove area in the west Pacific Ocean [88], displays attractive DHA levels, more abundant than EPA. In one report, it was demonstrated that the culture temperature and percentage of glucose in the nutrient medium influence DHA and biomass productivity. Growth was better at 25 °C than at 20 or 30 °C. DHA yields were enhanced (874 mg L^{-1}), by increasing glucose from 1 to 5 %; nonetheless, when 6 % glucose was used, the biomass decreased clearly, resulting in an overall decline of DHA [89]. Employing *Schizochytrium* sp., other authors showed that DHA production ranged from a maximum of 204 mg g^{-1} with a biomass of 13.2 g L^{-1} to a minimum of 158 mg g^{-1} with a biomass of 10.8 g L^{-1}. The maximum yield was attained with an optimal salinity of 25–30 ‰ [90]. Oxygen supply also affects the production of DHA in *Schizochytrium* sp. Optimizing the level of oxygen together with glucose resulted in a high cell density (71 g L^{-1}), high lipid content (35.75 g L^{-1}), and high-DHA percentage (48.95 %) that were achieved using a stepwise aeration controlled strategy. Thus, DHA productivity reached 119 mg L^{-1} h, 11.21 % larger than the best results obtained by constant aeration rate [91]. This approach was further optimized following a two-stage oxygen supply control strategy based on oxygen transfer coefficient employing a 50-L fermenter with a working volume of 35-L. Maximum concentrations of lipid and DHA reached 46.6 and 17.7 g L^{-1} with a productivity of 111 mg L^{-1} h^{-1}. Both results were higher than those attained at constant oxygen transfer coefficient processes. This strategy resulted in a considerable improvement in lipid and DHA concentrations, but more importantly, in a clear increase in DHA productivity [92]. This species has been approved for human use and is being largely exploited by different manufacturing companies for the production of mainly DHA, which is used for the preparation of infant food, other human nutraceutical products, and also as feeding additive in egg-laying hens enriched in this FA.

Different species of the genus *Thraustochytrium*, taxonomically related to *Schyzochytrium*, both from the same order Labyrinthulales, possess interesting FA profiles, which have been largely studied, and some are also commercially exploited. A *Thraustochytrium* strain, named KK17-3, showed high-DHA content (52.1 % of total fatty acids) and

wide range of PUFA (76.1 %), comprising arachidonic, EPA, and docosapentaenoic acids, together with DHA. Glucose and tryptone were the optimal carbon and nitrogen sources, in a medium with salinity at 75 % that of seawater. The PUFA contents in polar lipids (22.1 % of total lipid), in which the DHA content was 39.3 %, were higher than those in neutral lipids and glycolipids [93]. Analogously, a native Labyrinthulomycetes strain, *Thraustochytriidae* sp. TN5 (Thraustochytrium) from Japan, was scaled from shaken flask to a laboratory fermenter. Different growth media, particularly the influence of two discrete—carbon and nitrogen sources—and six continuous—concentrations of the carbon and nitrogen sources, yeast extract and artificial seawater, incubation temperature, and time factors, were assessed for DHA and lipid production. In the flask experiments, the best lipidic content was 25.2 % w/w of the biomass, with a DHA concentration of 0.48 g L^{-1} and a biomass production of 5.1 g L^{-1}. Fed-batch bioreactor experiments increased biomass concentration to 14 g L^{-1}, with the lipidic fraction between 16.2 and 34.8 % w/w, with lipids and DHA productivities of 50 and 23 mg (L^{-1} h^{-1}), respectively [94]. *Thraustochytrium* sp. ATCC 20892 strain was also investigated. Glucose and sodium glutamate were the preferred carbon and nitrogen sources, respectively, and the optimum condition for growth and DHA production was at pH 7.0 at 25 °C with 40 g glucose L^{-1} for 4 days. Under these conditions, the maximum DHA yields were 67.6 mg L^{-1} or 35 % of total fatty acids, relatively low amounts of 16:0 (29 %) and 18:1 (13 %) and insignificant amounts of other PUFAs [95].

Currently, several companies already exploit different species of microalgae for large-scale production of these omega-3 FA, especially for the manufacture of functional foods for human consumption. Many of them have already obtained approval by the various authorities for marketing them to the broad market in human medicine. For example, a company based in New Zealand, Protonz, is one of the largest producers of EPA by scaling and optimization of the fermentation of the microalga *Nitzschia laevis*. It has also established state-of-the-art infrastructure and technology for an industrial scale reactor of 7.5 tons, to meet mainly the demand of the cardiovascular healthcare sector market with figures of around 60 billion dollar and for which there is a high demand for EPA. Analogously, the company Martek native to the USA, and recently acquired by the Dutch multinational DSM, has also developed a line of business and has obtained approval to market the production of DHA and ARA, by fermentation of the microalgae *Thraustochytrium* and *Schizochytrium* mainly for the preparation of infant milk and for the preparation of omega-3 capsules and supply preparations for making multiple enriched end products with these FA for human consumption, with annual business figures of over a billion dollar. Nutrinova–Celanese, originally a Germany-based company, also exploits microalgae for DHA and EPA production, and Solarvest from Canada also produces and commercializes DHA.

In Table 9.2, a short summary of the most frequent microalgae species cultured for the production of the nutritionally important omega-3 FA, i.e., DHA and EPA, is

Table 9.2 Summary of the most frequent microalgae species cultured for the production of the nutritionally important omega-3 fatty acids, i.e, DHA and EPA

Microalga	Fatty acid						
	EPA	DHA	ARA	GLA	SDA	LA	ALA
Crypthecodinium	−	++	−	−	−	−	−
Isochrysis	+−	++	−	−	−	+	+
Nannochloropsis	++	−	+	−	−	+	−
Nitzschia	++	+	−	−	−	+−	+−
Odontella aurita	++	+−	−	−	−	−	−
Oocystis	+-	−	−	−	−	++	++
Pavlova	+	+	−	−	++	+	+
Phaeodactylum	++	+−	+	−	+	+	−
Porphyridium	+	−	+	−	−	+	−
Spirulina (*Arthospira*)	+	−	−	++	−	+	+
Schizochytrium sp.	+	++	−	−	−	−	+−
Tetraselmis suecica	+	−	−	−	−	+	++
Thraustochytrium	+	++	+	−	−	−	−
Trachydiscus minutus	++	−	−	+	−	+	+
Ulkenia	−	++	−	−	−	−	−

The appearance of other fatty acids for the same listed species is also displayed

presented. The appearance of other fatty acids for the same listed species is also displayed [65, 84, 96–106].

Fatty Acids from Seaweeds

Macroalgae are distributed worldwide; it is estimated that there exist about 9000 species; nonetheless, approximately about 200–220 are economically important and exploited mainly for the presence of other compounds, i.e., alginates, agar, polysaccharides, and pigments. These are also a source of PUFA, but, in spite of their accumulation, they are not highly exploited. Their PUFA contents also change according to seasonal period and geographical location. In a very recent report, lipid and fatty acid compositions were analyzed in 100 marine macroalgae. Almost all the lipid contents in macroalgae were low (2.3–20 mg g^{-1} fr. wt.), but regarding PUFAs, they showed high amounts of nutritionally important compounds such as LA, ALA, STA, ARA, EPA, and DHA. Up to 90 % of the species showed nutritionally beneficial n6/n3 ratios [107]. For instance, many species from different groups from various locations of Diu and Saurashtra, coast of Gujarat, India, have been studied [108]. The study of 9 members of Chlorophyta showed that this group was an attractive producer of omega-3 fatty acids. Some members of the genus *Ulva* produced higher amounts of DHA than EPA, although *Caulerpa racemosa and Caulerpa veravalnensis* were better EPA producer, with almost negligible amounts of DHA. Generally, they produced less amounts of ARA, with the exception of *C. veravalnensis.* An important fact of this group was that the ratio n6/n3 was between 1.5 and 2 % for almost all species, with *Ulva linza* showing the best value of 1.42. Regarding Phaeophyta, all the analyzed species produced EPA as the major omega-3 fatty acid, but with very low amounts of DHA. On the contrary, they produced high amounts of ARA, 32 % of total FAME (fatty acid methyl esters) in *Cystoseira indica.*

It is clear that here the ratio n6/n3 was higher than Chlorophyta, reaching 5.15 in *Sargassum tenerrimum*. It was also shown in this study how different species of Rhodophyta contained EPA as the major omega-3 fatty acids, with almost no detectable amounts of DHA. However, ARA was produced in higher amounts recording 46 and 58 % of total FAME in *Gracilaria debilis* and *Gracilaria dura*, respectively. Again the ratio n6/n3 was higher than in Chlorophyta, especially in these two species, reaching 18 and 27. Surprisingly, in *Grateloupia indica* and *G. wattii,* EPA was produced in higher amounts compared with ARA, and therefore, the n6/n3 ratio achieved by these two species was one of the best in this study with values of 0.61 and 0.74, respectively [108].

In another study, many different species from Chlorophyta, Phaeophyta, and Rhodophyta from the Algarve coast (Portugal) were analyzed in order to determine their fatty acid composition [109]. In the Chlorophyta group, all members showed EPA (20:5n-3) being detected at medium concentrations, ranging from 1 to 4 % of the total fatty acid content, and DHA was only detected in *Cladophora albida* (0.8 %). The only exception of a good omega-3 producer was *Ulva* sp., in which ALA was detected at high percentages (16 %), although the important EPA and DHA were very minor. In this group, the n6/n3 ratio varied notably, with the worst ratio of 31 with *Chaetomorpha* sp., and the best ratio achieved by *Ulva* sp. with 0.31. Similarly, species belonging to Phaeophyta were also analyzed. Accordingly, in all Phaeophyta, EPA was produced at relatively high amounts (6–14 %), except for *Dictyota spiralis*, in which EPA was not detected. DHA was only detected in *Halopteris scoparia, Taonia atomaria, and Sargassum vulgare* at low concentrations (0.8–1.5 % of total fatty acids). In this group, ARA amounts (11–18 %) were slightly higher than EPA. Again, the n6/n3 ratio was between 2 and 4 in all cases. Rhodophyta are considered as very interesting EPA producer as shown by Graeve et al. [110]. Accordingly, in this study, all analyzed species from Algarve coast produced high amounts of EPA (4 out of the 5 species showed higher than 15 % of total FAME) with *Peyssonnelia* sp. being the only representative of this phylum in which DHA (22:6n-3) was also detected. These are also attractive producers of ARA with amounts ranging from 1.79 to 26.6 of total FAME. This group also showed the best n6/n3 ratio, almost always under 1, with *Bornetia secundiflora* being the best one (0.29) [109].

In another report, species from Ireland, The Netherlands, France, and Norway were analyzed [111]. The best EPA producers were *Laminaria hyperborean* (Phaeophyta) and *Palmaria palmata* (Rhodophyta) with amounts of 26 and 59 % of total fatty acids. On the contrary, *Sargassum natans* (Phaeophyta) was the only DHA producer with a 13 % of total fatty acid content. Regarding omega-6 series, the best ARA producer was *Undaria pinnatifida* (Phaeophyta) with 16 % of total fatty acids. Here, the n6/n3 ratio, in almost all the cases, was less than 1, which is a ratio recommended by the World Health Organization, which should be less than 10 in order to prevent inflammatory, cardiovascular, and nervous system disorders [111, 112].

Nevertheless, there are also other species of algae that were poor producers of EPA, DHA, or ARA. This was the case of *Laurencia filiformis and L. intricata* collected in Espírito Santo State, Brazil [113]. In these two species, DHA was not detected, while EPA reached amounts of 0.8 mg g^{-1} dry weight in *L. filiformis* and 1.7 mg g^{-1} dry weight in *L. intricate.* Amounts of ARA were slightly lower, with 0.4 and 1.5 mg g^{-1} dry weight in *L. filiformis* and *L. intricate*, respectively. On the other hand, these species were good producers of palmitic acid, reaching amounts of 4.1

and 3.1 mg g^{-1} dry weight in *L. filiformis* and *L. intricata,* respectively.

It is known that the fatty acid content analyzed in different seasons changes among species [114, 115] and also that temperature affects clearly the fatty acid profile [116–118]. Heavy metals [119] and light [120, 121] are also external factors able to induce changes in growth and fatty acid content.

Recently, the total fatty acid content and profiles of 16 common Irish macroalgae, as a potential source of PUFAs, collected at two different seasonal sampling times, have been studied [122]. The data revealed that in the group of Phaeophyta, the most commonly distributed PUFA was ARA, with values ranging from 4.7 % of total fatty acid content in *Saccharina latissima*, to 17.6 % in *Himanthalia elongata*. EPA was also produced by all the Phaeophyta species obtaining the maximum amounts of 13 % in *Laminaria digitata.* Regarding the other valuable omega-3 fatty acid, DHA was detected in lower amounts or even not detected according to the species. The most dramatic change in any of the FA analyzed was observed in *S. latissima* with EPA fluctuating from 1.8 % in November to 10.8 % in June. In all these species, the n6/n3 ratio was nearly 1.0 or even less. Regarding Rhodophyta, the two main PUFAs were ARA with 30.8 % of total fatty acids in *Gracilaria gracilis*, and EPA with 41.2 % in *P. palmata* in which DHA was almost not detected. The highest EPA shift was observed in *G. gracilis* with no production in June, to reach an amount of 30.8 % in November. In most of all the species examined, the ratio of n6/n3 was about 1 with the lowest ratio of 0.04 observed for *P. palmata* due to its high amounts of EPA. The last group studied was Chlorophyta, in which only two species were analyzed. The most common PUFA was the essential omega-3 ALA with levels up to 11.3 % in *Ulva lactuca* and 19.9 % in *Codium fragile.* Percentages of EPA were low in both species. In *U. lactuca*, small amounts of DHA were observed as well. The ratio of n6/n3 fatty acids was low in both species, with values ranging from 0.2 to 0.6 [122].

Recently, Masatoshi et al. [123] studied the seasonal variations of total lipids and fatty acids composition of two macroalgae, *Sargassum horneri* (Turner) and *Cystoseira hakodatensis* (Yendo), from the northern seashore of Japan. It was observed that the maximum total lipid (TL) contents of *S. horneri* originated from Nesaki and Matsushima areas were 101.9 mg g^{-1} DW (January 2009) and 142.5 mg g^{-1} DW (January 2009), respectively, being palmitic (16:0), oleic (18:1n-9), SDA (18:4n-3), ARA (20:4n-6), and EPA (20:5n-3) the major fatty acids. Regarding *C. hakodatensis*, this macroalga showed the highest total lipids in May 2009 (122.9 mg g^{-1} DW) and in January 2010 (155.9 mg g^{-1} DW). On the other hand, the relative percentages of total n-3 PUFAs were generally larger in winter time, with the highest level of n-3 PUFAs corresponding to those on the total lipid fraction of *C. hakodatensis.*

Another study assessing the seasonal changes versus fatty acid contents was carried out with *U. pinnatifida,* from the Marlborough Sounds, New Zealand [124]. Significant differences in PUFA contents among winter, spring, and summer were observed. PUFA amounts significantly increased during the winter and decreased in spring. *Undaria* also produced more n-3 PUFAs in winter compared with n-6 PUFAs that were found to be the main PUFAs in summer, in which the temperature of water goes from 6 to 8 °C in July to 15–16 °C in December. Among the n-3 PUFAs, the major ones were ALA (18:3n-3), SDA (18:4n-3), and EPA (20:5n-3). On the other hand, the most abundant n-6 PUFAs were LA (18:2n-6), GLA (18:3n-6), DHGLA (20:3n-6), and ARA (20:4n-6) in December.

Similarly, the fatty acids profile of the Rhodophyta *Grateloupia turuturu* collected in Brittany, France, was investigated over four seasons [125]. The two important fatty acids ARA and EPA were produced at higher amount in summer, reaching ARA 11 % and EPA 16 % of total fatty acids. This season apparently was the best to produce large amounts of these two highly valuable PUFAs. Moreover, the ratio n6/n3 was lower than 1.0 in all seasons, which is considered a good nutritional ratio indicating higher amounts of omega-3 than omega-6 fatty acids.

In *Corallina pilulifera,* the maintenance and culture conditions were optimized. It was reported that the optimal temperature for incubation was 16 °C, the optimal light intensity was 40 μE m^{-2} s^{-1} with white fluorescent light, and the optimal light period was a 16 h light:8 h dark cycle. Under these conditions, it was found that from the fatty acids, 45.4 % were PUFAs, with EPA (20:5n-3) comprising 31.4 %, and also recording a very attractive n6/n3 ratio of 0.45 [126].

The influence of heavy metals and the changes produced in the lipid and fatty acid composition of algae has been researched [127]. The modes of action of heavy metals in these processes are not clear, but it is thought that they produce an oxidative stress and also an oxidative damage increasing the concentration of reactive oxygen species (ROS) in the cells [128]. In another recent research with cultures of *Gracilaria tenuistipitata*, the effect of heavy metals in the biosynthesis of fatty acids was evaluated, mimicking two different polluted environmental situations, adding low amounts of Cd^{2+} and Cu^{2+} to the cultures [129]. In a second scenario, and in order to establish how the light intensity variation affects fatty acid profiles, cultures were grown under 100 or 1000 μmol photons m^{-2} s^{-1} [129]. It was shown that the levels of the most important PUFAs, ARA, EPA, and DHA diminished, more significantly after treatment with Cd^{2+}, while high light intensity did not affect the amount of PUFAs in this species.

In *G. dura,* the effects of cadmium (Cd) in fatty acid profile, as well as the protective role of selenium (Se) and

Table 9.3 Summary of some seaweed species analyzed as producers of the nutritionally important omega-3 fatty acids, i.e., DHA and EPA

Seaweed	Fatty acid						
	EPA	DHA	ARA	GLA	SDA	LA	ALA
Ahnfeltiaplicata	++	+−	++	+−	−	+−	+−
Caulerpa racemosa	++	+−	+−	+−	−	++	+−
Caulerpa veravalnensis	+−	+−	++	+−	−	++	+−
Cystoseira indica	+−	+−	++	+−	−	+−	+−
Dictyota spiralis	−	−	++	−	−	+−	−
Georgiella confluens	++	+−	−	−	+−	+−	+−
Grateloupia indica	++	−	++	+−	−	+−	+−
Halopteris scoparia	++	+−	++	−	−	++	−
Myriogrammesmithii	++	−	−	−	−	+−	−
Peyssonnelia sp.	++	+−	++	−	−	+−	−
Sarconema filiforme	+−	−	++	+−	−	+−	+−
Spatoglossum asperum	++	+−	++	+−	−	+−	+−
Stoechospermum marginatum	+−	+−	++	+−	−	+−	+−
Ulva lactuca	+−	+−	+−	+−	−	++	+−
Ulva linza	+−	+−	+−	+−	−	++	+-
Ulva rigida	+−	++	+−	+−	−	++	+−

The appearance of other fatty acids for the same listed species is also displayed

polyamines, such as putrescine (Put) and spermine (Spm), were studied [130]. Treatments with Cd negatively affected the amounts of n-6 PUFAs, with ARA dropping from 51 % in control cultures to 21 % of total fatty acids in Cd-treated cultures. Surprisingly, the addition of Se or Spm had a positive re-establishing effect recovering the amounts of ARA even at the same initial levels. This fact shows the preventive role of both Se and Spm in controlling the Cd negative effect in *G. dura*.

Recently, a very curious experiment has been carried out in which the red algae *Gracilaria vermiculophylla*, a good producer of PUFAs, were transferred from Peter the Great Bay (Sea of Japan) and cultivated in the lagoon of Katba Island (Tonkin Bay in the South China Sea) [131]. It was shown that the amounts of ARA, with a 45 %, and EPA, with 4 % of total lipid in the original culture from Japan, that also showed a n6/n3 ratio of 10, were able to change the PUFA profile once cultivated in other conditions (South China Sea) and produced less amount of ARA (5.9 %), and 2.6 % of EPA adjusting the n6/n3 ratio to 2.3. This strategy appears as a valuable tool to change the fatty acid profiles and n6/n3 ratios far from 1.0.

In Table 9.3, a short summary of some macroalgae species analyzed as producers of the nutritionally important omega-3 fatty acids, i.e., DHA and EPA, is presented. The appearance of other fatty acids for the same species listed is also displayed [108–110].

Biotechnology of Fatty Acids from Marine Algae

The fast growing interests in the use of transgenic microalgae for industrial applications is powered by the rapid developments in microalgal biotechnology, particularly the advances made for biofuel research employing these organisms which can be extrapolated to many of these species. Nowadays, there are many different available genomes that have been successfully sequenced, for instance from the red alga *Cyanidioschyzon merolae* [132], the diatoms *Thalassiosira pseudonana* [133] and *P. tricornutum* [134], and the unicellular green alga *Ostreococcus tauri* [135]. Available tools for genetic modification and in silico predictive capacity have allowed the characterization of algal genomes and manipulation of different desirable pathways in an effort to develop and establish algal biofuel, and desirable fatty acids and PUFA production strains [136].

Microalgae are the natural initial EPA and DHA producers in the marine food chain and can grow rapidly under a variety of autotrophic, mixotrophic, and heterotrophic culture conditions with high PUFA production potential. Although large-scale cultivation facilities for microalgae exist, often their productivity is not fully optimized because of the lack of ideal strains that can be selectively manipulated for high biomass productivity, high TAG (triacylglycerol), or PUFA content [137]. Genetic engineering has

been used to altered fatty acid profiles in plants [26, 52, 138–140], so it could be easily considered as a promising tool to engineer fatty acids biosynthesis as a powerful strategy to create alternative algal strains with modified lipid yields and improved oil quality [141].

Genetically modified microalgae are emerging as alternative sources of TAG and PUFA [142] and represent progress toward the use of fermentation approaches to commercially exploit microalgae at large scale, thereby reducing the limitations often associated with photobioreactors, such as light dependency and lower growth [143]. However, the lack of efficient genetic modification systems so far has delayed the genetic engineering of microalgae, and not many prosperous attempts of genetically modified, for instance diatoms, have been reported. Genes encoding key enzymes participating in the fatty acid biosynthesis have been identified in *T. pseudonana* [144–146], *P. tricornutum* [147, 148], and in particular in the model organism *Chlamydomonas reinhardtii* [149]. At present, the mechanisms involved in the fatty acid biosynthetic pathways in microalgae have not been extensively studied, and most information has been gathered from studies on plant metabolism [150], and in not all the cases, the biosynthetic route and all the enzymes involved have been fully established yet. Nonetheless, clear advances have been attained which has permitted to draw the likely biosynthetic pathway taking place in these organisms [137, 151–154] (Fig. 9.2).

Numerous studies have been carried out applying the metabolic engineering strategy to boost the lipid accumulation in different species. These can be classified into five different approaches: (1) overexpressing enzymes of the fatty acid biosynthesis pathway; (2) overexpressing enzymes that enhance the TAG biosynthesis pathway; (3) regulation of related TAG biosynthesis bypass approaches; (4) partially blocking competing pathways; and (5) the multigene transgenic approach [155].

Some attractive examples of how PUFA biosynthesis could be improved through the regulation of genes involved in lipid metabolism have been published. For instance, one isoform of the enzyme diacylglycerol acyltransferase type 2 from the marine diatom *P. tricornutum,* which participates in TAG assembly, was overexpressed obtaining a higher productivity of oil bodies, as well as an increase of 35 % on neutral lipid content. Monounsaturated fatty acids showed an increase of 35.6 %, and PUFAs showed an increase of 34.8 %. It should be highlighted the increase of C20:5 (eicosapentaenoic acid, EPA) at 76.2 %, with the proportion increased from 4.21 to 7.42 %. Moreover, the growth rate of transgenic microalgae remained similar, thereby maintaining a high biomass. All these results clearly suggest that increased DGAT2 expression could alter fatty acid profile in the diatom, and these results represent a valuable strategy for PUFA production by genetic manipulation [156].

Another example of boosting EPA, which has been clearly demonstrated to play important roles in a number of aspects of human health and traditionally obtained from marine fish oils [151], was reported in *N. oculata* [157] which was subjected to *N*-methyl-*N*-nitrosourea-induced mutagenesis under the selection pressure of quizalofop, a well-known inhibitor of acetyl-CoA carboxylase (ACCase) activity, with the clear objective of generating genetically tractable mutants with modified fatty acid metabolism.

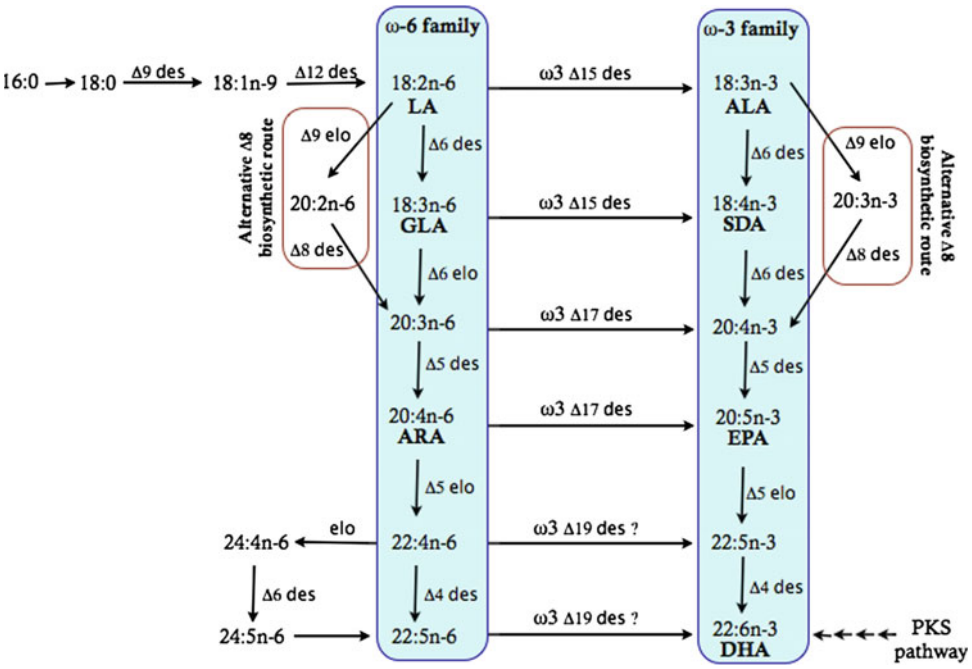

Fig. 9.2 Tentative biosynthetic pathway for the formation of long-chain polyunsaturated fatty acids (PUFAs) n-3 and n-6 in microalgae and microorganisms (*des* = desaturase, *elo* = elongase, *PKS* = polyketide synthase)

Two mutants, QUIZ1 and QUIZ2, showing resistance to quizalofop were isolated and partially characterized. The growth properties and morphology of the mutants appeared identical compared with the wild-type strain. Gas chromatographic analysis of fatty acids revealed how eicosapentaenoic acid (20:5n-3) (EPA) accumulated in higher amounts. The content of mutant strains QUIZ1 (28.29 mg g^{-1} dry matter) and QUIZ2 (25.2 mg g^{-1} dry matter) was higher than that of the parental strain (23.90 mg g^{-1} dry matter) under standard conditions (25 °C, a salinity of 31 %, pH 8.0, and a light intensity of 90 µmol photon m^{-2} s^{-1}, 12 h photoperiod). Mutant strains were rich in PUFAs (n-3 PUFAs), as well as total fatty acid contents; this was accompanied by a parallel increase in triacylglycerol (TAG), followed by linoleic acid (18:2) and arachidonic acid (20:4n-6). Random mutagenesis was shown to be a good tool to manipulate PUFAs and EPA in *Nannochloropsis*. The development of genetic manipulation as well as biochemical tools of this strain could be useful in understanding fatty acid metabolism and also for their direct use in mariculture.

An alternative approach consists of the elevation, and redirection of the endogenous accumulation of omega-3 long chain-PUFAs, and their incorporation into neutral lipids, such as triacylglycerol (TAG). Biolistic transformation has been used to engineer the diatom *P. tricornutum*, introducing and expressing, both combined or individually, the foreign Δ6-desaturase and Δ5-elongase from the picoalga *Ostreococcus tauri*. Some manipulated strains were able to accumulate the high-value omega-3 docosahexaenoic acid (DHA). Transgenic strains expressing the Δ6-desaturase showed no significant alteration of EPA and/or DHA, while the expression of the Δ5-elongase dramatically increased the amounts of DHA 8-fold compared with controls, representing a marked and valuable change in the fatty acid profile of this microalga. Importantly, DHA was shown to accumulate in triacylglycerols, with several novel triacylglycerol species being detected in the transgenic strains. In addition, the coexpression of both transgenes was carried out for the first time in a transgenic diatom, demonstrating further increases in DHA levels through simultaneous expression. Together, this demonstrates the high potential of using iterative metabolic engineering to optimize and overproduce omega-3 content in algae [153].

There are many other instances of how to manipulate microalgae to overproduce other desirable fatty acids apart from PUFAs. Overexpression of the endogenous PtTE (thioesterase) in the diatom *P. tricornutum,* using microparticle bombardment, did not lead to significant changes on fatty acid composition; nonetheless, the total fatty acid content increased by 72 % (from 78 to 133 mg g^{-1} dry cell weight) compared to control, especially both C16:0 and C16:1. This novel thioesterase gene shows its potential in metabolic engineering for enhancing lipid yield of microalgae [158]. In another attractive approach, the overexpression of the endogenous *C. reinhardtii* thioesterase (CrTE) increased the levels of short-chain fatty acids, although engineering the *Umbellularia californica* thioesterase (plant UcTE) into the *C. reinhardtii* chloroplast did not alter the fatty acid profile. These findings show how important and critical the interaction between proteins is in manipulating fatty acid biosynthesis in algae [159].

These methods are paving the way for a more thorough characterization of the algal lipid biosynthetic enzymes, which appear to be distinct from those found in terrestrial plants. In a similar fashion, transgenically overexpressing two different shorter chain length fatty acid acyl-ACP thioesterases, one from *Cinnamomum camphora* (myristic acid biased thioesterase) and another from *U. californica* (lauric acid biased thioesterase) in the eukaryotic microalga *P. tricornutum*, resulted in higher levels of lauric and myristic acids, which are more desirable as a fungible biofuel feedstock. This study shows the feasibility of using such an approach to modify algal oil composition, and how valid this strategy is for improving the fuel producing properties of algae [160].

Synechocystis sp. PCC6803 mutant strains where generated to study the effect of fatty acids activation, displaying either overexpression or deletion of the slr1609 gene, which encodes an acyl-ACP synthetase, which converts and activates free fatty acid (FFA) molecules into fatty acyl-ACP [161]. Similar growth rate was displayed by the wild-type and slr1609 deletion mutant strains, but the content of FFA showed substantial differences between both strains. In the slr1609 deletion mutant, the concentration of total FFA was 6.7 µg mL^{-1}/OD, while that of the wild type was 3.5 µg mL^{-1}/OD. FFA accumulation increased close to 2-fold after knocking out *slr1609*. This indicates that the dysfunction of FA activation caused by the deletion of *slr1609* results in an increase of FFA accumulation. Regarding unsaturated fatty acids with carbon chain length of C16 and C18, as in the case of FFA, the amount of unsaturated fatty acids was significantly higher in the slr1609 knockout mutant compared to the wild type. On the other hand, mutant strain GQ3, overexpressing *slr1609*, showed no significant changes on the production of FFA compared to the wild type. This approach showed how blocking FA activation by deleting *slr1609* gene in wild-type *Synechocystis* sp. PCC6803 led to doubling the amount of FFA. Similarly, other model cyanobacterium, *Synechococcus elongatus* PCC 7942, may be an advantageous host for biofuel production [162]. Unlike *Synechocystis* 6803, *S. elongatus* 7942 does not contain the pathway for polyhydroxybutyrate (PHB) synthesis [163]. As PHB synthesis is a competitive pathway of FFA synthesis for the available carbon flux, the PHB pathway is undesirable from a fuel production perspective, making *S. elongatus* 7942 an attractive host

candidate. In order to allow for FFA accumulation, the acyl-ACP synthetase gene was suppressed, and a truncated thioesterase, which allows removal of feedback inhibition of fatty acids biosynthesis, was expressed. The resulting engineered strain SE02 expresses a thioesterase, therefore reduces potential inhibition of fatty acids biosynthesis, and lacks the ability to metabolize FFA as a result of the *aas* knockout. As expected, FFA excretion by wild-type *S. elongatus* 7942 was negligible, whereas removing feedback inhibition of fatty acids biosynthesis through thioesterase expression in SE02 showed FFA production and excretion being further improved [162].

Another interesting approach consists of blocking competitive pathways. For instance, highly energetic compounds such as starch, which shares common carbon precursors with lipid synthesis, could be an interesting strategy for modifying TAG and fatty acid profiles in microalgae [164]. Several studies have been published indicating the interaction between the two pathways, although the regulation of carbon partitioning into starch and lipid synthesis pathways is not well understood. It could be hypothesized that controlling carbon precursors from the starch synthesis pathway might result in overproduction of fatty acids and TAG. Inactivation of ADP-glucose pyrophosphorylase in a *Chlamydomonas* starchless mutant led to a 10-fold increase in TAG, suggesting that shunting of photosynthetic carbon partitioning from starch to TAG synthesis may represent a more effective strategy than direct manipulation of the lipid synthesis pathway to overproduce TAG [165].

Likewise, it was shown that BAFJ5, one of the *C. reinhardtii* mutants defective in the small subunit of ADP-glucose pyrophosphorylase, accumulated neutral and total lipids up to 32.6 and 46.4 % of dry weight corresponding to 8- and 3.5-fold higher than the wild type, respectively [166]. Another example of this simple approach was studied in a novel starchless mutant of the unicellular green alga *Chlorella pyrenoidosa* [167] designated as STL-PI, which was isolated from this parental wild type after application of ultraviolet to induce mutagenesis [168, 169]. This starchless mutant had a 22 % higher growth rate and 24.5 % more protein than the parental strain, 82T. Furthermore, a very important fact was that the STL-PI mutant accumulated 20.4 % more PUFAs and 18 % less saturated fatty acids than the parental 82T. These results confirmed how promising these approaches are in order to increase lipid production through redirecting photosynthetically assimilated carbon away from starch synthesis into neutral lipid synthesis.

Another pathway that competes with lipids synthesis and should be taken into account is the conversion of phosphoenolpyruvate to oxaloacetate, catalyzed by the phosphoenolpyruvate carboxylase (PEPC) enzyme. TAG biosynthesis also requires phosphoenolpyruvate which converts successively to pyruvate, acetyl-CoA, malonyl-CoA, and then FA [170]. Some preliminary results also indicate that PEPC plays a role in the regulation of fatty acid accumulation and reduced PEPC activity by antisense expression which was correlated with an increase on lipid content in the cyanobacterium *Synechococcus* sp. [170].

A very interesting approach to altering fatty acid metabolism is to redirect carbon flux toward desired pathways, as was shown in *Schizochytrium* sp., a microalga that has been established as a candidate for commercial production of PUFAs, and able to produce large amounts of oil with 35 % dehydroacetic acid of total fatty acids [171]. During the aerobic fermentation of *Schizochytrium*, large amounts of acetate were produced, which is harmful to cell growth. Therefore, enhancing the capability of *Schizochytrium* to assimilate acetate would be useful in the fermentation process. The introduction of an *Escherichia coli* acetyl-CoA synthetase (ACS) gene was carried out, which carries an irreversible reaction that converts acetate into acetyl-CoA, in the marine microalga *Schizochytrium* sp. TIO1101. Some resulting mutants, such as ACS1 and ACS3, were able to produce a proportion of fatty acid content of 44.7 and 46.8 %, respectively, which were 1.06- and 1.11-fold larger than that of the wild-type strain (42.0 %). Finally, the fatty acid composition determined by GC-MS showed that both the wild-type strain and ACS transformants accumulated mainly six types of fatty acids. Furthermore, the dehydroacetic acid content of ACS1 and ACS3 was 43.4 and 43.7 %, slightly higher than that of the wild-type strain (43.3 %) [172]. On the contrary, it has been proposed that unlike disrupting carbohydrate pools, which are the primary carbon storage product of many microalgae, knockdown of lipid catabolism would have less impact on the primary carbon pathways associated with growth, increasing lipid accumulation while growth is not negatively affected. Only a few attempts have been made to engineer lipid metabolism in microalgae, and no targeted manipulation to date has significantly increased lipid yields without simultaneously decreasing growth. It has been reported that the targeted knockdown of a multifunctional lipase/phospholipase/acyltransferase version 3 protein ID 264297 (Thaps3_264297) increased lipid yields without affecting growth in the diatom *T. pseudonana*. Antisense-expressing knockdown strains 1A6 and 1B1 exhibited a comparable growth rate with the wild type, as well as an increased lipid content, under both continuous light and alternating light/dark conditions. Strains 1A6 and 1B1 contained 2.4- and 3.3-fold higher lipid content than wild type during exponential growth and 4.1- and 3.2-fold larger lipid content than wild type after 40 h of silicon starvation. These results clearly confirm that targeted metabolic manipulations can be employed to increase lipid accumulation in eukaryotic microalgae without affecting growth rates [173].

Analogously, it has also been demonstrated that multigene manipulation of different genes participating in TAG

metabolism is a very powerful tool to boost lipid yields. In *Chlorella minutissima,* five different genes involved in TGA biosynthesis, G3PDH: glycerol-3-phosphate dehydrogenase, GPAT: glycerol-3-phosphate acyltransferase, LPAAT: lysophosphatidic acid acyltransferase, PAP: phosphatidic acid phosphatase, and DGAT: diacylglycerol acyltransferase, were overexpressed [174]. Expression profiles using real-time analysis of the quintuple gene construct showed that the relative expression levels of G3PDH, GPAT, LPAAT, PAP, and DGAT were 27, 17, 16, 22, and 18 %, respectively. Furthermore, G3PDH showed the highest mRNA expression level, and thus, more glycerol-3-phosphate (G3P) should be introduced into the acyl pool to increase the metabolic flux for GPAT, LPAAT, PAP, and DGAT. After 14 days of cultivation of *C. minutissima* UTEX 2219 with this construct in MES-volvox medium including vitamin solution, the TAG accumulation achieved 44 wt%, 2-fold higher than control.

Another very attractive multigene approach was that in which the primary pathways toward FFA overproduction were manipulated. First by introducing an acyl-acyl carrier protein thioesterase gene (*E. coli* TE gene *tesA*), and then six successive generations of genetic modifications of the cyanobacterium *Synechocystis* sp. *PCC6803* [175], and at the same time, various enzymes belonging to competing pathways that uncouple the carbon flux from FFA overproduction, were successfully suppressed. It is known that long-chain acyl-ACP molecules are important feedback inhibitors of the activity of fatty acid synthesis (FAS) enzymes [176], such as acetyl-CoA carboxylase (ACC), the enzyme that catalyzes the conversion of acetyl-CoA into malonyl-CoA. Overproduction of enzymes like TE would reduce the cellular acyl-ACP concentrations, thus achieving the stimulation of FAS flow by decreasing feedback inhibition [176]. Except for one strain, SD225, most genetic modifications resulted in increased FFA secretion compared to the parent strains, reaching a fatty acid yield of 197 mg L^{-1} of culture in one improved strain at a cell density of 1.0×10^9 cells mL^{-1}. This result suggests that fatty acid secreting cyanobacteria could be considered as a feasible organism for renewable biofuel production [175].

In another study, *Synechocystis* 6803 was engineered for the accumulation of FFA, modifying different strains. One strain (F3), in which TE, a thioesterase catalyzing the liberation of FFAs from acyl-ACP, was overexpressed. Another strain (F16) in which inactivation of the AAS gene, encoding acyl-ACP synthetase, prevented the rethioesterification of FFA generated from membrane lipid recycling and led to elevated levels of intracellular FFA. And finally, strain (F3:16), in which both modifications were introduced and expressed [177]. Contrary to expectations, strain F3 displayed no increase in the amounts of intracellular FFA, while F16 showed a modest but notable increase. On the other hand, strain F3:16 demonstrated a dramatic 30-fold rise in FFA levels, being those FFA mostly stearic acid (octadecanoate; 18:0) and palmitic acid (hexadecanoate; 16:0), and the rest being monounsaturated octadecanoates, primarily oleic acid (18:1-Ω9), vaccenic acid (18:1-n11), and euric acid (22:1-n9). FFA secreted by the F3:16 cells differed significantly from the intracellular pool, with the majority being unsaturated fatty acids, chiefly linoleic acid (18:2n6), palmitoleic acid (16:1n9), vaccenic acid (18:1n11), and α-linolenic acid (18:3n3). Again, it has been demonstrated how a multigene modification is much more desirable and effective in order to achieve a higher production of fatty acids.

We need to take into account many other findings related to the metabolic engineering or manipulation of growing conditions, which could facilitate production of PUFAs, fatty acids, and biofuel [178]. Likewise, we could not disregard to positively use all the information available from other organisms, such as the cyanobacterium *Synechocystis* sp. PCC6803 [179], the yeast *Yarrowia lipolytica* [180, 181], and other findings, for instance related with new metabolic pathways [182].

All these results, together with the continuous important contributions, and new results on the field of PUFAs and biofuels, will shed light on how to develop and optimize different alternative sources of production of those long-chain n-3 fatty acids in high demand for human life, health, and food, compounds whose current sources, particularly fish, are being consumed at a high rate and therefore could result in a complete depletion in the near future.

Concluding Remarks

It is well demonstrated the tremendous importance of PUFA consumption for cell and body function, regardless of the organisms (animals, plants, fungi, algae, etc.). In humans, PUFA plays a high role in our well-being, and for attaining a healthy status, as has been presented in depth in different chapters of this book. Gradually, this is being understood and accepted by the health authorities and by the general public more concerned with their nutrition. These compounds cannot be synthesized de novo by the human biochemical machinery, although some of them can be produced through some intermediates but in a very inefficient manner, as encountered particularly with the vegan communities, and therefore must necessarily be taken up through diet.

The main source of PUFA for humans derives from fish oil, and very worrisome, this depends on extractive fishing practices that also contributes to the depletion of fish stocks, which, despite many efforts and policies, has not been managed to be exploited in a sustainable manner in order to provide a continuous and ordered supply of omega-3 fatty acids. The estate of fish stocks depicts an alarming scenario,

with 32 % of fish stocks overexploited, other 50 % highly exploited, 4 % already exhausted, and just 2 % on the process of being recovered [9]. Therefore, the demand for finding and establishing new sources of PUFA in a more sustainable fashion continues to be highly required, and thus, the search began a few decades ago, particularly in the oceans. Currently, many different species are being cultured and exploited for the controlled production of omega-3 fatty acids as presented in this chapter. Some show strong potential and attractive scale-up capabilities and are being exploited accordingly. These successful industrial examples provide clear evidences that one of the ways to accomplish a continuous supply of these metabolites is that offered by the microalgae, particularly autotrophic microalgae that do not demand an organic carbon source for their growth [150]. Furthermore, more research into the genetics of these organisms is required to gain full knowledge and understanding of their biochemical potential. On the other hand, the genetic engineering of the biosynthetic route leading to EPA and DHA accumulation has been attempted, particularly in microalgae, but its full potential has not been accomplished yet. Surely, this will generate fascinating and encouraging outcomes, permitting also a full understanding of this biosynthetic route in these organisms, ending up in establishing more manageable and productive systems.

Other alternatives, such as the ultimate advances made with higher plants, to which the complete biosynthetic route of the omega-3 fatty acids has been introduced and expressed, resulting in high yields of EPA and DHA, should be also considered [51, 52]. Macroalgae, although presenting interesting profiles and yields of fatty acids, seems to be less exploited for omega-3 fatty acid production than the microalgae, although they also accumulate an extensive array of very important bioactive compounds already under exploitation.

Regarding regulations on the production, use, and consumption of omega-3 fatty acids derived from these sources, this is defined by different governing bodies, but the most general and internationally applied is the Codex Alimentarius, which formulates and harmonizes food standards ensuring their global implementation [54]. Furthermore, it also allows them a role in the development of codes governing hygienic processing practices, and recommendations relating to compliance with those standards, and in general pursues consumer health protection. In Europe, the European Food Safety Authority is responsible to assess the safety of any new food and feed compound before they are authorized for production and commercialization. Similarly, in USA, the agency conducting all these policies and regulations is the Food and Drug Administration (FDA).

Recently, two meetings dedicated to the topic of biomarine advances, novel food from algae, aquaculture, etc., took place in Portugal (Alga biomass Novel Food Workshop; 5th BioMarine International Business Convention, Marine Bio-Resources for a New Blue Economy 2014). How the regulatory process and requirements, which are not always at pace with the fast scientific advances and discoveries, came out and were debated. Despite the need for assuring the safety on the consumption of food and food ingredients, a faster, agile, and clear regulatory framework that would make it possible to bring to the market a wide range of new species and related products and extracts, in a faster way, was demanded. This would be required for an optimal and rapid exploitation of food ingredients, such as, for instance, the omega-3 fatty acids obtained from newly determined novel microalga species with strong industrial potential.

Over the past decades, algae biotechnology has been growing increasingly with many successful examples of many enterprises exploiting different aspects of the biochemical potential of these organisms. Moreover, research continues, although most of the microalga species estimated to exist in our oceans (1–10 million) remains to be discovered and evaluated for their omega-3 production potential, as only 10,000–20,000 have been taxonomically described, and just a minor percentage of them has been screened for determining their omega-3 fatty acid profiles. Thus, the quest must continue, with many interesting and exciting results waiting to be accomplished.

In order to establish and offer an adequate continuous supply of these highly valuable metabolites needed for maintaining a healthy human population, we ought to consider all possible alternatives to thus satisfy the increasing world demand for these nutrients, including obviously algae.

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Emulsions of Omega-3 Fatty Acids for Better Bioavailability and Beneficial Health Effects

10

Sarang S. Puranik

Introduction

Role and benefits of omega-3 fatty acids in normal development as well as in variety of disease conditions have been proved by variety of clinical and laboratory studies. It was pioneering research by Dyerberg et al. [1], on very low or rare incidences of coronary heart diseases in Greenland Eskimos, which planted seeds of interest toward research on Polyunsaturated fatty acids (PUFA). Their study revealed that Greenland Eskimos consumed food which is fat rich; however, high amount of PUFA, mainly omega-3 fatty acids, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), present in fat avoided risk of cardiac ischemia. It was further confirmed by separate study by Dyerberg and Bang [2].

In cardiovascular diseases, omega-3 fatty acid beneficial effects include prevention of cardiac arrhythmia, sudden death from myocardial infarction, and lowering of blood triacylglyceride [3]. Omega-3 fatty acids have been found to be important for neuronal development and brain health. These benefits are applicable in a variety of age groups ranging from infants, to those adults with diseases and disorders like attention-deficit hyperactivity disorder (ADHD), dyslexia, depression, schizophrenia, and Alzheimer's disease [4–7]. Positive effects of DHA supplementations have been reported in the area of the inflammatory bowel diseases, Crohn's and ulcerative colitis [8–11], as well as in rheumatoid arthritis patients [12].

Due to benefits offered by PUFA, in the protection against metabolic diseases and disorders, they are considered as essential part of diet. However, humans cannot synthesize omega-3 fatty acids and therefore needs to be obtained through food rich in omega-3 fatty acids. Till date, fish oil remains major source of omega-3 fatty acids. It also exists in marine sources such as mussel, oyster, and shrimp [13] and in a wide range of plant products such as nuts, especially English walnuts, seeds, namely sesame [14], flax seed, and vegetable oils such as soybean, canola, and olive [15]. In recent years, marine algae have been successfully used for the production of PUFAs and considered as an alternative to fish oil. Currently, PUFAs (omega-3 fatty acids) are used in the form of nutritional supplements as well as additives in different types of food products ranging from infant formula to cookies. However, lipid peroxidation and fishy smell of PUFA are two major challenges, which affect stability and acceptability of omega-3s preparation in food industry.

Oxidation of PUFAs

Oxidation of PUFAs leads to inferior-quality foods and also affects its nutritional value. In addition to this, the animal origin of omega 3s makes vegan population averse of products fortified with them.

PUFAs are highly oxidizable and reactive, which is attributed to the presence of multiple double bonds in its structure. PUFA oxidation is equally dependent and influenced by both intrinsic and extrinsic factors, which include first and foremost composition of fatty acid, concentration of pro-oxidants and transition metals, enzymes, pH, ionic strength, exposure to oxygen, and temperature [16]. Exposure of oil to conditions such as increased temperatures, irradiation, oxygen and light speedup process of lipid peroxidation in PUFAs. This is a serious problem that results in the loss of shelf life, consumer acceptability, functionality and nutritional value of PUFA-fortified food products or supplements [17].

Peroxidation of lipids gives rise to free radicals, which may lead to the development of conditions like atherosclerosis [18]. Besides free radicals, oxidation process also forms intermediate compounds like hydroperoxides and a complex mixture of secondary lipid oxidation products such as alkanes, alkenes, aldehydes, and ketones [19, 20]. Some

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of these degradation products, e.g., aldehydes, which have an unpleasant smell and taste, have been implicated in aging, mutagenesis, and carcinogenesis [21, 22]. Moreover, ability of the secondary products of peroxidation process such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) to cross-link to proteins and bind covalently to nucleic acids leads to undesirable effects and toxicity [23]. The presence of naturally occurring, transition metals, especially iron, is sufficient enough to encourage oxidation process. Besides these pro-oxidants, such as lipoxygenases, exposure to oxygen accelerates process of oxidation in fish oil as well as food products fortified with it [24]. Exposure of PUFAs to oxygen initiates chain of autocatalytic oxidation reactions, with formation of free radicals, which can be categorized as induction stage, followed by propagation stage, and last termination stage. Induction stage of oxidation process gives rise to the formation of reactive substances, which acts as a seed and reacts with other lipid molecules, thus expanding oxidation process [25–27]. Process which starts with the formation of hydroperoxides ends up with the formation of huge number of intermediate and secondary products of oxidation in a complex mixture which includes non-volatile and volatile compounds, of different molecular weight and polarity, and bearing different oxygenated functions, such as hydroperoxy, hydroxy, aldehyde, epoxy, and ketone functions [28].

Considering oxidation-prone nature of polyunsaturated omega-3 fatty acids and considering health benefits offered, measures should be put in place. One method is preparation of emulsions to prevent oxidation and decomposition of polyunsaturated omega-3 fatty acids to harness their beneficial effects. It is a two-step process wherein at first an emulsion is prepared followed by drying of the emulsion.

What Is Emulsion?

Emulsions have garnered considerable attention in recent years due to its widespread applications ranging from food products to drug delivery. Emulsions can be employed for stabilization of oxidation-prone components like omega-3 fatty acids as well as for delivery of water-insoluble drugs. It offers good thermodynamic stability and increased drug solubilization and is easy to manufacture at large scale.

In simple terms, an emulsion is a mixture of two immiscible liquids. An emulsion is made up of three phases —a dispersed phase present as droplets, a continuous phase, and an interfacial region separating dispersed and continuous phase. It can either be oil in water (O/W) in which oil droplets are dispersed in water phase, e.g., milk, mayonnaise, and cream cheese, or water in oil (W/O) where water droplets are present in oil phase, e.g., butter and spreads.

Although microemulsions can be easily considered smaller version of emulsion, but micro- and nanoemulsions differ significantly from ordinary emulsions. Emulsions are cloudy in nature. They are thermodynamically unstable where phases eventually separate and preparation is energy intensive. On the contrary, microemulsions have excellent kinetic stability with clear or translucent appearance.

Danielsson and Lindman have defined microemulsions as "A system of water, oil and an amphiphile which is a single optically isotropic and thermodynamically stable liquid solution".

It was Hoar and Schilman who prepared first microemulsion by dispersing oil in an aqueous solution of surfactant where alcohol was used as cosurfactant. This formed stable, transparent oil-in-water formulation. The term "microemulsion" was coined by Schulman and coworkers in 1959.

Microemulsions formation is dependent on the type of surfactant and structure. In case of ionic surfactant containing single hydrocarbon chain, microemulsion formation occurs only when a cosurfactant or electrolyte is also present. Formation occurs spontaneously once conditions are correct. Average size of droplet, in microemulsion, is in the range of 10–140 nm [29] and has defined boundary at interface between oil and water.

Types of Microemulsion system:

Microemulsions are classified by Winsor into four types based on phases of microemulsions existing at equilibria and these are referred as Winsor phases as follows:

1. Winsor I: Oil-in-water (o/w) microemulsion
   This is two-phase system in which oil droplets surrounded by surfactant and cosurfactant are distributed in continuous phase formed by water.
2. Winsor II: Water-in-oil (w/o) microemulsion
   This is also two-phase system where oil forms continuous phase and water droplets are dispersed in it. It is also known as reverse micelles as polar head-groups of surfactant approach water.
3. Winsor III: Bi-continuous microemulsion
   In this type, both water and oil are present as continuous phase forming a sponge-like appearance. Oil-in-water and water-in-oil transitions occur in bi-continuous phase.
4. Winsor IV: Single-phase homogeneous mixture
   In this type as name suggests, oil, water, and surfactant are homogenously mixed to form a single-phase microemulsion.

To avoid any confusion, microemulsion is being referred as emulsion henceforth in this review.

Methods for preparation of emulsion:

In simple terms, emulsions are assumed to consist of three ingredients: oil, water, and surfactant. Different types of complex formation occur upon mixing of water, oil, and surfactants. These complexes include microemulsions and other entities like micelles, liquid crystals, and emulsion [30].

Omega-3 fatty acids emulsions are mainly used as supplements or food additives, and therefore, ingredients of emulsions system such as oil, surfactant, and solvents should fall under category of chemicals "Generally Recognized as Safe" (GRAS) and should be biocompatible, clinically safe, and non-toxic in nature. Common food emulsions generally falls under the type I, i.e., oil-in-water emulsion. It consists of two immiscible liquids where oil is dispersed in continuous aqueous phase in the presence of emulsifiers. In case of omega-3 fatty acids, fish oil has been widely used as a source of omega-3 fatty acids. Advances and development in algal fermentation process offer another source of omega-3 fatty acid.

Emulsions are prepared by maintaining oil–water interfacial tension at very low level and concentration of surfactant molecules sufficient enough to stabilize emulsion at low interfacial tension. Emulsions are usually made under high shear with, e.g., homogenizer, colloid mill, high shear mixer, or stirred vessel preferably equipped with baffles. However with advancement in understanding and technology, several methods have been developed and used for the preparation of highly uniform droplets of emulsions [31, 32]. Monodispersed emulsions may exhibit a more defined behavior and release pattern of entrapped active agent than polydispersed. Therefore, main objective of each method is to achieve smaller uniform droplet size and provide stability. It requires high amount of energy inputs to prepare emulsion with droplet size in nanometer range (100–600 nm) and this can be provided either by use of mechanical instruments or use of the inherent chemical potential of components [33]. Following methods are employed for the preparation of food emulsions.

Phase Inversion Method

In phase inversion method, fine dispersion of oil droplets is achieved by chemical energy , which is a result of phase transitions occurring during emulsification process. Required phase transitions are achieved by varying composition of system at constant temperature or by varying temperature keeping composition of system constant. This method is also known as phase inversion temperature (PIT), which was developed by Shinoda et al. using differences in solubility of surfactants with respect to temperature. At increased temperature, surfactant become lipophillic and hence promotes water-in-oil emulsion, whereas at lower temperature forms oil-in-water emulsion. Besides temperature, other parameters like pH and salt concentration may be considered more effectively instead of the temperature alone. Transition of phases can be brought by the addition of water, which initially forms droplets in oil, but upon increase in water volume fraction changes the spontaneous curvature of the surfactant from initially stabilizing a w/o microemulsion to an o/w microemulsion at the inversion point [34].

Phase Titration Method

This is a spontaneous emulsification method where oil is mixed with surfactant solution and then titrated with cosurfactant, e.g., alcohol until it forms a clear solution. Depending upon chemical composition of system and concentrations of components, emulsion is formed along with other species (such as micelles, lamellar, cubic, hexagonal structures as well as oily gels). Concentrations needs to be titrated to achieve control over emulsion formation. It was observed that using this method emulsion of long-chain oils can be obtained using surfactant having longer chain. Emulsification by phase titration is dependent on the type of cosurfactant used in system as different alcohols affect the formation of microemulsions in different ways. It was observed that best results, in terms of the greatest percent transmittance coupled with the widest range of oil (dispersed in water) concentration, were obtained when short or branched alcohols are used.

High-pressure Homogenizer

This method applies high pressure and energy provided by homogenizer over the system consisting of oil, water, and surfactant. As name suggests, emulsion is prepared by the application of high pressure by homogenizer. However, it is difficult to scale up production and component may deteriorate due to generation of heat during emulsion preparation. Another limitation of this method is it can prepare emulsion of oil in water only with less than 20 % oil concentration in system and difficult to achieve droplet size lower than 200 nm. Therefore, application of this method is limited to the production of small-scale food emulsions [35].

Sonication Method

Sonication is another energy-intensive material used for the preparation of emulsion. Oil, water, and surfactants are exposed to sonication waves resulting in the formation of emulsion. This can be employed to obtain smaller-sized droplets or even reduce droplet size of emulsion prepared using other methods. However, this is not suitable for large-scale preparations of emulsions [36].

During emulsion formation, oil droplets once dispersed in aqueous phase needs to be stabilized with stabilizing agents such as emulsifiers to prevent separation of phases. Emulsifiers decreases surface tension at interface between oil and water and thus total free energy of the system during homogenization process. It provides stability to oil droplets and inhibit flocculation and coalescence of oil droplets by steric or electrostatic interactions.

Emulsifiers

Emulsifiers used in food-grade emulsions are categorized as low molecular weight emulsifiers and high molecular weight emulsifiers. These can be employed either alone or together in combination with polysaccharides for food material emulsions.

Low Molecular Weight Emulsifiers

Class of low molecular weight emulsifiers consists of synthetic molecules as well as polar lipids isolated from natural sources. These are small and surface active and typically made up of a head-group which is hydrophilic and a tail which is hydrophobic in nature. Their hydrophilic head-group can be further classified as anionic, cationic, nonionic, and zwitterionic. Hydrophobic nature is imparted to tail by 1 or several hydrocarbon chains made up of 10–20 carbon atoms. Many food-grade surfactants, which include monoacyl and diacyl glycerols, polysorbates, polyoxyethylene sorbitan esters (Tweens), sorbitan esters (Spans), and mono- and diglycerides esters of sucrose, citric acid, lactic acid, and acetic acid are routines employed as emulsifier in food industry [37]. Lecithins a yellow brownish colored fatty substance, which represent other class, i.e., polar lipids, are widely employed emulsifier in food industry. They can be obtained from various natural sources such as soybeans, sunflower, and rapeseed. Lecithins are composed of a mixture of phospholipids (phosphatidylcholine and phosphatidylethanolamine), glycolipids, and sphingolipids and residual triglycerols.

High-Molecular Weight Emulsifiers

This category of emulsifiers, also known as wall materials, consists of biopolymers which are widely employed for stabilization of oil-in-water emulsion. Hydrocolloids such as starch, gum arabic, pectins, celluloses, and galactomannans as well as proteins such as casein, whey proteins, and soy proteins mainly represent high molecular weight emulsifiers [38]. Due to amphiphilic nature of these biopolymers, they readily form layer at oil–water interface by orienting hydrophobic part to interact with oil phase, while hydrophilic part faces water. Thus, molding them in conformation of oil droplets dispersed in water. It is well known and established fact that food preparations often have protein and or biopolymer aggregates, which readily get adsorb at interface and form stable emulsions. However, their ability to get adsorb at oil–water interface and formation of layer is totally dependent on intrinsic characteristics [39–43].

Emulsifiers, both low and high molecular weight, form thin layer at the interface between oil and water. Formation of layer is largely dependent on the type of emulsifier, its characteristics, and concentration used. Molecules of both low and high molecular weight emulsifiers arrange themselves in mixture of oil and water resulting in the formation of a monolayer at the oil–water interface. However, the formation of multilayers can occur in case of biopolymers such as caseins, when present in sufficient amounts at the interface [44–46]. In case of high molecular weight emulsifiers, especially when proteins are used as emulsifiers, only hydrophobic part of polypeptide chain interacts with oil, whereas remaining structure interacts with water resulting in approximately only 30–40 % coverage at interface [47]. On the contrary, surfactants, e.g., Tween-20, sorbitans, are more efficient in covering whole interface border with tightly packed structure whose thickness at interface ranges from 0.5 to 1 nm [46, 48]. Thickness of border increases in case of protein and biopolymers, which varies from 1 to 15 nm due to the formation of aggregates at surface [38, 49–52].

Physical stability of emulsion is highly dependent on surface properties of oil droplets as well as chemical nature of aqueous phase such as pH, ionic strengths, and ions. In case of PUFA emulsion process, surfactants as well as biopolymers are employed to obtain a stable emulsion.

Although surfactants forms thin layer at interface, they are generally considered as less efficient than proteins in avoiding the interfacial film-breaking and droplet coalescence. This is mainly attributed to the formation of thin film at boundary of oil and water, which provide weaker steric repulsion as compared to proteins [47, 48].

Proteins such as casein, soy protein, whey protein, lactoglobulin have been used as a wall material for

emulsification of PUFA. One of the advantages offered by biopolymers is that they can give rise to either steric (e.g., bovine serum albumin) and/electrostatic (e.g., casein) repulsion, which imparts stability to emulsion and restricts size by inhibiting coalescence of oil droplets [46–48, 53–56]. However, choice and concentration of biopolymers hold key, as in certain concentration ranges polymers can cause flocculation of oil droplets by bridging gap between droplets [46, 47, 53, 54]. It has been demonstrated in several studies that the emulsifier can protect the oil against lipid oxidation [57, 58]. Variety of biopolymers, such as proteins, carbohydrates, lipids, and gums, either alone or in combination are used as wall materials to achieve stable emulsion of omega-3 fatty acid rich oils. It has been reported that low molecular weight carbohydrates has poor encapsulation efficiency due to crystallization at temperature, which is above their glass transition temperature [59]. However, study by Buera et al. [60] showed that crystallization can be aborted or avoided by employing divalent cations or polymers along with low molecular weight carbohydrates. Encapsulation potential of maltodextrin in combination with starch, whey protein, and gum arabic as wall material was evaluated in a recent study for microencapsulation of flaxseed oil. It was observed that maltodextrin when used in combination with whey protein offered superior emulsion stability and oxidative protection as compared to others. In contrast, best encapsulation efficiency was achieved by using combination of maltodextrin and starch as compared to gum arabic and whey protein [61].

Milk proteins, e.g., whey protein and casein, are wall material of choice for the preparation of highly concentrated emulsions due to their antioxidative effect on lipids [62–64]. It has been attributed to their ability to form a physical barrier at oil–water interface and chelate metal ions such as iron, which promote lipid oxidation and have detrimental effect on emulsion stability [64, 65]. It was observed that not only the type of protein but also amino acid composition played an important role in emulsion stabilization and encapsulation process. Casein contains several phosphorylated serine residues, which have been involved in metal chelation, whereas the presence of more sulfhydryl groups imparts free radical scavenging property to whey protein [66–69].

Microencapsulation of Emulsion

Production of microencapsulated omega-3 fatty acids begins with selection of source of omega-3 oil, i.e., core material, wall materials, designing and optimizing formulation, i.e., ratio of materials used to achieve stable emulsion and selection of encapsulation method. Usefulness of encapsulation technique is evaluated based on intended application, efficiency of encapsulation, and stability of omega-3 fatty acids containing microcapsules, which is largely determined by type and composition of wall material used in emulsion. Type of wall material used significantly affects characteristics such as droplet size, droplet charge, and viscosity of resulting emulsion.

Spray Drying

Spray drying is most widely used process of drying of food- and pharmaceutical-grade emulsions because it is economical, easy to scale up, and ease of getting required instruments and provide good quality of finished product with large-scale production in continuous operation mode without altering properties of active ingredient [70–72]. It is preferred technique for drying of heat-labile and aromatic compounds to preserve aroma [73, 74]. Process starts with the preparation of stable emulsion of oil by dispersion of oil into polymer-containing solution, which forms wall material by coating oil droplets. This is followed by atomization of feed emulsion and dehydration of atomized droplets by spraying it in hot chamber to produce microcapsules. Thus, formed microcapsules are recovered using cyclone separator [73, 75, 76]. Size of microcapsules is dependent on concentration of solids in emulsion and operating condition used for drying. Size of microcapsules produced by spray drying can vary from very fine powder of 10–50 nm to 2- to 3-mm large particles [77, 78]. Encapsulation efficiency is dependent not only on wall material properties but also on methods used for emulsification of oil. Process of emulsion preparation determines the size of droplet, which has marked effect on encapsulation by spray drying. Decrease in the size of droplet emulsion increases efficiency of oil embedding within matrix of coating material. Thus, emulsification methods which generate smaller-sized droplets can accommodate higher amount of oil in encapsulation process without exposing it to outer surface. It was reported by Jafari et al. that microfluidizer was more efficient in the preparation of emulsion with small droplet size allowing maximum oil to get encapsulation during drying process [79, 80].

Although spray drying remains a method of choice for encapsulation of volatile compounds and oils including omega-3 fatty acids containing oils, it has certain limitations. Spray drying is performed at higher temperature in the presence of water, which promotes oxidation and degradation of omega-3 fatty acids. Carriers, which gives free-flowing texture to final product, used during spray drying process form porous particles, which exposes oil to oxygen and ultimately causing decrease in shelf life of final product. However, it was observed that employing carbohydrate and proteins as a wall material and cross-linking them circumvent this problem. Powder obtained after spray drying was found to be more stable using this approach [81–85].

Another shortcoming with spray drying for microencapsulation is the production of very fine powder in the range of 10–100 μm in diameter, which needs to be processed further by process called fluidized bed/spray drying.

Fluidized Bed Drying

In this process, coating is applied onto powdered particles either in continuous or batch operation mode. This is a three-stage process in which at first particles to be coated are suspended in air stream at specific temperature. Second step involved spraying of coating material onto particles to initiate film formation and then followed by subsequent wetting and drying process. Coating material spread on surface of particles and solvent was evaporated by hot air, which results in adhesion of coating material on particle surface [86, 87].

Powdered omega-3 fatty acid oil particles are generally coated with another lipid to avoid its contact air and prevent oxidation. Process can be made cost-effective by using melted wax and fats as no solvent is required, thus eliminating solvent exposure step. Fluidized spray drying is most suitable method for encapsulating aromatic and oxidation-prone compounds as it facilitates the formation of strong interparticle bonding upon redrying [88].

Fish oil emulsion prepared using casein as wall material was encapsulated by fluidized spray drying method using corn starch as coating material during spraying. In another study, fish oil emulsion was coated using liquefied palm wax to prevent oxidation and increase stability [89, 90].

Although fluidized bed drying offers well control on size of particles and ease of handling with low-cost instrumentation, its utility has been restricted to provide secondary coating on already formed microencapsulation and cannot be used for direct microencapsulation of omega-3 oils.

Freeze-Drying (Lyophilization)

Freeze-drying, also known as lyophilization, remains one of the most employed method for drying of thermosensitive materials. During lyophilization process, emulsion is dried by sublimation under vacuum after freezing at temperatures in the range of −90 to −40 °C. This technology is considered to be useful for microencapsulation of oxidation-prone and temperature-sensitive materials like omega-3 fatty acids because drying is performed at low temperature in the absence of oxygen.

In a study performed by Minemoto et al. [91], freeze-drying was shown to be better method in preventing oxidation of methyl linoleate as compared to spray drying. In another study, freeze-drying of fish oil emulsion was found to result in microencapsulated product with relatively slow oxidation rate [92]. However in subsequent studies, it was reported that microencapsulation of fish oil by freeze-drying resulted in porous, irregular, and flakes like structures in freeze-dried powder exposing fish oil to oxygen and causing oxidation [93, 94]. In addition, cost of freeze-drying is 50 times more than that of spray drying with longer processing time and storage and transport of particles is expensive too [86, 95, 96]. Therefore, freeze-drying technique remains less attractive for microencapsulation of omega-3 fatty acids.

Coacervation

In coacervation process, two liquids one rich in polymer and another poor in polymer are used for the formation of coacervate by liquid–liquid phase separation mechanism. Depending upon number of polymers used, process can be named as simple coacervation when only one type of polymer is used and complex coacervation when more than one type of oppositely charged polymers are used.

In microencapsulation of oil in water by complex coacervation, oil-in-water emulsion prepared using gelatin and gum arabic under heavy stirring is subjected to adjustment in pH from neutral to acidic while keeping temperature above melting point of gelatin. This leads to the formation of three immiscible phases of oil, polymer high, and polymer low, and polymer from polymer-rich phase gets adsorbed at interface on oil droplets forming coacervates [96–98]. Similarly for microencapsulation omega-3 fatty acids, oil is usually dispersed in gelatin solution and pH adjustment leads to the formation of coacervates of gelatin-coated oil droplets. Drop in temperature below melting point of gelatin caused hardening of coating. However, complex coacervation of omega-3 fatty acid oil looks attractive due to its ability to encapsulate and deliver high amount of oil, i.e., 40–60 %. Typical complex coacervation process of omega-3 fatty acid oils make use of whey protein or gelatin along with oppositely charged polymers such as gum arabic, starch, carboxy methyl cellulose. Coacervates are formed by adjusting pH causing coating of oil droplets with protein and polymer at interface which can be further cross-linked chemically using glutaraldehyde or transglutaminase to impart solidity to coating [97, 99–102].

Complex coacervation offers particles of smaller size in the range of 1–1000 μm and provides material with payload of up to 90 % for single core and 60 % for multilayer. This definitely offers an avenue to meet requirements for increasing daily diets of omega-3 fatty acids [103].

Being a batch process and time-consuming are some of the limitations of coacervation process. In addition, cross-linking of coating adds an extra step in process and some of cross-linking materials, e.g., glutaraldehyde, are not

allowed to use in food material in Europe, which restricts broader application of product. It also makes use of gelatin, which originated from animals thus not acceptable to vegetarian population. It suggests a need to research plant derived materials as an alternative to gelatin, which can impart structural and oxidative stability [99, 103, 104].

Extrusion

Extrusion is also one of the methods for microencapsulation of PUFA-rich oil emulsions. It was originally developed by a group in 1957 and patented subsequently [105]. Extrusion process was found to produce less porous material compared to spray drying; however, it increases production cost as compared to spray drying and use of screw extruders at high pressure are highly detrimental to omega-3 fatty acids. In addition, particle obtained by extrusion process are in the range of 500–1000 μm, which is too high to be included in food materials as it will affect the texture of final product [96, 106].

Factors Influencing Emulsion Formation

Emulsion formation and stability determined by following factors [107, 108].

Salt Concentration

Low concentration of salt causes increase in droplet size of oil-in-water emulsion due to increase in solubilization of oil. Upon increase in salt concentration to intermediate range system changes to bi-continuous type, whereas higher concentration leads continuous emulsion with decreased droplet size. In case of biopolymer emulsifiers, increased salt concentration may lead to precipitation causing coalescence or flocculation of oil droplets.

Alcohol Concentration

Alcohol acts as cosurfactant in emulsion preparation. It was observed that low molecular weight alcohol when used as cosurfactant at low concentration favors the formation of water-in-oil emulsion. Further increase in concentration phase transition occurs and leads to the formation of oil-in-water emulsion type. When high molecular weight alcohols are used as cosurfactant, exactly opposite phenomenon was observed.

Hydrophobic Chain Length of Surfactant

Surfactant with longer hydrophobic chain shows the change of o/w microemulsion to w/o via bi-continuous phase.

pH

pH is one the critical factor that determines fate of emulsion along with salinity in case of emulsion system where pH-sensitive surfactants are used. Its effect is more visible in case of acidic or basic surfactants. The presence of carboxylic acids and amines tend to cause phase transition with increase in pH.

Oil Properties

Highly aromatic nature of oil leads to phase transition from o/w to w/o and is opposite to that of increase in the alkane carbon number in oil.

Ionic Strength

As the ionic strength of the system increases, it passes from o/w microemulsion in equilibrium with excess oil to the middle phase and finally to w/o microemulsion in equilibrium with excess water.

Characterization of Emulsion

Physical Appearance by Visual Inspection

Emulsions appearance can be checked visually for its optical clarity, fluidity, and homogeneousness.

Size Determination

Method of choice for determination of size of droplets in emulsion is dynamic light scattering, which provide information about uniformity of droplets as well as zetapotential. Zetapotential must be neutral or negative, indicating no charge on droplets, and thus, system is stable. It is very useful for assessing and inhibiting flocculation because electrical charge on droplets influences rate of flocculation. Other techniques such as small-angle neutron scattering, small-angle X-ray scattering, and electron microscopy are also used for determination mono- or polydisperse nature of emulsion [109].

Limpidity Test

Limpidity test measures percent transmission of emulsion and it can be done using spectrophotometer [110].

Microscopic Examination

Emulsion systems are examined under crosspolarizing microscope for the absence of birefringence to exclude liquid crystalline systems [111, 112].

Rheological Properties

Viscosity of emulsion plays an important role in determining stability of emulsion. Rheological properties of emulsion are determined by digital viscometer [113].

Dilution Test

In case of oil-in-water emulsion, continuous phase, i.e., water is added to dilute emulsion tenfold to hundred fold and observed for separation of phases. Properly formed and stable emulsion does not show any sign of phase separation.

Staining Test

This is simple staining method where a water-soluble dye such as methylene blue or amaranth is either added in water and emulsion is prepared with oil and surfactant or added to a drop of emulsion. As dye is soluble in water when a drop of emulsions is observed under microscope, colored background is observed with colorless globules of oil.

Tests for Assessment of Emulsion Stability

Generally, stability studies involve time-consuming processes, and therefore, accelerated stability tests are preferred [114, 115].

Stress Testing Using Centrifugation

It is performed by centrifugation of emulsion 5000 and 10,000 rpm for 30 min to assess whether there are any of the physical instabilities like phase separation, phase inversion, aggregation, creaming, and cracking of the formulations. Previously thermally tested formulation are taken in centrifuge sample tubes and placed in the centrifuge basket at a well-balanced equilibrium position at ambient temperature conditions.

Repeated Freezing and Thawing

In order to access any change in stability of emulsion upon freezing and thawing, they are incubated at 25 °C for 24 h and followed by incubation for 24 h at −5 °C, and the cycle is repeated three times and the change was observed.

Long-term Stability

Along with accelerated stability studies, long-term stability needs to be performed as per ICH guidelines. Emulsion is subjected to storage at ambient conditions for 6 months, and periodically examined at interval of 1, 3, and 6 months by visual inspection and measurement of percent transmittance, pH, specific gravity, and rheological evaluation.

Determination of Thermal Stability

Thermostability of emulsion is determined by incubating emulsion at three different temperatures for a period of 1 month. Periodically samples are removed for visual inspection to observe any physical changes like loss of clarity, coalescence, and turbidity and also examined for the determination of the loss of aqueous phase that is an essential part of the emulsion stability [116].

Peroxides and Degradation Product Detection

Peroxides and secondary degradation products can be detected and estimated by gas chromatography–mass spectroscopy. This is equipment- and cost-intensive process and demands technical expertise. In contrast, relatively simple, reproducible, and cost-effective peroxide value (PV) and anisidine value (AV) assays were employed to determine oxidative stress on oil. Peroxide value estimates number of peroxide groups present, and anisidine value measures secondary degradation products, measurements are used to calculate total oxidative stress (Total Oxidative Stress = 2 × PV + AV), and guidelines about their acceptable levels are well documented [117].

Omega-3 Fatty Acid Emulsions and Microencapsulates: Bioavailability and Benefits

Emulsification and microencapsulation process prevents oxidation of omega-3 fatty acids and increases shelf life and consumer acceptability of product. Non-microencapsulated emulsions of omega-3 fatty acid are used for parental administration as well as fortification of foods, whereas use of microencapsulated is limited as food additives and preparation of over the counter supplements of omega-3 fatty acids. Potential of omega-3 emulsions to offer health benefits associated with omega-3 oil depends on their bioavailability after emulsification.

Importance of dietary lipids to human nutrition and physiology is firmly established. Continued research has confirmed potential health benefits offered by PUFAs, particularly from consumption of omega-3 fatty acids from sources such as fish and plant oils [118–120].

Consumption of food fortified with omega-3 emulsions containing high concentrations of EPA and DHA provides good option to increase levels of PUFAs in plasma.

Bioavailability of EPA and DHA is determined by intramolecular structure of triacylglycrol (TAG) and lymphatic transport. It has been reported that total lymphlipids of EPA and DHA are significantly higher if the PUFAs are esterified at the sn-2 position of the TAG compared to those at sn-1 or sn-3 positions and transesterification of fish oil increases bioavailability of EPA/DHA as compared to normal fish oil. It has been postulated that coproducts of transesterification might be involved in facilitation of intestinal digestion of EPA and DHA by acting as an emulsifying agent [121, 122]. In fat digestion process, emulsification of lipid in the stomach is a fundamental step, which forms lipid–water interface necessary to facilitate interaction between water-soluble lipases and water-insoluble lipids. Formation of lipid–water interface determines the bioavailability of dietary fats. Thus, emulsification of omega-3 fatty acid oil eliminates this normal physiological step and enhances bioavailability of oil [123, 124]. Studies has shown that pre-emulsification of fish oil indeed enhances absorption of PUFAs and also improves rate and extent of incorporation into plasma fatty acids. It also highlights effect of different molecular structures of EPA and DHA lipid sources on their absorption and metabolism [125, 126]. The health benefits of fish oil emulsions are attributed to the presence of high concentration of omega-3 fatty acids, including DHA and EPA, which are parent molecules for anti-inflammatory eicosanoids and proresolving lipid mediators [127]. Although relationship between lipid emulsion content and inflammatory homeostasis is still largely unknown, recent discovery of lipid mediators named as Specialized Proresolving Mediators has boosted our understanding on role of omega-3 fatty acids in anti-inflammatory effect. Molecules resolvins, protectins, and maresins were derived from omega-3 fatty acid, which are autacoids endogenous mediators of cellular programs to restore tissue homeostasis and resolve inflammation [128–132]. Two independently performed recent studies established that three weeks of oral supplementation of omega-3 fatty acid to healthy human subjects resulted in concentrations of Specialized Proresolving Mediators that are within the biological range known to have proresolving activity in human leukocytes and mice [133, 134].

In a clinical study on healthy volunteers, conducted by Raatz and colleagues, rate and extent of absorption of total n-3 and LCn-3 into the phospholipid fatty acid (PLFA) pool after emulsified fish oil compared to capsules of the fish oil used in its production was examined. It was observed that compared to a standard fish oil, consumption of an emulsified fish oil supplement resulted in an enhanced rate and extent of absorption of total n-3 fatty acids and EPA and a decline in the n-6/n-3 fatty acid ratio in plasma phospholipids over 48 h. Authors reported that emulsification of fish oils modifies the solubility of the supplement and has physical and chemical characteristics that differ from capsular or normal fish oil. The emulsified and water-soluble nature of fish oil increases exposure to lipase and reduces the gastric clearance time and improves the digestion and absorption of EPA and DHA. It was suggested that difference in absorption could be a result of wall material used for microencapsulation of fish oil.

Proteins and carbohydrates, which are mainly used as shell/wall material in emulsion and microencapsulation process, are susceptible to intestinal digestive enzymes and get easily digested in gastrointestinal tract. Microencapsulated omega-3 oils when used as food additive retained bioavailability and has been reported to be equivalent to fish oil. Although release was dependent on the nature of wall material used for microencapsulation, plasma triacylglycerol-lowering effect of microencapsulated omega-3 oil was equivalent to that of fish oil supplements [135–137]. Milk proteins, particularly whey protein, when used as wall material have been reported to enhance bioavailability of microencapsulated omega-3 fatty acids [138].

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Omega-3 Fatty Acids in Inflammatory Diseases

11

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Introduction

Fatty acids are straight chain hydrocarbons with a carboxyl group at one end and a methyl group at the other. The carboxylic acid (-COOH) end is considered as the beginning of the chain, thus designated as "alpha," and the methyl ($-CH_3$) end is considered the "tail" of the chain, designated as "omega." There are three major classes of fatty acids, namely saturated fatty acids, monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFAs). The saturated fatty acids do not contain any double bonds within the acyl chain, while unsaturated fatty acids contain at least one double bond. When a single double bond is present within the acyl chain, it is called as MUFA, and when two or more double bonds are present, they are referred to as PUFAs. The PUFAs can be further classified as omega-3 fatty acids (also called ω-3 fatty acids or *n*-3 fatty acids) and omega-6 fatty acids (also called ω-6 fatty acids or *n*-6 fatty acids) based on the location of the first double bond from the terminal methyl end of the molecule. Omega-3 fatty acids possess first double bond (C = C) at the third carbon atom from the methyl end of the carbon chain, while omega-6 fatty acids have first double bond (C = C) at the sixth carbon atom from the methyl end of the carbon chain. The human body can produce all except two of the fatty acids it requires, i.e., linoleic acid and α-linolenic acid, as the enzymes (desaturases) required to introduce double bonds in the n-3 and n-6 positions are not present in mammals. Linoleic acid (LA, C18:2n-6) is the precursor to the n-6 series of fatty acids and α-linolenic acid (ALA, C18:3n-3) is the precursor to the n-3 series of fatty acids. These fatty acids must therefore be obtained from the diet, and accordingly, they are known as essential fatty acids.

The essential fatty acids from omega-3 series involved in human physiology are alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Marine algae and phytoplankton are primary sources of omega-3 fatty acids. Common sources of plant oils containing ALA fatty acid include walnut, edible seeds, clary sage seed oil, algal oil, flaxseed oil, Sacha Inchi oil, *Echium* oil, and hemp oil, while sources of animal EPA and DHA fatty acids are mostly found in seafood, but fish do not actually produce these fatty acids. In fact, these compounds are produced by single-cell marine organisms that are consumed by fish. Other sources include egg oil, squid oils, and krill oil.

PUFAs regulate a wide variety of biological functions, depending on the location of the last double bond, which range from blood pressure and blood clotting to the correct development and functioning of the brain and nervous system [1]. In addition, lipid mediators generated from long-chain (LC-) PUFA (arachidonic acid (AA) in the n-6 series and EPA and DHA in the n-3 series) have important roles in immune regulation and inflammation [2]. This chapter is focused to give an insight about how dietary omega-3 fatty acids help in the management of inflammatory disorders.

Immune System and Inflammation

The immune system provides protection from an array of infectious agents while permitting tolerance to self-antigens and non-threatening agents such as food proteins and bacterial gut flora. The body's immediate response to infection or injury begins with inflammation. Inflammation functions to begin the process of elimination of invading pathogens and toxins and to repair damaged tissue. The immune

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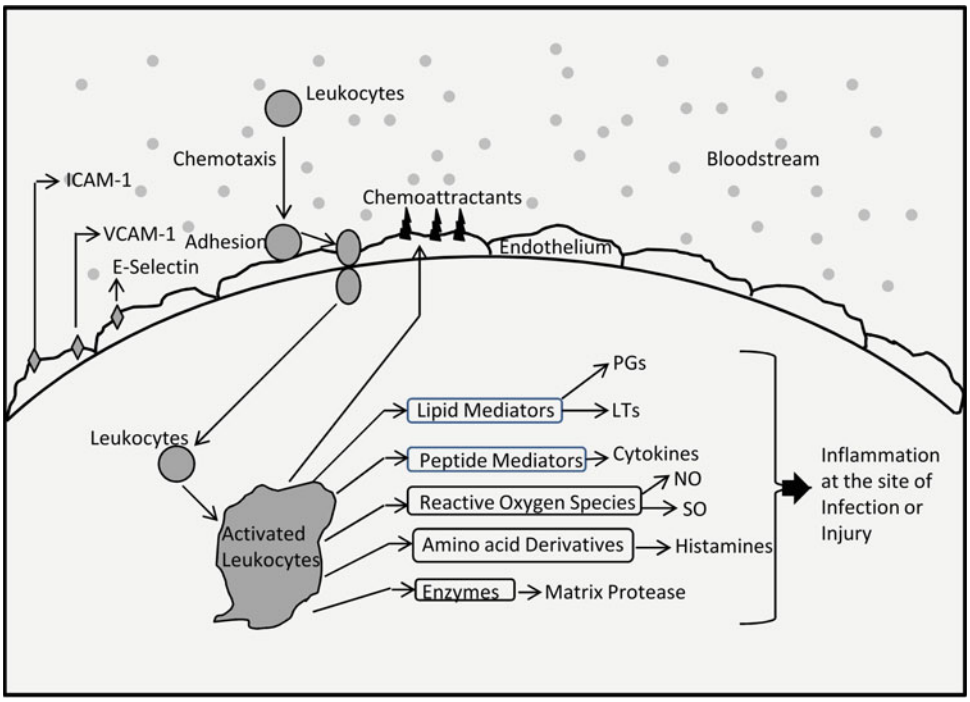

Fig. 11.1 Diagrammatic representation of the immunologic responses to infection and injury (Modified from [4]) © American Society for Nutrition. *PGs* Prostaglandins, *LTs* Leukotrienes, *NO* Nitric oxide, *SO* Superoxide, *ICAM-1* Intercellular adhesion molecule, *VCAM-1* Vascular Cell adhesion molecule

response involves a complexity of blood-borne factors and different immune cells with different roles but they act together to create a highly regulated and well coordinated immune response [3]. Clinical characteristics of acute inflammation include redness, swelling, heat, and pain. These occur as a result of increased blood flow to the site of inflammation; increased permeability across blood capillaries caused by retraction of endothelial cells, which allows large molecules (e.g., complement, antibodies, and cytokines) to leave the bloodstream and cross the endothelial wall; increased movement of leukocytes from the bloodstream into the surrounding tissue and then to the site of inflammation as depicted in Fig. 11.1. This movement is induced by release of chemoattractants and by the upregulation of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin on the surface of endothelial cells allowing leukocyte binding and subsequent diapedesis. The last stage involves release of mediators from leukocytes at the site of inflammation. These may include lipid mediators (e.g., prostaglandins [PGs], leukotrienes [LTs]), peptide mediators (e.g., cytokines), reactive oxygen species (e.g., superoxide), amino acid derivatives (e.g., histamine), and enzymes (e.g., matrix proteases) depending upon the cell type involved, the nature of the inflammatory stimulus, the anatomical site involved, and the stage during the inflammatory response. Several of these mediators may act to amplify the inflammatory process by acting as chemoattractants. Some of the inflammatory mediators may escape the inflammatory site into the circulation and from there they can exert systemic effects. Thus, inflammation and the inflammatory response are part of the normal, innate immune response [4].

Although inflammation is a normal response, when it occurs in an uncontrolled or inappropriate manner excessive damage to host tissues and disease can ensue. Such uncontrolled or inappropriate inflammatory responses are characterized by hyperexpression of endothelial and leukocyte adhesion molecules, appearance of soluble forms of adhesion molecules in the circulation, sequestration of leukocytes to sites where they are not usually found, production of inflammatory mediators, and damage to host tissues [4].

Role of PUFAS in Inflammation

The major substrates for energy production are fatty acids; however, they are also involved in the formation of cellular structures as well as in the transmission of cellular signals. Dietary lipids are absorbed and distributed to essentially every cell membrane in the body where they perform important structural and functional roles. They are known to modulate the immune system by various means, such as altering membrane fluidity, regulating eicosanoid metabolites, oxidative stress, producing lipid peroxides, regulation of gene expression, apoptosis or modulation of gastrointestinal microbiota, and interacting directly with cellular activation processes [3]. Polyunsaturated fatty acids and

their metabolites are crucial to the physiologic and pathophysiologic processes in inflammation. Altering fatty acid type and their composition in phospholipids of immune cells through diet supplements for beneficial outcomes in disease has been of major interest to the community. The types of fatty acids being esterified in membrane phospholipids provide a characteristic fatty acid composition of the phospholipids which can dictate the characteristics of the inflammatory response depending on the types of metabolites of polyunsaturated fatty acids formed through the lipoxygenase (LOX) and cyclooxygenase (COX) pathways, either promoting or inhibiting the inflammatory process, by controlling intracellular signaling pathways, such as protein kinase C (PKC), mitogen-activated protein (MAP) kinases, and phosphoinositol 3 (PI3) kinase [5]. Certain membrane fatty acids also have specific roles in regulation of cell and membrane functions. This is exemplified by gamma linolenic acid (GLA), AA, and EPA which act as precursors for synthesis of an important class of immunoregulatory molecules called eicosanoids. Eicosanoids are a family of 20 carbon-oxygenated derivatives of AA, GLA, and EPA, and include prostaglandins (PGs), thromboxanes (TX), leukotrienes (LTs), and other oxidized derivatives, which are generated from arachidonic acid by the metabolic processes. Eicosanoids are involved in modulating the intensity and duration of inflammatory responses, have cell- and stimulus-specific sources, and frequently have opposing effects. Thus, the overall physiologic (or pathophysiologic) outcome will depend on the cells present, the nature of the stimulus, the timing of eicosanoid generation, the concentrations of different eicosanoids generated, and the sensitivity of the target cells and tissues to the eicosanoids generated [6].

Mechanisms by Which Omega-3 Fatty Acids Influence Inflammation

Polyunsaturated fatty acids (PUFAs) are important constituents of the phospholipids of all cell membranes. They can influence inflammatory cell function and so inflammatory processes by the following ways (Fig. 11.2) [7]:

Altering the Physical Properties of the Membrane

PUFAs can be incorporated into the phospholipids of inflammatory cell membranes where they play important roles assuring the correct environment for membrane protein function, maintaining membrane fluidity, and influencing lipid raft formation [8].

Exerting Effects on Cell Signaling Pathways

It is achieved either through modifying the expression, activity, or avidity of membrane receptors, or through modifying intracellular signal transduction mechanisms that lead to altered transcription factor activity and changes in gene expression. Membrane phospholipids are substrates for the generation of second messengers such as diacylglycerol, and it has been demonstrated that the fatty acid composition of such second messengers, which is determined by that of the precursor phospholipid, can influence their activity [9]. In addition, membrane phospholipids are substrates for the release of (non-esterified) PUFAs intracellularly—the released PUFAs can act as signaling molecules, ligands (or precursors of ligands) for transcription factors, or precursors for biosynthesis of lipid mediators which are involved in regulation of many cell and tissue responses, including aspects of inflammation and immunity.

Altering the Pattern of Lipid Mediators Produced

PUFA intake can influence complex lipid, lipoprotein, metabolite, and hormone concentrations that in turn influence inflammation. Non-esterified PUFAs can act directly on inflammatory cells via surface or intracellular "fatty acid receptors"—the latter may include transcription factors such as peroxisome proliferator-activated receptors (PPARs). PUFAs can be oxidized (enzymatically or non-enzymatically) and the oxidized derivatives can act directly on inflammatory cells via surface or intracellular receptors—oxidation can occur to the non-esterified form of the PUFA or to PUFAs esterified into more complex lipids including circulating or cell membrane phospholipids and intact lipoproteins such as low-density lipoprotein (LDL). The membrane phospholipids of inflammatory cells taken from human-consuming Western-type diets typically contain approximately 10–20 % of fatty acids as arachidonic acid, with about 0.5–1 % EPA and about 2–4 % DHA [10–17], although there are differences between the different phospholipid classes in terms of the content of these fatty acids. The eicosanoid family of inflammatory mediators is generated from 20-carbon polyunsaturated fatty acids (PUFAs) liberated from cell membrane phospholipids. Thus, arachidonic acid is usually the dominant substrate for eicosanoid synthesis. Eicosanoids include PGs, thromboxanes (TXs), leukotrienes (LTs), and hydroxyeicosatetraenoic acids (HETEs). Arachidonic acid in cell membrane phospholipids can be mobilized by various phospholipase enzymes, most notably phospholipase A2, and the free acid can subsequently act as a substrate for the

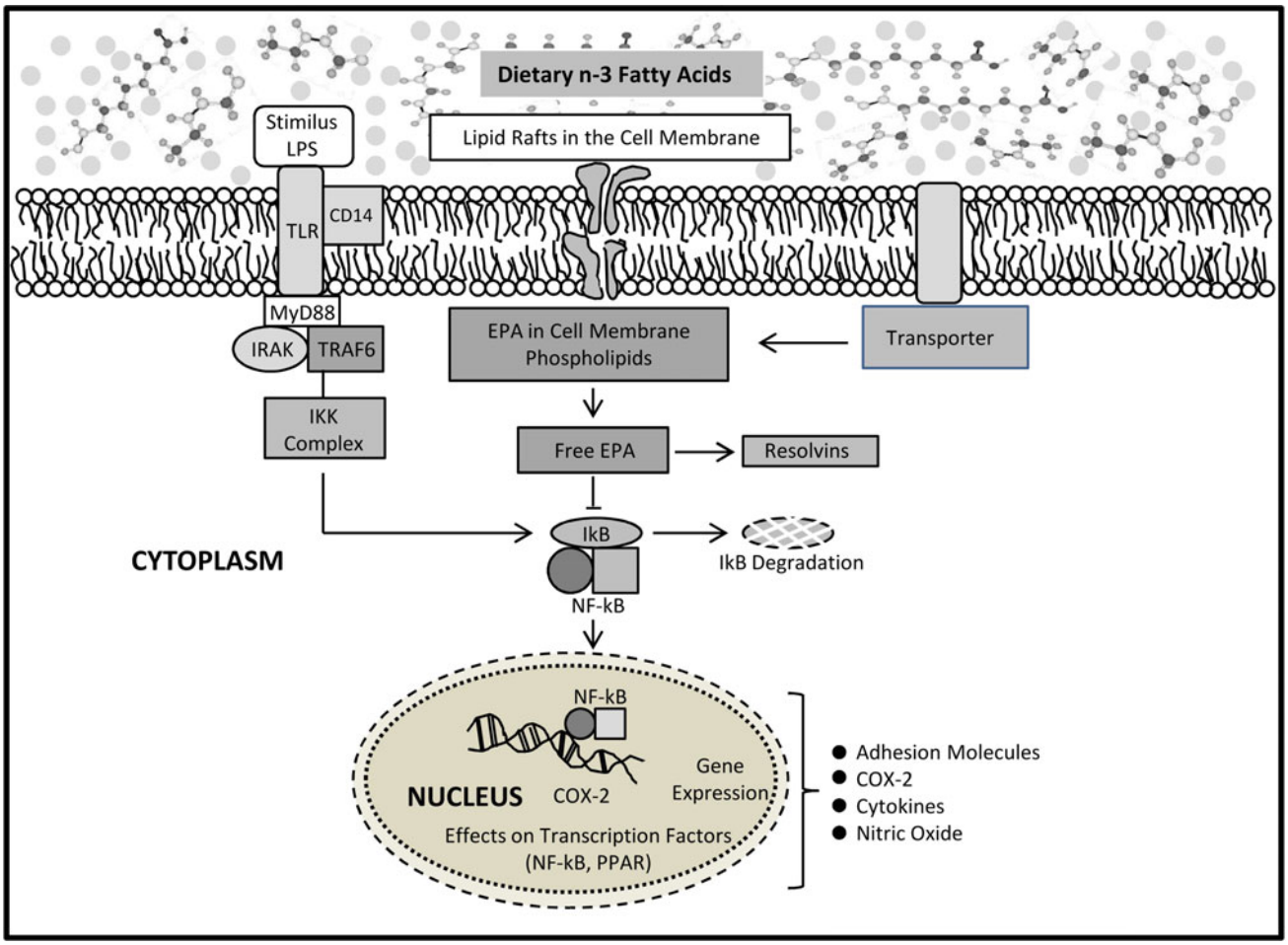

Fig. 11.2 Schematic representation of mechanisms by which omega-3 fatty acids modulate immune response (Modified from [7]) © Springer Science+Business Media, LLC 2010. *COX2* Cycloxygenase2, *EPA* Eicosapentaenoic acid, *LPS* Lipopolysaccharide, *CD14* cluster of differentiation 14, *TLR* Toll-like receptor, *PPAR* Peroxisome proliferator-activated receptor, *NFkB* Nuclear factor kappa B, *IkB* Inhibitory unit of NFkB, *IKK* IkB kinase, *IRAK* Interleukin-1 receptor-associated kinase, *TRAF6* TNF receptor-associated factor 6, *MyD88* Myeloid differentiation primary response gene 88

enzymes that synthesize eicosanoids. Metabolism by cyclooxygenase (COX) enzymes gives rise to the 2-series PGs and TXs. COX-2 is induced in inflammatory cells as a result of stimulation and is responsible for the markedly elevated production of PGs that occurs upon cellular activation. Monocytes and macrophages produce large amounts of PGE2 and PGF2, neutrophils produce moderate amounts of PGE2, and mast cells produce PGD2. Metabolism of arachidonic acid by the 5-lipoxygenase (5-LOX) pathway gives rise to hydroxy and hydroperoxy derivatives (5-HETE and 5-HPETE, respectively), and the 4-series LTs, LTA4, B4, C4, D4, and E4. Neutrophils, monocytes, and macrophages produce LTB4, while LTC4, D4, and E4 tend to be produced by mast cells, basophils, and eosinophils. PGE2 has a number of proinflammatory effects including inducing fever, increasing vascular permeability and vasodilatation, and enhancing pain and edema caused by other agents. PGE2 has been shown to induce COX-2 mRNA expression in cultured fibroblasts and so to upregulate its own production and to induce production of the inflammatory cytokine IL-6 by macrophages [18]. LTB4 increases vascular permeability, is a potent chemotactic agent for leukocytes, induces release of lysosomal enzymes, and enhances generation of reactive oxygen species and production of inflammatory cytokines such as TNF-α, IL-1, and IL-6. The cysteinyl-LTs (LTC4, D4, and E4) are bronchoconstrictors, increase vascular permeability, and promote hypersensitivity. In inflammatory conditions, increased rates of production of arachidonic acid-derived eicosanoids occur and elevated levels of these eicosanoids are observed in blood and tissues from patients with acute and chronic inflammatory conditions. Despite the ongoing emphasis on the proinflammatory effects of arachidonic acid-derived eicosanoids, some of these mediators, for example lipoxin A4, are actually anti-inflammatory [19]. Recent studies have shown that PGE2 inhibits 5-LOX and so decreases the production of inflammatory 4-series LTs and induces 15-LOX promoting the formation of lipoxins that are found to have anti-

Table 11.1 Some diseases with an inflammatory component in which omega-3 fatty acids have beneficial effect

Disease	Conditions
Rheumatoid arthritis	Inflammation of joints
Ulcerative colitis	Inflammation of the mucosa of the colon
Crohn's disease	Inflammation of the ileum and the colon
Asthma	Inflammation of respiratory tract
Multiple sclerosis	Autoimmune disease of brain and spinal cord
Psoriasis	Inflammatory autoimmune disease
Systemic lupus erythematosus	Autoimmune disease affecting any organ system
Chronic obstructive pulmonary disease	Chronic inflammation of the peripheral airways and lung parenchyma
Neurodegenerative disease of aging	Inflammation of central nervous system

inflammatory effects [20]. These findings demonstrate that PGE2 have both proinflammatory and anti-inflammatory actions.

Membrane fluidity and eicosanoid synthesis are the two realms in which lipids have their most potent effects. The effect of dietary fatty acid intake on immune function can be modulated by intake, offering the potential of a dietary management tool in its regulation. Some diseases and conditions that are recognized to having an inflammatory component are listed in Table 11.1. This chapter describes the role of omega-3 fatty acids in rheumatoid arthritis, inflammatory bowel disease, asthma, and multiple sclerosis in detail.

Role of Omega-3 Fatty Acids in Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic, progressive, inflammatory autoimmune disease associated with articular, extra-articular, and systemic effects. The pathobiology of RA is multifaceted and involves T cells, B cells, and the complex interaction of many proinflammatory cytokines TNF-α and IL-6 [21, 22]. The cytokines most directly implicated in this process are TNF-α and IL-6 [20]. These cytokines are messengers that activate and differentiate effector cells that cause local and systemic symptoms associated with this disease.

The cause of rheumatoid arthritis remains unknown, but insights into pathogenic pathways have accumulated over the past two decades [23]. Recent findings suggest a genetic basis for disease development in RA. Environmental factors, such as smoking and infection, may also influence the development, rate of progression, and severity of RA [24, 25]. Various immune modulators (cytokines and effector cells) and signaling pathways are involved in the pathophysiology of RA [22]. The complex interaction of immune modulators is responsible for the joint damage and begins at the synovial membrane [22]. Synovitis is caused by the influx or local activation, or both, of mononuclear cells (including T cells, B cells, plasma cells, dendritic cells, macrophages, and mast cells) and by angiogenesis [22]. The synovial lining then becomes hyperplastic, and the synovial membrane expands and forms villi [22]. The osteoclast-rich portion of the synovial membrane, or pannus, destroys bone, whereas enzymes secreted by neutrophils, synoviocytes, and chondrocytes degrade cartilage [22].

In addition to joint symptoms, many patients experience extra-articular or systemic manifestations, or both [26]. Extra-articular manifestations include rheumatoid nodules, vasculitis, pericarditis, keratoconjunctivitis sicca, uveitis, and rheumatoid lung [26]. Systemic manifestations include acute-phase protein production, anemia, cardiovascular disease (CVD), osteoporosis, fatigue, and depression [27, 28].

Increased understanding of the pathobiology of RA has led to the development of biologic agents that target various immune mediators involved in the disease process [29–42]. Therapies targeted against TNF-α, IL-1, and IL-6, in addition to T- and B cell inhibitors, when used alone or in combination with MTX, have resulted in favorable clinical outcomes in patients with RA [42].

Mode of action of TNF-α inhibitors

TNF-α inhibitors bind with high affinity to soluble and membrane-bound TNF-α and inhibits its effect by blocking TNF-α receptor interactions. It selectively neutralizes membrane-associated and soluble TNF-α and forms high-affinity, stable complexes with soluble and transmembrane bioactive forms of TNF-α, preventing the binding of TNF-α to its receptors [29–38].

Mode of action of other cytokine inhibitors

They neutralize activity of both IL-1a and IL-1b by binding specifically to soluble IL-6 receptor (sIL-6R) and membrane-bound IL-6 receptor (mIL-6R) and inhibiting sIL-6R and mIL-6R-mediated signaling [39, 40]

Mode of action of B- and T- cell inhibitors

B cell inhibitors act by binding CD20 domain expressed on mature B and pre-B cells thereby depleting peripheral B cells temporarily and T cell inhibitors act by selectively blocking the specific binding of receptors of CD80/CD86 on the membrane of the antigen presenting cells with the CD28 receptor on T cells, which is, pathophysiologically, a block of the second signal for activation of T cells. [41, 42].

However, although biologic agents are promising, they are not without limitations [43]. During the 1980s and 1990s, several studies in patients with rheumatoid arthritis showed the beneficial effects of *n-3* PUFA on the development of RA. Several authors reported that fish oils reduce the

Table 11.2 Overview of clinical outcomes in studies using n-3 PUFAs in patients with rheumatoid arthritis

Study and design	Duration of study and no of patients	Placebo	Dose of EPA and DHA (g/d)	Clinical outcomes that improved with intake of n-3 PUFAs	Ref.
DB, PC, P	12 weeks, n = 38	Paraffin oil	1.8 + 1.2	Intake of n-3 PUFAs improved NTJ and DMS	[45]
DB, PC, CO	14 weeks, n = 33	Olive oil	2.7 + 1.8	Intake of n-3 PUFAs improved NTJ, NSJ, TTF and PhyGA	[46]
DB, PC, P	12 weeks, n = 46	Olive oil	3.2 + 2	Intake of n-3 PUFAs improved NTJ and GS	[47]
DB, PC, CO	12 weeks, n = 16	Coconut oil	2 + 1.3	Intake of n-3 PUFAs improved NSJ and DMS	[48]
DB, PC, P	24 weeks, n = 49	Olive oil	Low-dose EPA 1.7 + 1.2 High-dose EPA 3.5 + 2.4	Intake of n-3 PUFAs improved NSJ, NTJ, and GS in low- and high-dose groups and improved DMS and PhyGA in high-dose groups only	[49]
DB, PC, P	12 weeks, n = 27	Coconut oil	2 + 1.3	Intake of n-3 PUFAs improved NSJ and DMS	[50]
DB, PC	24 weeks, n = 43	Mixed oils	1.8 + 1.2	Intake of n-3 PUFAs improved NSJ, NTJ, GS, DMS, and PhyGA	[51]
DB, PC, P	12 weeks, n = 43	Mixed oils	2 + 1.2	Number and severity of tender joints	[52]
DB, PC, P	12 weeks, n = 51	Vegetable oil	2 + 1.2	Intake of n-3 PUFAs improved NTJ, DMS, and CRP	[53]
DB, PC, P	16 weeks, n = 67	Corn oil	3.8 + 1.2	Intake of n-3 PUFAs improved NSJ, STJ, and DMS	[53]
DB, PC	52 weeks, n = 64	Air	1.7 + 1.1	Intake of n-3 PUFAs reduced use of NSAIDs	[54]
DB, PC, P	52 weeks, n = 60	Olive oil	1.7 + 0.4	Intake of n-3 PUFAs improved PtG, and reduced use of NSAIDs	[55]
DB, PC, P	52 weeks, n = 60	Olive oil	0.8 + 0.2	Intake of n-3 PUFAs improved PtG, and reduced use of NSAIDs	[56]
DB, PC, CO	26-30 weeks, n = 49	Corn oil	4.6 + 2.5	Intake of n-3 PUFAs improved NSJ, STJ, PtG, PhyGA, and DMS	[57]
DB, PC	15 weeks, n = 50	Mixed oils	40 mg/kg 2.3 g/d n-3 fatty acids	Intake of n-3 PUFAs improved NSJ, STJ, PtG, HAQ, PhyGA, and DMS	[58]
DB, PC, CO	8 months n = 62	Corn oil	30 mg n-3 fatty acid/kg body wt	Intake of n-3 PUFAs improved NSJ, NTJ, and reduced CRP in those on MTX	[59]
DB, PC, P	16 weeks n = 66	Liquid supplement without added PUFA	1.4 + 0.2 (+0.5 GLA in liquid supplement)	Study did not show superior clinical benefit of daily nutrient supplementation with EPA, GLA at the doses tested as compared to placebo.	[60]
Parallel randomized	24 weeks n = 43	Soybean oil	Total 3 g/d	Intake of n-3 PUFAs improved PtG, JP, GS, RAI, PhyGA, and DMS	[61]
DB, PC,	1 yr, n = 49	Inert oil	240 mg/d EPA with GLA	Intake of GLA with or without EPA reduced use of NSAIDs and improved patient symptoms	[62]

DB Double blind, *PC* Placebo controlled, *CO* Crossover, *P* Parallel, *DHA* Docosahexaenoic acid, *EPA* Eicosapentaenoic acid, *GLA* Gamma Linolenic acid, *HLA* Histocompatibility antigen, *PUFA* Polyunsaturated fatty acid, *LTB4* Leukotriene B4; *NK* Natural killer, *TB3* Thromboxane B3, *NTJ* Number of tender joints, *DMS* Duration of morning stiffness, *NSJ* Number of swollen joints, *TTF* Time to fatigue, *PhyGA* Physician's global assessment, *GS* Grip strength, *CRP* C-Reactive Protein, *PtG* Patient's global assessment, *HAQ* Health assessment by questionnaire, *MTX* Methotrexate, *NSAIDs* Non-steroidal anti-inflammatory drugs, *RAI* Ritchie articular index, *JP* Joint pain

production of inflammatory mediators such as LTB 4 by neutrophils and monocytes [44]. A number of randomized, placebo-controlled, double-blind studies of fish oil treatments for RA have been reported which are listed in Table 11.2 and each concluded the benefit of *n-3* PUFA in RA and suggest that use of n-3 PUFAs as standard therapy for management of RA.

Role of Omega-3 Fatty Acids in Inflammatory Bowel Diseases

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic idiopathic inflammatory disorders of the gastrointestinal tract, collectively termed as inflammatory bowel diseases (IBD). While UC involves exclusively the mucosa of the colon in a variable continuous extent, CD may occur in any part of the digestive tract in a segmental transmural fashion, with the ileum and colon being the most often involved segments [63].

Two transcription factors that are likely to play a role in inflammation of the gastrointestinal tract are nuclear factor kappa B (NFkB) and peroxisome proliferator-activated receptor (PPAR)-γ. NFkB is the principal transcription factor involved in upregulation of inflammatory cytokine, adhesion molecule, and COX-2 genes [64, 65]. NFkB is activated as a result of a signaling cascade triggered by extracellular inflammatory stimuli and involving phosphorylation of an inhibitory subunit (inhibitory subunit of NFkB (IkB)) which then allows translocation of the remaining NFkB dimer to the nucleus [66]. Thus, expression of inflammatory genes is upregulated. NFkB is a recognized target for controlling intestinal inflammation [67–69].

The second transcription factor, PPAR- γ, is also expressed in intestinal tissue [70] where it is believed to act in an anti-inflammatory manner. Colonic biopsies of patients with ulcerative colitis show lowered PPAR-γ expression [71], PPAR-γ knockdown mice show enhanced susceptibility to TNBS-induced colitis [72] and PPAR-γ agonists reduce colitis in murine models [73, 74]. Thus, upregulation of PPAR-γ is also a recognized target for controlling intestinal inflammation [74]. While PPAR-γ directly regulates inflammatory gene expression, it also interferes with the activation of NFkB creating an intriguing interaction between these two transcription factors [75].

There is no curative therapy for these IBDs (except for total proctocolectomy in UC), as its precise etiology remains elusive. IBDs are thought to occur as a result of an inadequate and sustained immune response against luminal (most probably bacterial) antigens, and patients should receive medical treatment for both controlling the inflammatory flares and preventing further bouts of the disease, since they typically have a relapsing and remitting course [76]. Drugs such as aminosalicylates, corticosteroids, immune suppressants (such as thiopurines, cyclosporin, or methotrexate), and biologic agents (mainly anti-TNF monoclonal antibodies) are effective for inducing and/or maintaining remission in IBD [77, 78] but encompass an increased risk for infections and possibility of developing malignancies.

The anti-inflammatory properties of n-3 PUFAs have prompted a series of studies to investigate their efficacy in animal models of inflammatory bowel disease. The primary studies involved chemically induced colitis. The outcomes of these studies are summarized in Table 11.3 and suggest some benefits including improved sigmoidoscopic score, lower relapse rate, and decreased use of corticosteroids. Therefore, dietary management is sought as an alternative approach to IBD therapy.

Role of Omega-3 Fatty Acids in Asthma

Asthma is a chronic inflammatory disorder of the airways leading to airways hyper-responsiveness and associated symptoms such as wheezing and coughing, and is also typically associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment [101]. The inflammatory response is complex and involves a variety of inflammatory cell types including mast cells, alveolar macrophages, neutrophils, eosinophils, lymphocytes, platelets, and a variety of inflammatory mediators [102, 103]. Since airway inflammation is multifactorial, involving various cell types and mediators, the drugs used to decrease inflammation may act at several different steps in the inflammatory process [103, 104]. Various therapeutic strategies have been developed to manage asthma, including the use of short acting beta-2 agonist bronchodilator medications as symptom relievers and anti-inflammatory preventer medications such as inhaled corticosteroids and oral leukotriene antagonists [103, 104]. While pharmacological medications have proven highly effective and have facilitated the management of asthma, prolonged use of some medications may result in reduced efficacy or tachyphylaxis [105, 106]. There is accumulating evidence that dietary modifications have the potential to influence the severity of asthma and reduce the dose requirements of drug treatment. Therefore, various studies to relate the effect of n-3 PUFA supplementation on patients suffering from bronchial asthma have been conducted and have demonstrated different levels of benefit. Though there are inconsistency among study results which may be attributed to the heterogeneity in definitions of the study populations (e.g., age, gender, clinical picture of asthma including its severity), and the type of intervention (e.g., amounts of oil and omega-3 fatty acid contents). Only few data are available on the effect of n-3 PUFA supplementation on patients with asthma which are listed in Table 11.4.

Table 11.3 Overview of clinical outcomes in studies using n-3 PUFAs in patients with inflammatory bowel disease

Disease (Study and design)	Duration of study and no. of patients	Placebo	Dose of EPA and DHA (g/d)	Clinical outcomes that improved with intake of n-3 PUFAs	Ref.
Ulcerative colitis (DB, PC, CO)	4 months n = 11	Mixed oils (Oleic + Palmitic + Linoleic)	2·7 + 1·8	56 % mean reduction in DAI with fish oil versus 4 % with placebo, decreased use of corticosteroids	[79]
Ulcerative colitis (DB, PC, CO)	4 months n = 18	Mixed oils (Oleic + Palmitic + Linoleic)	3·2 + 2·2	Greater improvement in histology index with fish oil (P = 0·002). No differences in clinical and endoscopic response	[80]
Ulcerative colitis (DB, PC, P)	4 months n = 53	Sunflower oil	1·5 g EPA/day + 2·1 g GLA/day	n-3 and n-6 (alone or in combination) had no steroid sparing effect.	[81]
Ulcerative colitis (DB, PC, P)	6 months n = 18	Sunflower oil	3·2 + 2·4	Improvement in clinical score with fish oil ($p < 0·05$) but not with placebo. Lower endoscopic and histological scores at 6 months with fish oil	[82]
Sulfasalazine-controlled, crossover study	2 months treatment and 2 months washout n = 10	Sulfasalazine 2 g per day	3·2 + 2·2	Increase in C-reactive protein, ESR and platelet count in the fish oil group. In spite of that endoscopic score improved with fish oil	[83]
Ulcerative colitis (DB, PC, P)	6 months n = 51	Sunflower oil	5·6 g n-3 PUFA/day	Clinical and endoscopic improvement in the EFA group	[84]
Ulcerative colitis (DB, PC, P)	6 months n = 121	Liquid supplement based on sucrose alone	Nutritional liquid supplement with <2·5 g EPA and <1·0 g DHA per day (plus prebiotics and antioxidant micronutrients	Improvement in clinical, endoscopic and histological indices. Faster reduction in steroid dose with active therapy	[85]
Ulcerative colitis (DB, PC, P)	12 months n = 87	20 ml/d of olive oil	20 ml/d (5·0 g EPA, + 2·1 g DHA)	EPA decreased serum leukotriene B4 levels, reduced the need for corticosteroids but only modestly improved clinical parameters	[86]
Ulcerative colitis (DB, PC, P)	24 months n = 64	Corn oil	5·1 g n-3 PUFA per day	n-3 FAs demonstrated little long-term benefits as assessed by colonoscopy, histology	[87]
Ulcerative colitis (DB, PC, P)	12 months n = 50	20 ml/day olive oil	20 ml/day (3·2 g EPA + 2·2 g DHA)	No effect on relapse rate.	[88]
Ulcerative colitis (DB, PC, P)	12 months n = 63	Sunflower oil	EFA capsules: 1·62 g GLA*, 0·27 g EPA, 0·05 g DHA per day	55 % relapse rate with EFA versus 38 % with placebo	[89]
Crohn's disease (DB, PC, P)	9 weeks n = 31	Nutritional supplement with 7·8 g Linoleic acid (n-6 FA) per day	Nutritional supplement enriched with 3·0 g n-3 FA (EPA, DHA, ALA), 11·4 g L-Arginine, and 1·2 g RNA per day	Significant decreases in CDAI and C-reactive protein in active and control groups	[90]

(continued)

Table 11.3 (continued)

Disease (Study and design)	Duration of study and no. of patients	Placebo	Dose of EPA and DHA (g/d)	Clinical outcomes that improved with intake of n-3 PUFAs	Ref.
Crohn's disease (DB, PC, P)	9 weeks n = 41	Elemental diet with 0·4 % of energy as n-3 FA (ALA) and 5·4 % as n-6 FA	Polymeric diet with 1·5 % of energy as n-3 FA (ALA) and 3 % as n-6 FA (LA)	71 % remission rate with active versus 70 % with control formula	[91]
Crohn's disease (DB, PC, P)	12 weeks n = 78	Enteric-coated MCT capsules with 2·7 g caprylic and 1·8 g capric acid per day	Enteric-coated capsules with 1·8 g EPA/day + 0·9 g DHA/day	28 % relapse rate with fish oil versus 69 % with placebo	[92]
Crohn's disease (DB, PC, P)	12 weeks n = 135	Corn oil (All patients on a high-fiber, low arachidonic acid diet)	Fish oil capsules: Omega-3 PUFA (3·3 g EPA and 1·8 g DHA per day) as ethyl esters	57 % relapse rate with fish oil versus 55 % with placebo	[93]
Crohn's disease (DB, PC, P)	12 months n = 50	Not described	Fish oil capsules: Enteric-coated capsules with 1·8 g EPA/day and 0·9 g DHA/day	8 % clinical recurrence rate with fish oil versus 21 % with placebo	[94]
Crohn's disease (DB, PC, P)	12 months n = 38 pediatric patients	Olive oil	Fish oil capsules: Enteric-coated capsules with 1·2 g EPA and 0·6 g DHA per day	61 % relapse rate with fish oil versus 95 % with placebo	[94]
Crohn's disease (DB, PC, P)	52 weeks n = 363 adult patients	Enteric-coated capsules with MCT (4 g/d)	Enteric-coated capsules with 2·0–2·4 g EPA and 0·6–1·0 g DHA per day	31·6 % relapse rate with fish oil versus 35·7 % with placebo	[95]
Crohn's disease (DB, PC, P)	52 weeks n = 375 adult patients	Enteric-coated capsules with MCT (4 g/d)	Enteric-coated capsules with 2·0–2·4 g EPA and 0·6–1·0 g DHA/day	47·8 % relapse rate with fish oil versus 48·8 % with placebo	[96]
Ulcerative colitis	12 weeks n = 6	Not described	3–4 g/d EPA	Lower levels of LTB4	[97]
Ulcerative colitis	8 weeks n = 10	Not described	2.7 g/d EPA 1.8 g/d DHA	Amelioration of disease markers	[98]
Crohn's disease	2 years n = 38	Not described	100–250 g fish	Remissión of major symptoms	[99]
Ulcerative Colitis (PC)	6 months n = 43	Primrose oil/olive oil	Max-EPA	EPA produced minimal changes in clinical outcome as assessed by sigmoidoscopy, rectal biopsy, and symptoms	[100]

DB Double blind, *PC* Placebo controlled, *CO* Crossover, *P* Parallel, *DHA* Docosahexaenoic acid, *EPA* Eicosapentaenoic acid, *GLA* Gamma Linolenic acid, *PUFA* Polyunsaturated fatty acid, *LTB4* Leukotriene B4; *DAI* Disease activity index, *ESR* Erythrocyte sedimentation rate, *TB3* Thromboxane B3, *NSAIDs* Non-steroidal anti-inflammatory drugs *MCT* Medium chain triglycerides, *CDAI* Crohn's disease activity index

Role of Omega-3 Fatty Acids in Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). The target cells in the pathogenesis of MS are oligodendrocytes, the myelin-forming cells of the CNS. At present, the cause of onset of MS is unknown, but activated T cells and macrophages are thought to be involved in demyelination through various mechanisms. The important pathological mechanisms involved in MS include immune-mediated inflammation [117], oxidative stress [118–120], and excitotoxicity [121]. These mechanisms

Table 11.4 Overview of clinical outcomes in studies using n-3 PUFAs in patients with asthma

Study and design	Duration of study and no. of patients	Placebo	Dose of EPA and DHA (g/d)	Clinical outcomes that improved with n-3 PUFA supplementation	Ref.
Parallel RCT	10 week n = NR	Olive oil placebo	3.2 g/d EPA + 2.2 g/d DHA	Improved PEK	[107]
Parallel RCT	8 week n = 23	600 mg/d olive oil	200 mg/d EPA/DHA + 400 mg/d	There was a significant decrease in daytime wheeze, the concentration of exhaled H_2O_2 and an increase in morning PEF in the lipid extract group compared to the placebo group	[108]
Parallel RCT	8 week (n = 6)	0.1 g/d EPA ethyl ester (+trace DHA)	4.0 g/d EPA ethyl ester (+trace DHA)	None identified	[109]
Crossover RCT	10 week (n = 15)	15 g/d olive oil placebo	2.7 g/d EPA + 1.8 g/d DHA	None identified	[110]
RCT	4 week (n = 7)	10–20 g/d corn oil	10–20 g/d Perilla seed oil (ALA: NR)	Supplementation of Perilla seed oil-rich diet suppressed generation of LTB4 and LTC4 by leukocytes and improvement of pulmonary function	[111]
Crossover RCT	10 week (n = 36)	20 ml/d olive oil/20 ml/d evening primrose oil	20 ml/d fish oil (EPA + DHA)	Moderate doses of evening primrose oil or fish oil are ineffective as a supplementary treatment of bronchial asthma	[112]
Parallel RCT	26 week (n = NR)	Olive oil placebo (dose: NR)	3.2 g/d EPA + 2.2 g/d DHA	None identified	[113]
Parallel RCT	26 week (n = NR)	n-6: 1.8 g/d safflower oil + 1.8 g/d palm oil + 0.4 g/d olive oil + sunflower diet (dose: NR)	0.72 g/d EPA + 0.48 g/d DHA + ALA (dose: NR) via canola diet	None identified	[114]
Parallel RCT	10 month (n = 15)	300 mg/d olive oil placebo (n = 15)	17.0–26.8 mg/kg/d EPA; 7.3–11.5 mg/kg/d DHA (300 mg/d fish oil)	Decreased asthma symptom scores; decreased bronchial hyper-responsiveness to acetyl choline challenge	[115]
Parallel RCT	9 month (n = NR)	"Placebo" (type and dose: NR)	1.0 g/d EPA + DHA	Improved FEV_1	[116]

DB Double blind, *PC* Placebo controlled, *CO* Crossover, *P* Parallel, *RCT* Randomized controlled trials, *DHA* Docosahexaenoic acid, *EPA* Eicosapentaenoic acid, *ALA* Alpha-linolenic acid, *GLA* Gamma-linolenic acid, *PUFA* Polyunsaturated fatty acid, *LTB4* Leukotriene B4, *LTB4* Leukotriene C4, *TB3* Thromboxane B3, *NR* Not reported; *FEV_1* Forced expiratory volume at 1 s, *PEK* Peak expiratory flow

may all contribute to oligodendrocyte and neuronal damage and even cell death, hence promoting disease progression.

At present, no therapy exists that can confer prolonged remission in MS and therapeutic agents are only partially effective. Their long-term beneficial effects are uncertain and often detrimental side effects have been reported [122, 123]. In a recent survey, 37 % of 1,573 patients with MS revealed that they had used omega-3 unsaturated fatty acids at some point in their lives [124]. Several small studies have demonstrated a reduction in PUFA content in serum, cerebral white matter, erythrocytes, and lymphocytes in patients with MS compared with controls [125–128]. However, these observations do not help to clarify the exact nature of the relationship between PUFA intake and MS, as no data were provided on the dietary habits and clinical characteristics of the study participants.

In an attempt to provide a proper assessment of the efficacy of PUFA supplementation in MS, multiple controlled studies have been performed, some of which date back to the 1970s. These studies, however, generally produced inconclusive results. The results of the controlled trials performed to date are summarized in Table 11.5.

Summary and Conclusion

Inflammation is the root cause of a number of degenerative diseases such as rheumatoid arthritis, inflammatory bowel disease, asthma, multiple sclerosis, and atherosclerosis. Although steroidal anti-inflammatory drugs (SAID) and non-steroidal anti-inflammatory drugs (NSAIDs) are used effectively to manage the acute inflammatory reaction, their

Table 11.5 Overview of clinical outcomes in studies using n-3 PUFAs in patients with multiple sclerosis

Study and design	Duration of study and no. of patients	Dietary	Dose of EPA and DHA (g/d)	Clinical outcomes	Ref.
DB, P	24 months n = 87 patients with DSS scores from 0–6	Oleic acid (7.6 g/day) emulsion	Linoleic acid (17.2 g/day) emulsion	Significant improvement in relapse severity and nonsignificant trend toward lower annualized relapse rates in the linoleic acid group; no differences in disability between the two groups	[129]
DB, P	24 months n = 152	Oleic acid (4.8 ml/day)capsules and oleic acid (4 g/day) spread	Four treatment arms: linoleic acid (0.36 g/day) + GLA (3.42 g/day) capsules and linoleic acid (11.5 g/day) spread	No significant differences in disability (measured on the DSS), relapse rates or relapse severity score among the four groups	[130]
DB, P	24 months N = 116 patients with relapsing MS	Oleic acid (4.0 g/day) capsules and oleic acid (16 g/day) spread	Four treatment arms: linoleic acid (0.34 g per day) + GLA (2.92 g/day) capsules and linoleic acid (23 g/day) spread	Linoleic acid plus linolenic acid group had briefer and less-severe relapses compared with placebo group, but accumulated more disability than placebo group	[131]
DB, P	30 months n = 96 patients with relapsing and progressive MS	Oleic acid 21 g/day	Linoleic acid 17 g/day	No differences in disability, rates, or severity of relapse, or timed functional tests between the two groups; significant increase in serum concentrations of linoleic acid in the active arm	[132]
DB, P	24 months, n = 312 patients with relapsing MS	Oleic acid (7.2 g/day) capsules	Fish oil (mixture of EPA 1.71 g/day and DHA 1.14 g/day) capsules	Fish oil group showed a nonsignificant trend toward less disability progression	[133]
DB, P	12 months n = 31 patients with relapsing MS	Oleic acid (1.0 g/day) capsules	Fish oil (EPA 1.98 g/day and DHA 1.32 g/day) capsules	No differences seen in relapse rates between the two groups; fish oil group had improvements in quality-of-life measures	[134]
DB, P	18 months n = 36 patients with active MS	Polyethylene glycol	High-dose GLA (14 g/day) versus low-dose GLA (5 g/day)	High-dose GLA group had significantly reduced relapse rates and disability progression (measured on the expanded DSS) compared with low-dose GLA and placebo groups	[135]

DB Double blind, *P* Parallel, RCT, *DHA* Docosahexaenoic acid, *EPA* Eicosapentaenoic acid, *ALA* Alpha-linolenic acid, *GLA* Gamma-linolenic acid, *PUFA* Polyunsaturated fatty acid, *LTB4* Leukotriene B4, *LTB4* Leukotriene C4, *TB3* Thromboxane B3, *NR* Not reported, *MS* Multiple Sclerosis, *DSS* Disability Status Scale

use for chronic inflammation is followed by severe adverse effects. This has given an impetus to search for alternate natural and safe anti-inflammatory agents.

The knowledge that dietary nutrients can act as drugs for ameliorating disease captured attention of researchers to figure out the active component of the particular dietary source responsible for protective effect. Population studies revealed the anti-inflammatory and cardioprotective effects of omega-3 fatty acids, with subsequent clinical studies (prospective randomized placebo-controlled trials) supporting their therapeutic role in chronic inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease, asthma, and cardiovascular disease.

Omega-3 fatty acids act by increasing production of anti-inflammatory eicosanoids and inflammation resolving resolvins from EPA and DHA, downregulating adhesion molecule expression on leukocytes and on endothelial cells, reducing intercellular adhesive interactions and production of proinflammatory cytokines induced via the NFkB system and decreasing chemotactic responses of leukocytes.

The supplementation trials in patients with rheumatoid arthritis appear to be the most successful with most trials reporting several clinical benefits. In most other inflammatory diseases and conditions, there are either too few studies or unequivocal results to draw a clear conclusion of the possible efficacy of omega-3 fatty acids as a treatment. Hence, additional studies are needed to conclude about the effective dosage and duration of omega-3 fatty acid administration for best possible clinical benefit in a particular inflammatory condition.

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Omega-3 Fatty Acids in Cancer: Insight into the Mechanism of Actions in Preclinical Cancer Models

12

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Abbreviations

ALX/FPR2	Lipoxin A4 receptor/formyl peptide receptor 2 (ALX/FPR2)
AP1	Activator protein 1
Bak	Bcl-2 homologous antagonist killer
BAX	Bcl-2-associated X protein
bcl-2	B-cell lymphoma 2
BCRP	Breast cancer resistance protein
bFGF	Basic fibroblast growth factor
BLT1	Leukotriene B4 receptor 1
Bv8	Prokineticin-2
CCL2	Chemokine (C–C motif) ligand 2
CCR5	C–C chemokine receptor type 5
ChemR23	Chemerin *Receptor* 23
COX	Cyclooxygenase
cPLA2	Cytosolic phospholipases A2
CXCL12	C–X–C motif chemokine 12
CYP450	*Cytochrome P450*
DCA	Dichloroacetate
DHA	Docosahexaenoic acid
DRM	Detergent-resistant membranes
EDPs	Epoxydocosapentaenoic acids
EETs	Epoxyeicosatrienoic acids
EEQs	Epoxyeicosatetraenoic acids
eFoX	Electrophile oxo-derivative
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFR	Epithelial growth factor receptor
EPA	Eicosapentaenoic acid
EMT	Epithelial–mesenchymal transition
ER	Estrogen receptor
ETC	Electron transport chain

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FA	Fatty acids
FASN	Fatty acid synthase
GPR120	G protein-coupled receptor 120
GPR32	G protein-coupled receptor 32
HEPEs	Hydroxyeicosapentaenoic acids
HER-2	Human epidermal growth factor receptor 2
HETEs	Hydroxyeicosatetraenoic acids
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
HMGB1	High-mobility group box-1
HMGCoAR	3-hydroxy-3methylglutaryl-coenzyme A reductase
HUVECs	Human umbilical vein endothelial cells
HEPE	Hydroxyeicosapentaenoic acid
ICAM-1	Intercellular adhesion molecule 1
IGF-1R	Insulin-like growth factor 1 receptor
IL-1	*Interleukin 1*
IL-10	*Interleukin 10*
IL-6	*Interleukin 6*
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinas
LA	Linoleic acid
LC-PUFA	Long-chain polyunsaturated fatty acid
LOX	Lipoxygenase
LTB4	Leukotriene B4
MAPKs	Mitogen-activated protein kinases
MCP-1	Monocyte chemotactic protein 1
M-CSF	Macrophage colony-stimulating factor
MMPs	Matrix metalloproteases
MRPs	Multidrug resistance-related proteins
MTP	Mitochondrial permeability transition
w-3	Omega-3
w-6	Omega-6
NF-kB	Nuclear factor-kappaB
NO	Nitric oxide
nrf2	Nuclear factor (erythroid-derived 2)-like 2
OSM	Oncostatin M
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
Pgp/ABCB1	P-glycoprotein/ATP-binding cassette subfamily B member
PI3K/mTOR	Phosphoinositide 3-kinase/mammalian target for rapamycin
PKC	Protein kinase C
PMN	Polymorphonuclear
PPARγ	Peroxisome proliferator-activated receptors
PPREs	PPAR response elements
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SOCI	Store-operated calcium influx

SREBP1	Sterol regulatory element-binding protein-1
STAT-3	Signal transducer and activator of transcription 3
TAK1	Transforming growth factor β-activated kinase-1
TAM	Tumor-associated macrophages
TAN	Tumor-associated neutrophils
TCA cycle	Tricarboxylic acid cycle
TGF-β	Transforming growth factor-beta
TNF α	*Tumor necrosis factor-alpha*
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
ALA	α-linolenic acid

Introduction

Cell–cell interaction, cell–extracellular matrix interaction, and proliferative and anti-proliferative signals such as tumor suppressor proteins are critical in tissue homeostasis and cellular quiescence. For normal cell to proliferate, stimulatory signals are essential which are activated by growth factors, extracellular matrix components, and cell–cell interaction molecules [1]. In addition to this, a mammalian cell has limited capacity to proliferate with some exceptions such as stem cells and germ cells which are known as Hayflick limit. When cells grow old or insulted by physicochemical stimuli, they enter senescence phase, dead cells are removed, and new cells take their place. When this regulated cascade is disturbed, uncontrolled cell growth leading to cancer may arise. Majority of the cancers are linked with somatic mutations in proto-oncogenes and/or tumor suppressor genes and/or DNA repair genes. Cancers are also linked with environmental factors. Cancer-causing environmental exposures include substances, such as the chemicals in tobacco smoke, and radiation, such as ultraviolet rays from the sun. Among various cancer-causing factors, chronic inflammation has been shown to play major role [1–3]. Besides being pro-tumorigenic, inflammatory factors also influence immune response toward tumors.

Cellular functions such as membrane structure and fluidity, cellular metabolism and energy production, cell signaling, and cellular interactions are attributed to fatty acids [1, 4]. Dietary inclusion of w-3 FA from marine and/or plant origin has beneficial effect on various health conditions. Omega-3 fatty acids (w-3 FAs) are known to help reduce the risk of heart disease, lower triglycerides, and relieve inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease. Emerging evidence from both experimental and epidemiological studies suggests w-3 FAs may play a role in cancer prevention [5]. Several clinical and preclinical studies have pointed out that w-3 FA consumption is associated with decreased cancer risk of the breast, prostate, colon, and kidneys [6]. The main FAs in this group are α-linolenic acid (ALA), and the long-chain FAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Like linoleic acid (LA, parent FA from w-6 PUFA family), ALA is an essential FA for humans, as it is required for normal function and body is unable to make it. EPA and DHA can be synthesized in the body from ALA. LA and ALA are converted to w-6 (AA) and w-3 (EPA and DHA) LC-PUFAs, respectively, by series of reactions conducted by desaturases and elongases in endoplasmic reticulum. Both these pathways share same set of enzymes for the conversion to respective PUFA leading to competition between w-6 and w-3 PUFAs for their metabolic conversion [1]. Synthesis of DHA takes place in peroxisomes by β-oxidation of 24:6w-3.

Experimental data show that w-3 PUFAs may act on the hallmarks of cancer cells, including the newly added criteria by Hanahan and Weinberg [7]. Evidence accumulated from numerous experimental systems indicates that w-3 FAs may exert an antitumor action by altering the cell membrane phospholipid composition and, consequently, affecting the expression and function of numerous receptors, proteins, and lipid-derived signaling molecules.

Various mechanisms have been explained for the effect of w-3 PUFA's beneficial effect in cancer [8]. Here we will look into major mechanisms involved in anticancer effect of w-3 PUFA in cancer.

Role in Lipid Peroxidation

Among many anti-cancer mechanisms studied for w-3 PUFA, major one is lipid peroxidation [9–11]. w-3 PUFAs get incorporated into the tumor cell membranes, which results in the increased susceptibility of these cells for lipid peroxidation and accumulation of lipid peroxidation products (reactive lipid hydroperoxides) to cytostatic or cytotoxic levels.

Oxidative damage is key mechanism for many known chemotherapeutic agents such as doxorubicin and mitomycin. Supplementing the diet of tumor xenograft-bearing

mice with w-3 PUFAs results in increased efficacy of these chemotherapeutic agents. The increased efficacy of these drugs has been assigned to increase the susceptibility of tumor cell membranes to lipid peroxidation [12].

w-3 PUFA-promoted cell death is mainly through ROS-induced apoptosis as shown in various cell lines where intracellular ROS levels were elevated and treatment with antioxidants abrogates effect of w-3 PUFA-induced cell death. Cell death is mainly via apoptosis where caspases 8, 3, and 9 are shown to execute the death process in human pancreatic cancer cells [13].

In vivo xenograft data support the conclusion that EPA-induced cell death is mainly mediated through the formation of intracellular ROS where animals were fed with fish oil diet and 3-nitroso-cysteine was used as a marker for protein nitration and cellular oxidative stress in vivo [13].

Role in Mitochondrial Membrane Lipid Peroxidation

Epidemiological data suggest that while high levels of saturated fatty acids are positively associated with cancer risk, w-3 FAs seem to prevent cancer [14–17].

Protective action of w-3 FAs against cancer has also been supported by in vitro and in vivo studies. Consumption of dietary fiber enhances butyrate production resulting in enhanced protective action [18, 19]. Due to enhancement of unsaturation of mitochondrial phospholipids, especially cardiolipin, w-3 FAs prime butyrate to initiate apoptotic cascade. So there is a synergy between w-3 FAs and butyrate.

The role of DHA in mediating mitochondrial membrane lipid oxidation causing apoptosis in colonocytes has been elucidated [20]. It has been observed that (i) DHA and butyrate synergistically enhance lipid oxidation, (ii) mitochondrial-targeted antioxidant protects cells from oxidative stress, (iii) DHA enhances mitochondrial membrane potential, and (iv) mitochondrial-targeted antioxidant suppresses butyrate-induced apoptosis.

DHA, via mitochondrial ROS over production, simultaneously induces autophagy and apoptosis in cancer cells [21]. Autophagy and apoptosis are self-destructive processes that share many key regulators. Cytotoxicity is restricted to w-3 PUFAs as arachidonic acid had no effect. DHA incorporation into mitochondrial membrane phospholipids and enhanced mitochondrial lipid oxidation and ROS generation seems to be contributing to the antitumor activity of w-3 PUFAs. Mitochondrial ROS generation induced by w-3 PUFAs that inhibit PI3K/mTOR may be responsible for the autophagy and apoptosis.

There is evidence that dietary fiber that increases butyrate levels in colon is protective against colorectal cancer [22]. It has also been shown that butyrate and omega-3 fatty acids work coordinately and protect against colon tumorigenesis which is primarily by increasing apoptosis [23]. Interestingly, it has been shown that DHA alters colonocyte mitochondrial membrane composition, thereby creating permissive environment for apoptosis [19]. Further, it has also been shown that mitochondrial lipid oxidation products, membrane phospholipid-derived hydroperoxides (LOOH), play an important role in DHA and butyrate-induced apoptosis [24].

It is known that finely tuned intracellular homeostasis and compartmentalization of Ca^{2+} can lead to cell death. It is now recognized that mitochondria play a key role in both apoptosis and necrosis by regulating intracellular Ca^{2+} homeostasis, activation of caspases, and the release of ROS [25]. Mitochondria can be regarded as critical checkpoint in Ca^{2+} signaling, and in certain situations mitochondrial Ca^{2+} accumulation is a trigger for cytochrome C release and the induction of apoptosis [26]. DHA and butyrate synergistically induce colonocyte apoptosis by enhancing mitochondrial Ca^{2+} accumulation [23]. DHA along with butyrate enhances ROS and LOOH production and causes a change in mitochondrial transition permeability. DHA amplifies the butyrate action up to 43 %. DHA alone, however, has no significant action. The synergistic action of enhancement of apoptosis is possibly due to Ca^{2+}-mediated intrinsic mitochondrial pathway [27].

Cancer cell mitochondria are hyperpolarized [28], along with decreased levels of mitochondria-derived ROS and also decreased expression of Kv channels. The decreased ROS production can close redox-sensitive MTP. Increased hexokinase levels support the compensatory aerobic glycolysis in cancer cells. In summary, hyperpolarization of mitochondria, blocking entry of pyruvate into mitochondria, increased glucose uptake, and aerobic glycolysis are some of the measures taken by the cancer cells to avoid apoptosis. Targeting mitochondria by modifying mitochondrial membrane composition by omega-3 fatty acids, depolarization of the mitochondrial membrane to allow entry of pyruvate into mitochondria, restoring normal TCA cycle and apoptotic pathways, can be a very effective strategy to treat all types of cancer. In this respect, there exists a lot of excitement about dichloroacetate (DCA). DCA reverses cancer cell abnormal metabolism from aerobic glycolysis to glucose oxidation by reducing the activity of mitochondrial pyruvate dehydrogenase kinase 1 (PDK1). DCA reduces high mitochondrial membrane potential and increases mitochondrial reactive oxygen species (ROS) in malignant, but not in normal cells.

Figure 12.1 represents various key modifications or adaptations cancer possesses to avoid apoptosis.

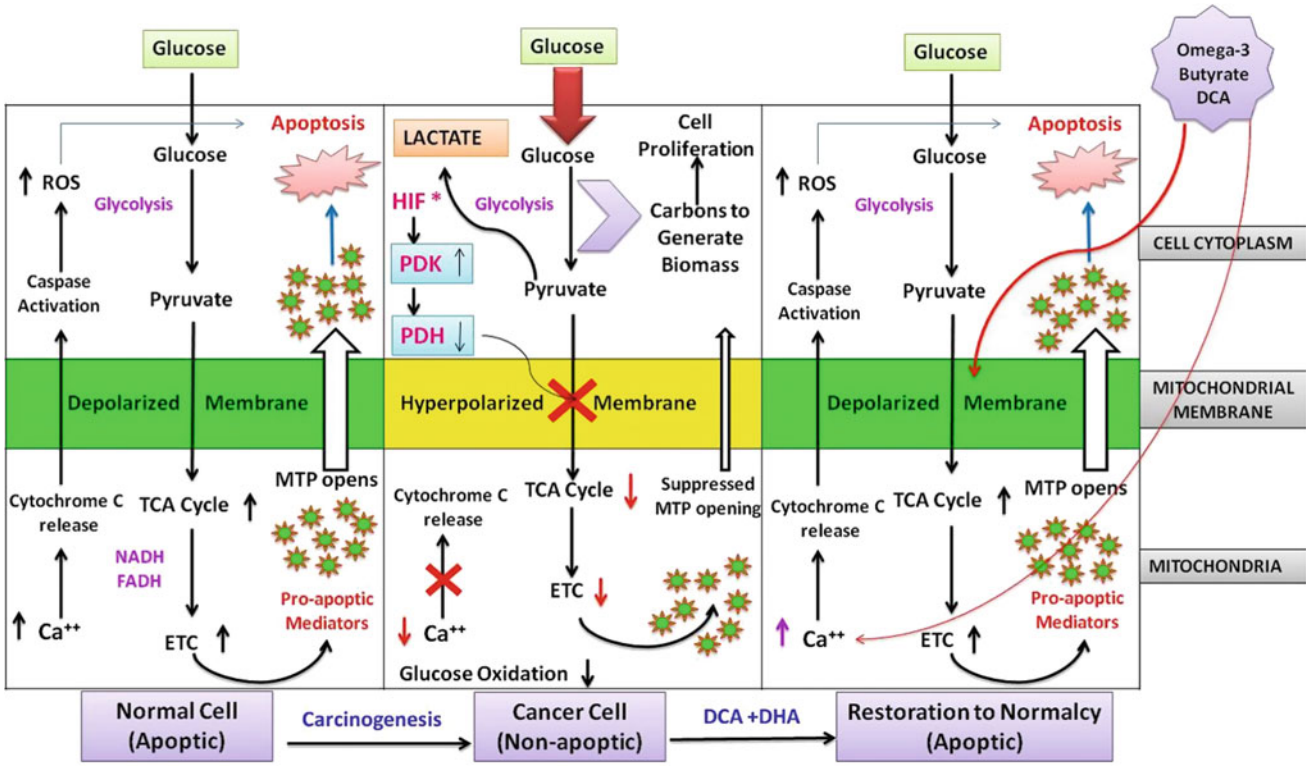

Fig. 12.1 Illustration depicts various strategies of cancer cells to avoid apoptosis. Mitochondrial hyperpolarization, aerobic glycolysis in association with upregulation of hexokinase, and inhibition of PDH. Various agents that target these modifications alone or in combination resulting in cancer cell apoptosis are marked in bold letters

Role in Inflammation

Inflammation has been shown to play very crucial role in tumorigenesis [29] and is involved in the every single step of tumorigenesis, right from tumor initiation, tumor promotion to metastatic progression. Various types of immune and inflammatory cells are frequently present within tumors. Microenvironment of the tumor is influenced by these cells through the production of cytokines, chemokines (such as TNF α, IL-1, and IL-6), growth factors, prostaglandins, and reactive oxygen and nitrogen species. Most of the tumors trigger inflammatory cascade that in turn modulates tumor microenvironment in favor of cancer cells [29]. Signaling pathways involved in the pro-tumorigenic effects of inflammation are often subjected to a feed-forward loop in tumor cells. For example, activation of NF-kB in immune cells induces production of cytokines that activate NF-kB in cancer cells to induce chemokines that attract more inflammatory cells into the tumor [30]. The cytokine IL-6 promotes oncogenesis by stimulation of NF-kB and STAT-3 signaling [31].

Transcription factors, NF-kB and STAT3, are at the center stage in various inflammatory processes as they control various cytokines involved in the inflammatory process [32]. Both these transcription factors are shown to be involved in the tumorigenesis and are considered cancer therapy targets [2]. Figure 12.2 represents the impact of inflammation on tumor initiation and progression.

w-6 and w-3 PUFAs are stored in the phospholipids of the cytosolic leaflet of cell and organelle membrane in esterified form and can be mobilized by phospholipase A_2 [33]. Free or membrane-released FAs are acted upon by cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome p450 (CYP450). Resulting metabolites are known as eicosanoids, which act as short-lived hormone-like lipids. These eicosanoids are involved in the various processes such as platelet aggregation, cellular growth, and cell differentiation [6]. As mentioned earlier, there exists competition between w-3 and w-6 PUFAs for desaturases and elongases [34]. The eicosanoids from w-6 PUFA (AA) are pro-inflammatory (series 2 prostaglandins and thromboxanes, and series 4 leukotrienes and lipoxins) while those derived from w-3 PUFA and CYP450 metabolites of w-6 PUFA (series 3 prostaglandins and thromboxanes, E-series and D-series resolvins, series 5 leukotrienes, EEQs, HEPEs, merlines, EDPs, EETs, nHETEs, and DiHETEs) are considered anti-inflammatory or immunologically less reactive. That is why, a higher level of ALA results in reduced

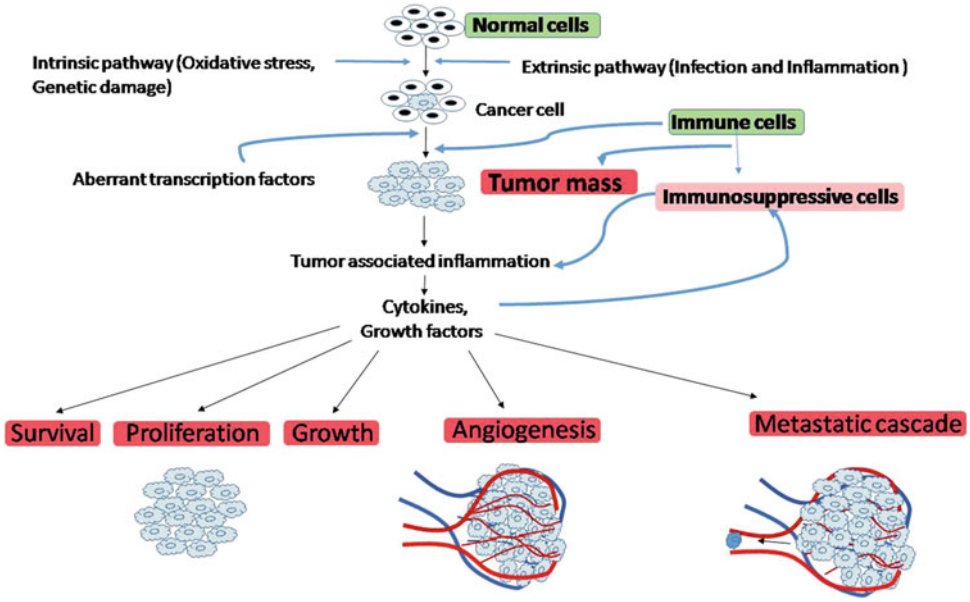

Fig. 12.2 Tumor cell mass and tumor-associated various cells such as stromal cells, tumor-associated macrophages show feed-forward association resulting in the microenvironment that will allow sustained survival, continuous proliferation, angiogenesis, and metastasis

synthesis of AA from LA, in turn resulting into low levels of pro-inflammatory AA eicosanoids [35].

NF-kB mediates inflammatory process by controlling cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [32]. COX2 [36–40] and/or iNOS levels are elevated in many cancer types [41]. PGE2 (AA metabolite of COX) is shown to induce tumor cell proliferation. PGE2 stimulates the expression and activation of aromatase enzyme which coverts androgens to estrogens. A significant correlation has been established between aromatase and cyclooxygenase enzyme systems at gene and protein levels indicating autocrine and paracrine mechanisms may be involved in hormone-dependent cancer development via growth stimulation from local estrogen biosynthesis [42, 43]. A strong epidemiological evidence associates the estrogen to breast, endometrial, and uterine cancers [44]. 3-series and 5-series prostaglandins and leukotrienes derived from EPA and DHA are shown to have anti-inflammatory activity. These are shown to reduce PGE2 production by 60 % and LTB4 by 75 % in human peripheral blood mononuclear cells [45]. Thus, increasing w-3 PUFA levels with lowering w-6 levels may play important role in managing tumor-associated inflammation.

Apart from inhibiting expression of COX-2 and iNOS target genes, DHA treatment in Caco-2 cell line results in simultaneous reprogramming of genes involved in differentiation such as $p21^{(Waf1/Cip1)}$, p27, and apoptosis by activating caspases such as caspase-3 [46].

Supplementation of w-3 PUFA to neuroblastoma cells reduced the AA-derived eicosanoids [8, 47]. In these cells, DHA and its metabolites induced apoptosis by intrinsic pathway. 17-hydroperoxy-DHA showed the highest cytotoxic potency. Additive or synergistic effect has been seen with DHA and COX-2 inhibitor celecoxib in neuroblastoma cells [48] and other human cancer cell lines [49]. Mechanism seems to be COX2-dependent (inhibiting AA metabolism by blocking COX-2) and COX-2-independent (modulation of heat shock proteins and NF-kB activity and steroid receptors) [1, 49].

In cultured pancreatic cells and human monocytes, w-3 PUFAs inhibit NF-kB activity directly. Inhibitory effect is mediated by decreased degradation of IkB, inhibitory subunit of NF-kB [30, 33]. w-3 FAs are converted by COX-2 into OH derivatives, which are then metabolized to eFoX. eFoX inhibits pro-inflammatory responses and induces nrf2-dependent gene expression (nrf2 regulates major antioxidant pathways including glutathione) [50] and PPARγ activation and adduct to protein and glutathione [51].

In colon xenograft model, COX-2 has been shown to decrease apoptosis by modulating expression of bcl-2 gene expression. In this model, tumor growth was restricted by w-3 PUFA supplementation through downregulation of COX-2. In prostate tumor model, beneficial effect is mediated by downregulating bad phosphorylation resulting in the upregulation of tumor cell apoptosis [1].

Tissue-associated macrophages play important role in the inflammatory cascade. Macrophages express GPR120, an orphan G protein-coupled receptor which works as a w-3 FA receptor or sensor. In the Toll-like receptor signaling pathway and TNFα inflammatory pathway, TAK1 phosphorylation is essential for downstream JNK and NF-kB pathway activation as TAK1 is converging point. Binding of DHA to GPR120 results in the inhibition of TAK1 phosphorylation and inhibition of downstream NF-kB and JNK activation in β-arrestins-dependent manner [52]. Both these w-3 PUFAs inhibit NF-kB activation and TNF-α secretion in macrophages.

An opposing effect of EPA was reported by Wu et al. In contrast to the inhibitory effect of w-3 PUFA in prostate

cancer, FFA4 (GPR120) and its agonist EPA have oncogenic role in colon cancer. In Caco-2 colon cancer cell line, EPA activates p70S6K through FFA4-mediated signaling [53].

Enzymes 5-LOX, 15-LOX, or acetylated COX act on the EPA and/or DHA to produce E-series and D-series resolvins. Protectins from DHA are also formed by these enzymes. Discovery of resolvins and protectins in 2002 helped to understand the mechanism underlying the w-3 PUFA beneficial effects [9–11].

Lipoxins, resolvins, and protectins also increase the expression of CCR5 receptors on T cells and aging PMN, which help clearing local chemokine depots from the inflammatory site. Apoptotic neutrophils are then phagocytized by macrophages, leading to neutrophil clearance and release of anti-inflammatory and inflammation resolution cytokines such as transforming growth factor-beta1 [54, 55].

Best studied eicosanoid from w-3 PUFAs is EPA-derived resolvin E1 (RvE1). In colorectal cancer cell lines, RvE1 shows anticancer activity by inhibiting NF-kB inflammatory pathway through G protein-coupled receptors ChemR23 and BLT1 [56, 57].

Role in Physicochemical Properties of Lipid Rafts and Signal Transduction

Lipid rafts are important membrane domains for cell signaling and drug accumulation inside the cell as they are enriched with many regulatory proteins, some growth factor receptors, and drug efflux pumps [58]. Lipid rafts are highly ordered and less fluid than the surrounding membrane due to presence of cholesterol and sphingolipids mainly sphingomyelin (containing saturated fatty acids) [58, 59]. Cholesterol maintains the microdomains of the lipid rafts. Supplementing growth medium with EPA or DHA results in the alteration in the structure and composition of the lipid rafts as cholesterol and sphingomyelin levels go down. Due to long carbon chain and unsaturated nature, incorporation of EPA or DHA results in disturbance in lipid raft organization and structure resulting in suppression of the raft-associated cell signaling [46, 60–64].

Wu et al. have shown that EPA and DHA activate neutral sphingomyelinase that hydrolyzes sphingomyelin to ceramide which has apoptotic function [65].

PUFAs act on membrane-associated signal transduction. Upregulation of pro-survival pathways such as EGFR, Ras/Raf, and PI3K/mTOR pathway is seen in many malignancies. Unsaturated FAs particularly w-3 PUFA [66] (mainly DHA) are able to modulate the activity of various intracellular signaling pathways mediated by calcium, protein kinase C (PKC), mitogen-activated protein kinases (MAPKs), epithelial growth factor receptor (EGFR), and Ras [1, 4].

Schley et al. have shown in the MDA MB231 cells that the incorporation of EPA or DHA in the lipid rafts results in the depletion of cholesterol followed by expulsion of EGFR from the microdomains and its phosphorylation. p38MAPK (downstream of EGFR) gets phosphorylated after EGFR phosphorylation. p38MAPK phosphorylation is associated with apoptosis in MDA MB231 cells [67].

Activation and overexpression of EGFR have been seen in many malignancies including breast cancer. In ER-ve breast cancer cell lines, DHA treatment results in inhibition of EGFR activation and slight decrease in the expression of EGFR. Inhibitory effect is mediated through caspase-3 or caspase-8 pathways [36, 60, 68, 69].

In MCF7 cell line, ALA alone was able to downregulate HER-2 expression by 79 % which was further confirmed by downregulation of expression of Ki-67 and PCNA [45].

Nuclear receptors, peroxisome proliferator-activated receptors (PPARs), function as ligand-activated transcription factors and regulate PPAR response elements (PPREs). These PPREs are found in regulatory regions of variety of genes involved in lipid metabolism and homeostasis, cell proliferation, cell differentiation, and inflammatory responses. PUFA and eicosanoids are naturally occurring PPAR ligands. In in vitro studies on breast cancer cell lines, PPARγ activation induces apoptosis via syndecan-1 [1, 70, 71]. In rats with induced mammary carcinogenesis, dietary supplementation with a low w-6/w-3PUFA ratio (1:14.6) was shown to increase PPARγ protein content, which was paralleled with a reduction of tumor burden. All these evidences indicate PPARγ activation is beneficial for controlling breast cancer and a potential role for PPARγ ligands (w-3 PUFA) in the treatment of breast cancer [45].

Ca^{+2} is a major intracellular factor playing role in signaling pathways that are involved in various cellular processes such as growth, proliferation, gene expression, contraction, secretion, and metabolism. Majority of these processes require sustained increase in the intracellular-free calcium. Store-operated calcium influx (SOCI) pathway governs the influx of calcium from extracellular matrix into the cells. In an aggressive thymoma rat models, w-3 PUFA-containing diet resulted in low levels of SOCI and reduced tumor growth [72].

Once taken up by cells or released from the cell membrane, DHA acts as a ligand to different nuclear receptors, such as the PPARγ and the RXR receptor. In conjunction with these receptors that act as transcription factors, DHA helps regulate various biological functions ranging from homeostasis and lipid metabolism to cell differentiation and cell death [6, 56, 73].

Figure 12.3 represents major cellular processes modulated by w-3 PUFA.

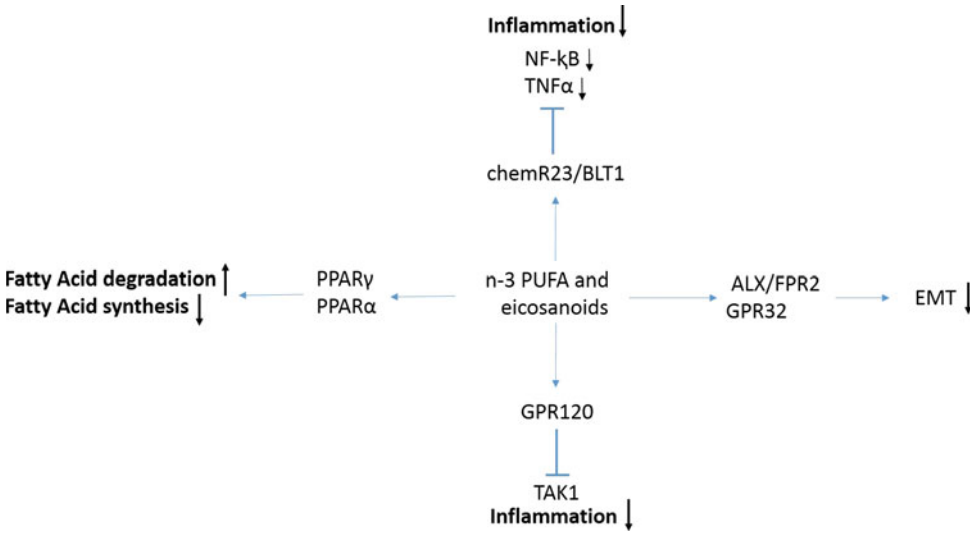

Fig. 12.3 w-3 PUFA metabolites, i.e., eicosanoids, modulate cellular processes by interacting with various receptors or altering signaling pathways. Overall, modulation of signaling pathways results in downregulating inflammatory cascade, enhanced fatty acid degradation in association with lowered fatty acid synthesis, and low expression of EMT markers ultimately resulting in the cell death

Role in Limitless Cell Proliferation

Limitless proliferation is one of the hallmarks of cancer cell. w-3 PUFAs, EPA, and DHA may inhibit mitosis by at least three different mechanisms such as both these w-3 PUFAs inhibit AA-induced activation of PKC and further mitosis, by down-regulating Ras and AP1 oncoproteins which are associated with many cancers and are known to stimulate mitosis and by inhibiting synthesis of pro-mitosis eicosanoids derived from AA. These mechanisms have been demonstrated in the breast and colon cancer cell lines. In colon cancer animal models, w-3 PUFA inclusion in the diet resulted into prevention of K-Ras mutation and Ras membrane expression [1, 74].

Role in Apoptosis

bcl2 family proteins regulate important mechanism of cell death, i.e., programmed cell death by apoptosis. Vast amount of clinical and preclinical data indicates that, in majority of cancers, expression of pro- or anti-apoptotic bcl2 family proteins is altered. These proteins promote or inhibit apoptosis by the release of effectors and executors from mitochondria.

In in vitro studies in breast cancer, colon cancer cell lines highlight that treatment with EPA and/or DHA shifts the balance between pro- and anti-apoptotic bcl2 proteins in favor of cancer cell death. EPA and DHA may downregulate anti-apoptotic bcl2 and Bcl-xL proteins and upregulate pro-apoptotic Bak and Bcl-xS protein level [1]. Both these FAs are shown to initiate apoptosis process by cytochrome C release from mitochondrial membrane and direct binding with $PPAR_{\gamma}$. Similar mechanisms were illustrated in the rat models of various cancers such as in colon cancer rat model, feeding animals with fish oil resulted into lower levels of bcl2 and apoptosis of cancer cells [75, 76].

Role in Adhesion and Angiogenesis

Adhesion and angiogenesis are first two critical steps in the tumor establishment. Cytoskeletal rearrangement is essential for cellular adhesion which is controlled by Rho GTPase. DHA has also been shown to decrease TNFα-induced monocyte rolling, adhesion, and transmigration. Probably by down regulating Rho GTPase, ICAM-1, and VCAM-1, DHA can inhibit adhesion [6].

Experimentally, it has been shown that, once the tumor is established, for a tumor to grow beyond the size 1–2 mm^3, substantial new blood vessel development is essential to cater the increasing nutrient demand [77]. As the tumor grows, core of the tumor becomes necrotic resulting into cell death and release of pro-inflammatory factors such as IL-1 and high-mobility group box-1 (HMGB1) which in turn trigger neo-angiogenesis [78, 79].

It is known that inflammation, hypoxia and mechanical stress may result in the endothelial cell activation or release of cytokines involved in abluminal sprouting. Basement membrane proteolysis for capillary sprouting is mediated by MMPs. EPA has been shown to downregulate MMP2 and 9. In in vivo experiments, similar findings were observed where mouse xenograft models for colon and breast cancer showed low tumor microvessel density and VEGF levels in EPA-fed mice against control group [77]

Tumor-associated macrophages secrete pro-inflammatory and pro-angiogenic factors which are directly regulated by NF-kB, STAT3, and AP1 [30]. Inhibition of NF-kB, STAT3, CCL2, CXCL12, and depletion of TAM result in reduced angiogenesis and tumor growth [80].

NO and COX-2 also regulate VEGF-mediated angiogenesis. Nitric oxide (NO) promotes tumor growth (by enhancing invasiveness), endothelial cell survival, and proliferation, and inhibits apoptosis. Inducible nitric oxide synthase (iNOS)

stimulates NO production which in turn increases PGE2 production, which is implicated in tumor growth and progression. EPA and DHA have been demonstrated to inhibit NO production and iNOS expression in murine macrophages and downregulate NO and nuclear factor-kappaB (NF-kB) in human colon cancer cell lines.

DHA inhibits angiogenesis probably by decreasing levels of VEGF, PDGF, and platelet-derived endothelial cell growth factor [6].

In CYP450-mediated metabolism, epoxyeicosatrienoic acids (EETs) are derived from w-6 AA and epoxydocosapentaenoic acids (EDPs) from w-3 DHA. In terms of angiogenesis and cancer, EETs are pro-angiogenic and have been shown to accelerate tumor growth and metastasis by stimulation of tumor angiogenesis. In angiogenesis model, Matrigel plug assay where inducers were VEGF and bFGF, EDP inhibited vascularization. One of these EDP metabolites, 19,20-EDP dramatically inhibited endothelial tube formation after a 6-h treatment in human umbilical vein endothelial cells (HUVECs) by inhibiting migration of the HUVEC cells. 19,20-EDP directly targets endothelial cells to suppress angiogenesis, primarily via suppression of endothelial cell migration. The 19,20-EDP also inhibited the activity of matrix metalloproteinase 2 (MMP-2) but with a weak activity. 19,20-EDP inhibited angiogenesis via blocking VEGF–VEGFR2 signaling. Overexpression of CYP epoxygenases in cancer cells or endothelial cells accelerates tumor growth and metastasis, which are largely attributed to AA-derived EETs [81, 82]. Thus, lowering the ratio of w-6/w-3 FA may result in the lowered synthesis of pro-inflammatory and pro-angiogenic EETs and higher levels of anti-inflammatory and anti-angiogenic EDPs.

VEGFR2 is the therapeutic target of numerous angiogenesis inhibitors. But, VEGF-VEFGR2 inhibitors are routinely associated with induction of hypertension. EDPs are extremely potent vasodilators which give them unique advantage in antiangiogenic agent that can be used in cancer therapy avoiding hypertension [81].

Role in Metastasis

AA-derived eicosanoids are involved in the every step of metastatic cascade such as intravascular tumor cell–tumor cell interaction, interactions with platelets and forming aggregates, and secretion of type IV collagenase essential for invasion [83]. As mentioned earlier, eicosanoids derived from EPA and in particular DHA [72] are biologically less active and inhibit the actions of AA-derived eicosanoids [81]. CD44 is pro-metastasis molecule, expressed by aggressive cancer cells, and is essential for the EMT. DHA may downregulate the expression of CD44. Anti-metastatic properties of DHA are highlighted in many animal models such as breast cancer xenograft model and colon cancer models. Preoperative and/or postoperative DHA dietary supplementation in these models results in low bone and lung metastasis, respectively [84].

Role in Immunomodulation

Among the multiple subtypes present in macrophages, M2-type macrophages have tumor growth-promoting properties, higher expression of immunosuppressive cytokines such as TGF-β, induce invasion and angiogenesis, and promote metastasis. TAMs are induced to M2 polarization by cytokines such as IL-4 and IL-13(TH-2 cytokines), which assists in tumor promotion and development. Macrophage recruitment and polarization to M2 type are regulated by growth factors such as M-CSF and chemokines such as MCP-1. Both M-CSF and MCP-1 expressions are upregulated in many cancer types such as prostate cancer. In vitro studies using PC3 prostate cancer cell line and THP1 cells (induced to show M2-type functional properties) gave similar findings. Supplementation of EPA/DHA resulted in lowered macrophage recruitment by downregulating M-CSF and MCP-1. Data from similar experimental system suggest involvement of $PPAR_{\gamma}$ and NF-kB [85]. w-3 PUFA from fish oil is shown to decrease TH-1 and TH-2 cytokine responses at mucosal level [86]. Tumor promotion functions of TAM include extracellular matrix remolding, promotion of tumor cell invasion, angiogenesis, lympho-angiogenesis, metastasis, and immunosuppression [87].

Similar to TAM, tumor-associated neutrophils (TANs) show tremendous plasticity. The N2-type TAN phenotype driven by TGF-β is negatively correlated with clinical outcome. Tumor microenvironment influences the pro-tumorigenic functional properties of TANs. These properties include release of elastases, MMPs, oncostatin M, etc., and enzymes or growth factors that ultimately result in tumor cell proliferation, invasion, and angiogenesis [87, 88].

Figure 12.4 represents functional molecules from TAM and TAN involved in tumor progression

Role in Combination with Chemotherapeutic Agents and Drug Resistance

Majority of current chemotherapeutic treatments are associated with adverse side effects and resistance development against single targeted therapeutic drug. This unwanted chemotherapy-related toxicity is mainly due to requirement of high dose to achieve clinically significant efficacy.

In vitro combination studies with w-3 PUFA and various standard chemotherapeutic agents show additive or synergistic effects. In the colon cancer cell lines, synergistic

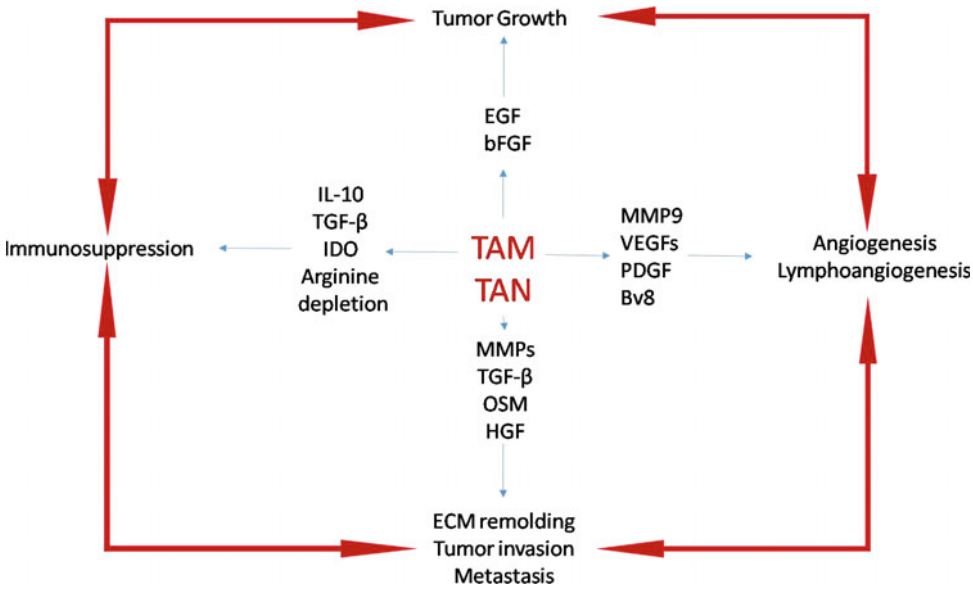

Fig. 12.4 TAM and TAN modulate tumor survival, angiogenesis, and metastasis by virtue of various factors such as interleukins, growth factors, and proteases

combination effect of DHA with 5FU was independent of p53 status and was executed by intrinsic apoptotic pathway [89–94]. Apart from involvement of mitochondrial apoptotic pathway, distinct gene expression profile suggests role played by some other pathway [95]. Among various mechanisms elucidated for the beneficial combination effects, lipid peroxidation and ROS generation have been major players along with modulation of lipid rafts resulting into drug efflux [96–98]. It has been shown that lipid peroxidation initiated by DHA is required for this beneficial combination effects. The preclinical cell line-based data indicate DHA pretreatment reverts chemo-resistance for doxorubicin, taxol, docetaxel, arsenic trioxide, vincristine, and cisplatin. It has been shown in various resistant cell lines that degree of resistance is lowered after DHA treatment [99].

Drug resistance is influencing the mortality rates in various cancers. ABC transporters (P-glycoprotein (Pgp/ABCB1), multidrug resistance-related proteins (MRPs/ABCCs), and breast cancer resistance protein (BCRP/ABCG2)) limit the intracellular accumulation of several anticancer agents by inducing the efflux of chemotherapeutic drugs. High level of cholesterol synthesis has been linked with multidrug resistance especially in colon cancer. The activity of Pgp and BCRP is directly related to the amount of cholesterol in the plasma membrane. Significant amount of these drug efflux pumps is embedded in cholesterol-rich domains of plasma membranes especially detergent-resistant membranes (DRMs). DHA acts as a strong DRM-interrupting agent. Due to long carbon chain and high level of unsaturation, DHA poorly fits in the cholesterol-rich DRM increasing degree of lipid unsaturation and altering the physicochemical properties of these compartments [100].

3-hydroxy-3methylglutaryl-coenzyme A reductase (HMGCoAR) is a crucial enzyme in the biosynthesis of cholesterol. EPA and/or DHA in cell line-specific manner has been shown to reduce HMGCoAR activity and restore ubiquitination level in drug-resistant cell lines. It has been shown that the inhibitory effect of EPA and DHA is cell line specific and controversial, for example, in SW480 cells, DHA has very little effect on de novo cholesterol synthesis and increases SREBP-2 which induces several genes involved in cholesterol synthesis.

Maheo et al. have reported contrasting data. Accordingly, irrespective of the breast cancer cell line studied, DHA incorporation in the cellular membrane did not result into intracellular doxorubicin accumulation [101].

High levels of prostaglandins and COX2 are associated with many human cancers such as breast, cervix, lung, skin, colon, and prostate. Celecoxib (COX-2 inhibitor) shows potential in cancer treatment, but use is limited because of dose-induced toxicity. In colon cancer cell line, combination of celecoxib and DHA resulted in induction of apoptosis at much lower doses compared to individual treatment has been shown that combination with DHA reduces side effects (toxicity) allowing increase in the dose [46, 61–64, 102–104]. Underlying mechanism is associated with NF-kB p65 translocation, and PPARc and RXRa are involved in the mechanism [48]. Similar synergistic effect was seen in the combination of curcumin and DHA. The combinations were also found to suppress iNOS, COX-2, 5-lipoxygenase (5-LOX), and cPLA2 activities [105]. The anticancer effect of 1a,25(OH)2D3 is well documented [47], but the doses required to achieve clinically significant effect may result in the hypercalcemia. Combination of 1a,25(OH)2D3 with

DHA has synergistic effect in glioblastoma and lung cancer cell line [99].

Experimental Evidence from Animal Studies

Various xenografts models have been studied to explore effect of dietary PUFA on tumor growth or tumor burden. The inhibitory effects of n-3 PUFA on tumor growth have been well documented for breast cancer in rodent xenograft models such as MDA Mb 435, MDA MB231, MCF7, and R3230AC [106]. When these xenograft-bearing mice were fed with fish oil or menhaden oil showed slow tumor growth and low tumor volume compared to control group, xenograft models fed with w6-rich diet. Modification of cell membrane structure and composition, high incorporation of w-3 PUFA into the tumor phospholipids, which further reduced pro-inflammatory eicosanoid synthesis from AA, and lipid peroxidation in the tumor cells are underlying this beneficial effect of w-3 against tumor progression. The antitumor property of w-3 PUFA was supported by Ki-67 immunohistochemical staining (absent in the resting cell). Similar observations were seen in case of prostate cancer fed with ALA-rich flaxseed oil.

The fat-1 transgenic mouse model provides evidence that DHA and DHA-derived compounds may have significance in cancer development. These mice carry a gene that encodes a desaturase that catalyzes conversion of w-6/w-3 FAs, a feature that is lacking in most mammals, including humans. In this mouse model, melanoma formation and growth, colitis-associated colon cancer growth, and prostate cancer growth were all inhibited compared to tumor growth in non-transgenic animals [48].

In another study, aggressive HER-2-positive BC model (MMTV-neu-YD5) was crossed with fat-1 mice yielding mice that were able to convert w-6 PUFA to w-3 PUFA and susceptible to mammary tumor growth were fed with w-3 FAs-containing or w-6 FA-containing feed. In both these approaches, tumor incidence was dramatically reduced along with prolonged tumor latency. Effect of the w-3 PUFA was dose-dependent [107].

In line with the previous study, the observed dose-dependent effects of w-3 PUFA were associated with a dose-dependent change in the fatty acid profile, reflecting a decreased w-6/w-3 ratio in the mammary glands and an increased EPA and DHA in tumor phospholipid classes [45].

Various chemical carcinogen [e.g., 7,12-dimethylbenz (α) anthracene]-induced tumor models were used to determine effect of w-3 FAs or ration of w-6/w-3 FAs on tumor growth, tumor burden, and apoptotic index. As seen in these models, tumor burden and tumor multiplicity went down simultaneously increasing apoptotic index after w3-containing feed. Mechanistically, oncogenic pathway proteins such as HER2, pAkt, and bcl2 were downregulated, and pro-apoptotic proteins such as Bax and p53 were upregulated [45].

Conclusion

Effects of w-3 PUFA are mediated by displacing AA in the cell membrane phospholipids and inhibiting pro-inflammatory, pro-angiogenic, and pro-metastatic AA eicosanoids. Lipid peroxidation and oxidative stress are the possible major mechanisms for w-3 PUFA-induced cell death. EPA and DHA are shown to attenuate pro-survival and anti-apoptotic pathways

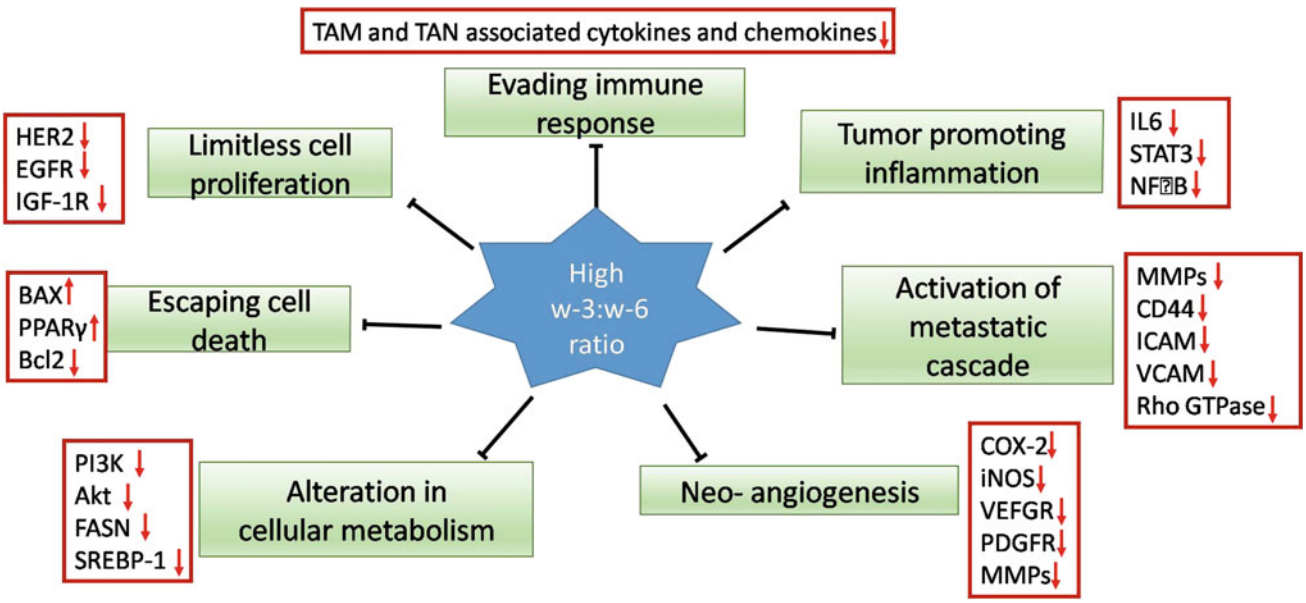

Fig. 12.5 High w-3/w-6 FA ratio downregulates cell survival and proliferation by inducing apoptosis or autophagy, and alters tumor cell metabolism and tumor microenvironment by immunomodulation. It restricts neo-angiogenesis and metastasis. Downregulation of inflammatory mediators in favor of tumor mass reduction and induction of apoptosis in cancer cells seems to be important mechanism

which results in inhibition of cancer cell proliferation and induction of apoptosis. EPA and DHA both act as immune modulators controlling activity of tumor-associated immune cells and microenvironment.

Figure 12.5 summarizes various signaling pathways mitigated by high w-3/w-6 PUFA ratio.

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Linseed as a Functional Food for the Management of Obesity

13

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Obesity and Functional Foods

In the short term, energy from food is stored predominantly as glycogen for rapid release as glucose; in the longer term, fats are favoured for energy storage. This storage allows the energy from times of excess to be used in times of famine. Continued excess energy intake means that stores keep increasing, resulting in obesity, especially in the abdomen. Obesity is an enormous cost to the community as it greatly increases the risk of chronic diseases including diabetes, disability, depression, cardiovascular disease and some cancers, as well as mortality [1]. Food is essential for nutrition, but non-nutritive components of food can promote health and reduce the risk of disease. This combination of nutrition and health benefits defines functional foods and includes bioactive fatty acids, phenolic compounds, plant sterols, dietary calcium and dietary fibre [2].

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History of Linseed

Wild flax fibres were possibly used for making cords by early hunter-gatherers 32,000-26,000 years ago in Dzudzuana cave, Georgia [3], but the evidence for identification as flax is argued [4]. Flax or linseed (*Linum usitatissimum* L.) is now known only as a cultivated plant with domestication in the Near East as one of the original crops in agriculture more than 8000 years ago, with pale flax as the progenitor [5–7]. Linseed is the emblem of Northern Ireland, and common linseed is the national flower of Belarus. Linseed has been widely used to make linen for cloth since ancient times. Linseed oil has been used for medicinal purposes in both ancient and modern societies and has been discussed as a modern functional food [8].

Linseed is found in two varieties with shiny yellow or dark brown seed. The seed is oblong in shape, flattened with a pointed tip, smooth glossy surface, comprising an embryo with two cotyledons surrounded by a thin endosperm [9]. Linseed is mainly grown for its oil as a major source of α-linolenic acid (ALA, Fig. 13.1), the essential n-3 fatty acid, but there are also industrial, medicinal and nutritional uses. Linseed yields vary depending on the season, weather and location, with best yields in moderate-to-cool climates [10]. Canada is the world's largest producer of linseed with 2013 production of 712,000 tons or around 32 % of the world production of 2.2 million tons; other countries producing more than 100,000 tons in 2013 were China, Russia, Kazakhstan, India and Ethiopia [11, 12]. Australia is one of many minor producers with about 7000 tons in 2013. The average yield in Canada in 2013 was 1728 kg/ha with lower average yields in Australia of 1133 kg/ha and India of 435 kg/ha [12].

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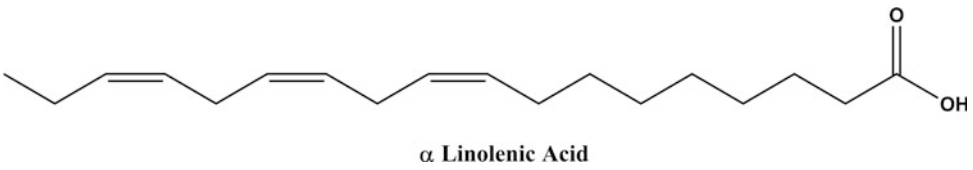

Fig. 13.1 Chemical structure of ALA

Linseed Composition

Canadian linseed contained an average of 41 % oil, 20 % protein, 29 % total carbohydrate mainly as fibre (28 %) with some sugars and starches (1 %), 7.7 % moisture and 3.4 % ash [13]. Comparisons of protein, fat and carbohydrate content of linseed, linseed meal, de-oiled linseed meal and processed fractions of linseed oil are available [14]. The 2012 survey of Canadian linseeds showed that the oil contained 56.7 % ALA, 19.2 % oleic acid, 14.7 % linoleic acid, 5.0 % palmitic acid, 3.4 % stearic acid and 0.2 % non-esterified fatty acids [14]. Linseeds contain plant lignans, especially secoisolariciresinol diglucoside (SDG), which act as precursors of mammalian lignans also known as enterolignans (Fig. 13.2), as well as vitamins and minerals (Table 13.1) [8].

Isolation of Linseed Oil

High-quality linseed oil is produced commercially by processes that include seed cleaning, flaking, cooking, pressing, solvent extraction using *n*-hexane and solvent removal [15]. These methods will influence the quality of oil because of the heat involved during the procedures. In addition, the use of *n*-hexane in defatting linseed meal enriches cyanogenic glycosides such as linustatin (Fig. 13.3), neolinustatin and linamarin which can result in goitrogenic problems in humans [16]. Cold press isolation is preferred to obtain high-quality oil but this process consumes more energy with lower yield [17]. The lack of refining processes of cold press oil is a marketing advantage by producing "first press" oil [18]. Alternative extraction methods that improve yield and maintain quality and stability of the oil include supercritical fluid extraction, accelerated solvent extraction, aqueous enzymatic extraction, ultrasound-assisted extraction and microencapsulation of oil by spray-drying techniques [17, 19]. The oil yields obtained by supercritical fluid and accelerated solvent extraction of 36.49 g and 41.90 g/100 g seeds, respectively, compared well to conventional solvent extraction of 42.40 g/100 g [15].

Pharmacology and Therapeutic Benefits of ALA

Linseed is high in the essential n-3 fatty acid, ALA. The Australian Heart Foundation recommends the consumption of at least 2 g/day of ALA to reduce the risk of heart disease [20].

ALA is highly bioavailable with oral absorption in adults of 96 % [21] while a small proportion may also be converted to conjugated linolenic acid primarily in the caecum and colon by the gut microbiota [22]. In preterm infants, absorption of [U-(13)C] labelled ALA from preterm formulae ranged from 74 to 98 % with higher absorption with increasing birth gestation [23]. Higher plasma ALA concentrations were observed after whole linseed supplementation compared to milled seed or linseed oil supplementation [24, 25]. Further, in vivo experiments in rats suggest that emulsification of linseed oil increases the rate and extent of ALA recovery in the thoracic lymph duct when compared to non-emulsified oil (C_{max} = 14 mg/ml at 3 h and 9 mg/ml at 5 h, respectively) with higher area under the curve in the emulsion group (48 mg × h/ml and 26 mg × h/ml, respectively) [26].

Once absorbed, ALA has at least three metabolic fates. It may undergo β-oxidation and carbon recycling, be deposited as ALA predominantly in adipose tissue, skin and muscle, or undergo a series of elongation and desaturation steps to produce eicosapentaenoic acid (EPA; 20:5n-3), docosapentaenoic acid (DPA; 22:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) [27–29]. In vivo tracer studies have shown that the oxidation of ALA in rats is efficient and comparable to lauric, oleic and linoleic acids, the most highly oxidisable fatty acids [30]. Of the total ingested ALA, 58.9 % was assumed to be removed by oxidation, 0.4 % was excreted in faeces, 10.6 % accumulated as ALA in adipose tissue, 9.7 % accumulated in the carcass-skin compartment, 4 % accumulated as ALA in organ compartment and 17.2 % accumulated as long-chain derivatives (14 % as DHA and 3.2 % as EPA + DPA) [31]. Similar results were obtained for disappearance, excretion and total accumulation with DHA [31].

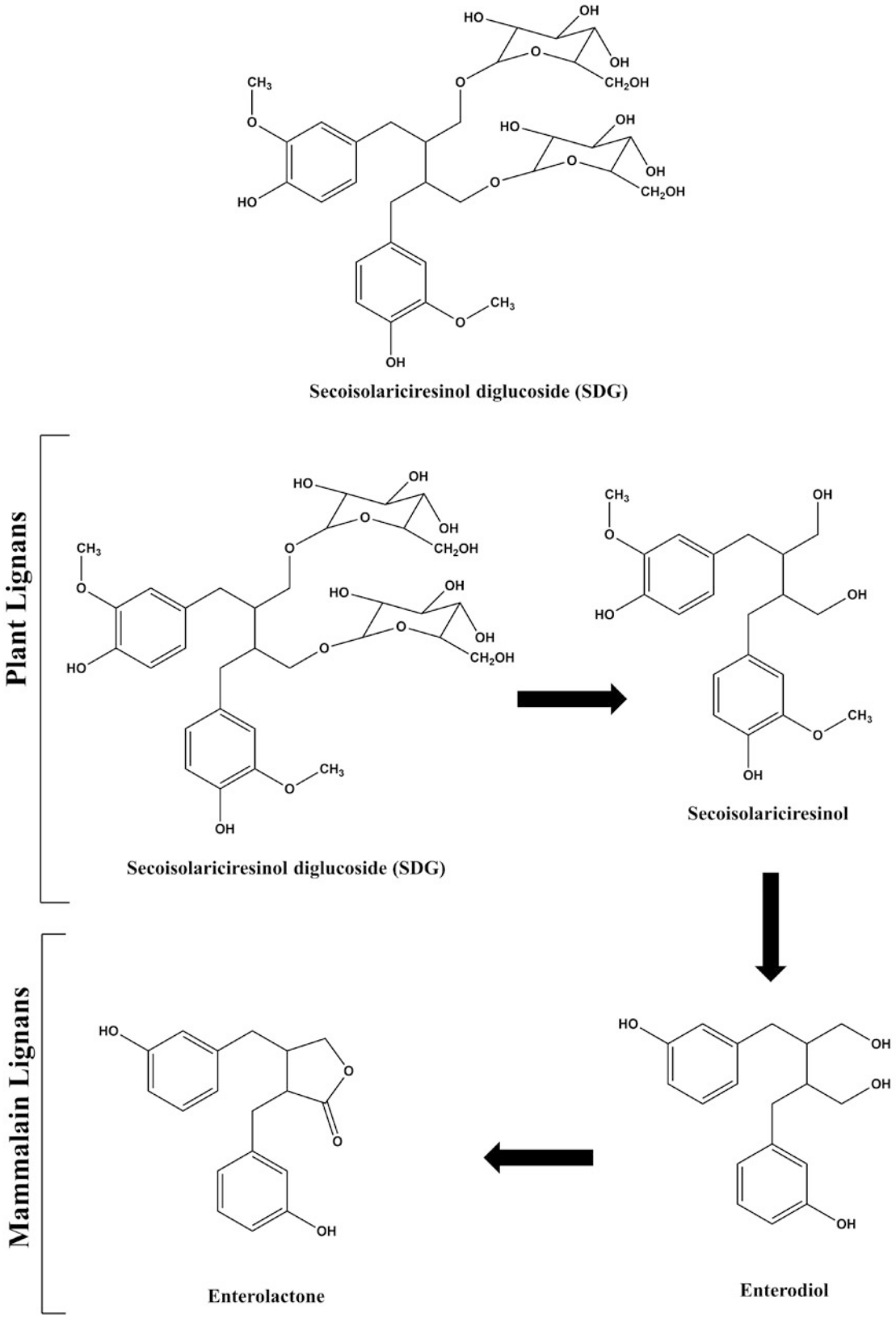

Fig. 13.2 Structures of SDG and its metabolites

Table 13.1 Vitamins and minerals in linseed

Linseed components	Quantity/100 g of seed	Proportion of recommended dietary intake (%)	
		Men	Women
Vitamins			
Ascorbic acid	0.50 mg	1.1	1.1
Thiamine	0.53 mg	44.2	48.2
Riboflavin	0.23 mg	17.7	20.9
Niacin	3.21 mg	20.1	22.9
Pyridoxine	0.61 mg	46.9	46.9
Pantothenic acid	0.57 mg	9.5	14.25
Folic acid	112 µg	28	28
Biotin	6 µg	20	24
γ-Tocopherol	8.5–39.5 mg	–	–
Minerals			
Calcium	236 mg	23.6	23.6
Copper	1 mg	58.8	83.3
Magnesium	431 mg	107.8	139
Manganese	3 mg	54.5	60
Phosphorus	622 mg	62.2	62.2
Potassium	831 mg	21.9	21.9
Sodium	27 mg	2.9–5.9	2.9–5.9
Zinc	4 mg	28.6	50

Sources Flax Council of Canada (2007) [13] and Nutrient reference values for Australia and New Zealand from National Health and Medical Research Council [135]

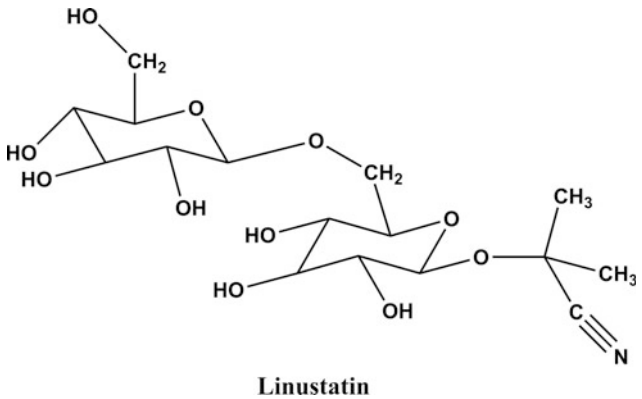

Fig. 13.3 Chemical structure of linustatin

The extent of conversion of ALA to EPA and DHA in humans has been a matter of great debate [29, 32, 33]. Healthy young men consuming a diet providing 6.3 % calories from ALA (linseed oil diet containing 28.8 % energy from fat) increased ALA concentrations in the serum and peripheral blood mononuclear cells lipids along with increase in the EPA and DHA contents of mononuclear cell lipids [34]. A physiological compartmental model of ALA metabolism derived from plasma concentration-time curves for radiolabelled ALA, EPA, DPA and DHA after consumption of 1 g oral dose of an isotope tracer ALA suggests that only about 0.2 % of the plasma ALA was destined for the synthesis of EPA, but approximately 63 % of the plasma EPA was accessible for the production of DPA and 37 % of DPA was available for the synthesis of DHA [35]. However, in plasma phospholipids, which may more closely resemble hepatic n-3 fatty acid metabolism, 7 % of dietary ALA was incorporated into plasma phospholipids, 99.8 % of which was converted into EPA and 1 % of the EPA was converted into DPA and subsequently into DHA [36]. This study suggests that the limited incorporation of dietary ALA into the hepatic phospholipid pool contributes to the low hepatic conversion of ALA into EPA [36]. A low conversion of ALA-derived EPA into DPA might be an additional obstacle for DHA synthesis [36]. However, in women, estimated net fractional conversion of ALA to EPA was 21 %, to DPA was 6 % and to DHA was 9 % with approximately 22 % undergoing β-oxidation [37]. These results suggest that women may possess greater capacity for ALA conversion than men.

Obese Patients

Whether ALA-rich linseed promotes weight loss in obese humans is controversial. Short-term (2–8 weeks) dietary intervention studies with linseed oil or flour supplementation in obese patients did not provide any evidence of weight loss [38, 39]. However, 12 weeks of linseed supplementation (30 g/day) in patients with metabolic syndrome decreased the prevalence of metabolic syndrome along with reduced central obesity, body weight, serum glucose, total- and LDL-cholesterol, apolipoprotein (Apo) B, ApoE and blood pressure [40]. However, it should be noted that the patients in the 12-week study were also undergoing lifestyle counselling using the American Heart Association guidelines [40].

Nevertheless, clinical studies consistently report beneficial effects of linseed in obese patients. In overweight or obese men and postmenopausal women with pre-diabetes, 13 g of ground linseed for 12 weeks decreased glucose and insulin concentrations and insulin sensitivity (HOMA-IR) [41]. Similar improvement in HOMA-IR was observed in obese glucose-intolerant patients [42]. Further, in middle-aged men and postmenopausal women, 40 g/day of ground linseed-containing baked products for 10 weeks improved insulin sensitivity suggesting that these effects are also observed in non-obese patients [43].

However, both short-term (3 weeks) and long-term (3 months) intervention studies with ALA from different sources including linseed oil did not improve glycaemic control in healthy or diabetic patients [44, 45]. ALA (6 g/day) from fortified rapeseed oil for 3 weeks in healthy, non-obese subjects did not affect fasting serum concentrations of glucose, insulin, fructosamine or hemoglobin A1c (HbA1c) [44]. ALA (5.5 g/day from 10 g/day linseed oil) in type 2 diabetic patients for 3 months also did not affect fasting serum glucose, insulin or HbA1c concentrations [45]. Together, these studies suggest that non-ALA components from linseed may be responsible for the improved insulin sensitivity in response to linseed supplementation.

Obesity-related inflammation is a key determinant of insulin sensitivity [46] with increased pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) and decreased anti-inflammatory adipokines such as adiponectin linked to the development of insulin resistance and type 2 diabetes [47]. It is possible that the improved insulin sensitivity in response to linseed noted above may be mediated by suppressing the pro-inflammatory state. However, this effect may be dependent on the form of linseed supplementation. A total of 13 g/day ground linseed (2.9 g/day ALA) for 12 weeks did not affect plasma C-reactive protein (CRP), adiponectin or IL-6 concentrations [41]. Markers of systemic inflammation did not change at the higher dose of 40 g/day (8.4 g/day ALA) ground linseed for 10–12 weeks [42, 43]. In contrast, linseed flour may have anti-inflammatory effects in obese patients since 30 g/day linseed flour supplementation (5 g/day ALA) for 2 weeks decreased CRP, serum amyloid A and fibronectin concentrations [39]. In morbidly obese patients, a higher dose of 60 g/day linseed flour (10 g/day ALA) for 12 weeks decreased neutrophil count and prevented further increases in fibrinogen and complement C4 concentrations and prothrombin time [48].

Improved insulin sensitivity and decreases in the pro-inflammatory state may be further assisted by reduced apparent digestibility of fat in response to dietary linseed supplementation [49]. Linseed supplementation in rye bread for 8 weeks increased faecal dry weight and excreted fat suggesting decreased fat digestibility without affecting average transit time [49]; further, linseed does not affect satiety [50]. In women in the late postoperative period following Roux-en-Y gastric bypass, whole or defatted linseed did not affect hunger, satisfaction, fullness or desire to eat for up to 180 min after ingestion of test meals [50]. However, defatted linseed increased serum leptin concentrations [50].

Obesity is also a key risk factor for breast cancer, particularly in postmenopausal women [51]. Elevated oestradiol, oestrone and testosterone concentrations are important predictors of breast cancer in postmenopausal women [52–54]. In postmenopausal women, consuming 7.5 g/day of ground linseed for the first 6 weeks and 15 g/day for an additional 6 weeks decreased oestradiol, oestrone and testosterone concentrations with pronounced effects in overweight/obese women [55]. The decreases in these hormone concentrations in response to linseed do not seem to worsen osteoporosis. A systematic review of 30 human and animal studies suggested that ALA from linseed oil, but not the lignan fraction, benefited osteoporotic bone, especially together with oestrogen therapy [56].

Further, in obese insulin-resistant patients, ALA supplementation (20 g/day for 4 weeks) from margarine products based on linseed oil increased systemic arterial compliance without changing mean arterial pressures [57]. Many animal and human studies have confirmed the blood pressure lowering effects of linseed but only after 12 weeks of treatment [58].

Other sources of ALA, such as rapeseed oil, perilla oil and chia seed, reduced blood pressure with greater reductions in diastolic blood pressure [59–62], serum inflammatory biomarkers such as CRP, plasminogen activator inhibitor-1 (PAI-1) and TNF-α, as well as YKL-40 (chitinase-3-like protein 1), an inflammatory biomarker for coronary artery disease [60, 62, 63], and reduced triglycerides [59, 63] in obese or overweight patients with metabolic syndrome but with no effect on body weight.

In summary, whole linseed improves insulin sensitivity, linseed flour but not oil attenuates the pro-inflammatory state and linseed oil but not the lignan fraction benefits osteoporotic bone in obese or overweight patients.

Obese Rodents

Studies in obese rats and mice suggest that linseed may protect from obesity-associated heart, kidney and liver damage, supporting the beneficial effects of linseed in obese patients. We have previously demonstrated that linseed and chia seed oil supplementation for 8 weeks, both containing about 59 % ALA, did not reduce total body fat in a high-carbohydrate, high-fat diet-induced model of metabolic syndrome in rats but induced lipid redistribution away from the abdominal area and improved glucose tolerance, insulin sensitivity, dyslipidaemia, hypertension and left ventricular dimensions, contractility, volumes and stiffness [64, 65]. Additionally, both linseed and chia seed oil supplementations reversed inflammation in the heart and the liver, cardiac fibrosis and hepatic steatosis [64, 65].

The obese spontaneously hypertensive/NIH-corpulent (SHR/N-cp) rat is a genetic model of obesity and type 2 diabetes that consistently develops nephropathy resembling human diabetic nephropathy [66]. Addition of 20 % linseed meal to the diets of obese SHR/N-cp rats for 6 months did not affect body weight or plasma glucose but decreased plasma insulin concentrations [66]. Measures of renal function such as plasma creatinine, creatinine clearance and urinary urea excretion also did not change but urinary protein excretion was lower in rats fed linseed meal diet [66]. Further, the percentage of abnormal glomeruli with mesangial expansion and the tubulointerstitial score as an index of severity of tubulointerstitial damage were reduced in rats fed linseed meal [66]. Linseed oil supplementation reduced concentrations of fasting serum insulin, urinary thiobarbituric acid reactive substances, hepatic triglycerides and cholesterol with higher hepatic mRNA expression of PPAR-γ as early as 4 weeks in SHR/N-cp rats fed a high-fat diet [67]. Further, linseed meal (20 % of energy) lowered plasma total, LDL- and HDL-cholesterol, and plasma triglyceride concentrations and lowered hepatic fat deposition [68]. Consistent with these findings in the liver, dietary linseed oil supplementation for 12 weeks in high-fat diet-fed rats prevented hepatic steatosis with increased EPA and DHA and lowered arachidonic acid incorporation into hepatic phospholipids [69]. These effects may be mediated, at least in part, by SDG as discussed in subsequent sections.

In Zucker fatty rats, linseed supplementation for 9 weeks did not affect obesity, oral glucose tolerance, pancreatic function or molecular markers related to insulin, glucose and lipid metabolism [70]. However, ALA-rich linseed oil supplemented diet for 8 weeks decreased adipocyte size and adipose tissue concentrations of MCP-1, IL-10 and TNF-α [71]. Linseed oil supplementation reduced T-cell infiltration into the adipose tissue but did not alter macrophage infiltration [71].

Some of the effects of linseed oil may also be neurally regulated. In a mouse model of diet-induced obesity, partial substitution of the fatty acid component of the diet by linseed oil attenuated hypothalamic inflammation, hypothalamic and whole body insulin resistance and whole body adiposity [72]. In addition, after intracerebroventricular injection in obese rats, pure ALA reduced spontaneous food intake and body mass gain [72]. These effects were accompanied by the reversal of functional and molecular hypothalamic resistance to leptin/insulin and increased pro-opiomelanocortin and cocaine- and amphetamine-mediated upregulation of transcript expressions [72]. Additionally, ALA inhibited the AMP-activated protein kinase/acetyl-CoA carboxylase (AMPK/ACC) pathway and increased carnitine palmitoyltransferase 1 (CPT1) and stearoyl-CoA desaturase-1 (SCD-1) expression in the hypothalamus [72]. Finally, acute hypothalamic injection of ALA activated signal transduction through the recently identified G protein-coupled receptor (GPR) 120 unsaturated fatty acid receptor [72].

Possible Mechanisms of Action of ALA

The mechanisms of action of ALA can be broadly classified into three categories depending on its metabolism. Firstly, ALA may act directly, as shown in studies on insulin secretion and sensitivity. ALA is considered to be the endogenous ligand of GPR40 and GPR120, and these receptors are the predominant mediators of ALA-induced insulin secretion [73]. The insulinotropic effects of ALA may be augmented by ALA-induced expression and secretion of insulin-like growth factor-I from hepatocytes [74]. Further, the effects of ALA could be mediated by reduced production of pro-inflammatory cytokines from adipose tissue [71] leading to improved insulin sensitivity in both animal and human studies as discussed in preceding sections. Further, ALA supplementation may suppress the expression and activity of SCD-1 [64, 65, 75–77]. Suppressed SCD-1 activity reduces body adiposity, increases insulin sensitivity, provides resistance to diet-induced obesity and may produce anti-inflammatory effects such as reduced macrophage inflammatory response, reduced expression of MCP-1, TNF-α, PAI-1 and vascular cell adhesion molecule 1 (VCAM-1), as well as chemokine receptor 2, colony stimulating factor 1 (CSF-1) receptor and decreased DNA-binding activity of NF-κB p65/50 [78–82].

Secondly, assuming adequate conversion, some of the effects of ALA could be mediated by EPA and DHA. EPA and DHA limit both hypertrophy and hyperplasia of adipocytes, increased lipolysis and increased fat oxidation, assisted by increased expression and activity of CPT1 and peroxisomal acyl-CoA oxidase gene that regulate the

alternate pathway of fatty acid oxidation [29]. Further, EPA and DHA can also directly act on GPR120 in the enteroendocrine L cells to secrete glucagon like peptide-1, a potent insulin secretagogue [83].

Thirdly, metabolites of EPA and DHA could also mediate the effects of ALA. 5-hydroxy-eicosapentaenoic acid (5-HEPE), a direct metabolite of EPA, is a potent agonist for GPR119, expressed in the pancreas and the intestine, and enhances glucose-dependent insulin secretion [84]. Further metabolism of EPA by the action of lipoxygenase and cyclooxygenase produces eicosanoids that induce vasodilatation and are potent anti-inflammatory and anti-thrombotic molecules [29, 85]. EPA-derived 15-hydroxy-eicosapentaenoic acid (15-HEPE) may have anti-inflammatory effects through the inhibition of 5-lipoxygenase, a major enzyme that generates pro-inflammatory lipid mediators [86]. Additionally, ω-hydroxyl fatty acids such as 7,18-epoxyeicosatetraenoic acid and 19,29-epoxy docosapentaenoic acid derived by the action of cytochrome epoxygenases on EPA and DHA, respectively, have potent anti-inflammatory effects and reduce blood pressure [86]. Effects on renal function may be mediated by ALA-derived oxylipins associated with reduced glomerulomegaly in diet-induced obese rats fed a linseed oil supplemented diet [87].

DHA metabolites including resolvins, protectins, maresins, docosatrienes and neuroprotectins are important resolution phase mediators of inflammation [88–90] that could mediate important anti-inflammatory responses to ALA. The production of DHA-derived 17-hydroxy-DHA, the precursor of resolvin D1 and protectin D1, is decreased in adipose tissue of obese mice [91]. Further, in these obese mice, 17-hydroxy-DHA decreased the expression of pro-inflammatory cytokines (TNF-α, F4/80 and IL-6), increased adiponectin expression and improved glucose tolerance and insulin sensitivity [91]. In *db*/*db* mice, treatment with resolvin D1 (2 μg/kg) improved glucose tolerance, decreased fasting blood glucose concentrations, increased insulin-stimulated Akt phosphorylation in adipose tissue, increased adiponectin, decreased IL-6 and reduced $F4/80^{+}CD11c^{+}$ macrophages in adipose tissue indicating the important role of resolvin D1 in resolution of inflammation [92]. These studies provide evidence for the role of DHA-derived resolvin D1 in attenuation of inflammation, and hence, ALA-derived DHA could produce the same responses in obesity.

Isolation and Pharmacology of SDG from Linseed

Linseed is one of the richest sources of plant lignans with SDG as the major component at 1–26 mg/g of seeds or about 75–800 times greater concentrations than in other fibre-rich plants [13, 93]. SDG content depends on the linseed cultivars, growing region and method of analysis [94]. SDG is stored in plants as a complex macromolecular structure with an ester linkage to 3-hydroxy-3-methyl glutaric acid to give a molecular weight of ~4000 Da [95]. Other lignans in linseed include isolariciresinol and pinoresinol, also found in similar concentrations in sesame seeds [96], and matairesinol [97]. The most common extraction method for SDG uses aliphatic alcohol extraction followed by alkaline hydrolysis and chromatographic separation [16]. This was less efficient than direct alkaline hydrolysis in aqueous or alcoholic solutions as an initial alcoholic extraction did not completely recover the lignan from the plant matrix [98].

The biological responses to SDG from linseed are related to the gut bacteria-mediated conversion to the enterolignans, enterodiol and enterolactone (Fig. 13.2) [99, 100]. Interactions between dominant and subdominant intestinal anaerobes were involved in lignan breakdown by deglycosylation, demethylation, dehydroxylation and dehydrogenation of SDG [101, 102]. The oral bioavailability of lignans was influenced by the microflora present in the intestine, antibiotic use, food matrix, type and form (aglycone or conjugated) of plant lignan, chronic exposure and other host-related factors such as age and gender [103]. Enterodiol and enterolactone are absorbed by the colonic epithelium either as glucuronides or sulphate conjugates or as free forms into the blood stream or directly excreted via faeces [104]. Conjugated enterolignans are absorbed from intestinal tract and transported to the liver, while free forms are conjugated before being released into the bloodstream [105]. Both forms undergo enterohepatic circulation with further metabolism in the colon and excretion in the urine in conjugated form [106]. The enterolignans are excreted via urine with conjugated enterolignans excreted mainly as monoglucuronides with only small amounts excreted as monosulphates or free aglycones [107, 108].

Pharmacokinetic studies on oral SDG have been reported in both animals and humans. SDG was administered to male and female Sprague–Dawley rats by gavaging ^{3}H-SDG (3.7 kBq/g body weight) and unlabelled SDG (5.3 μg/g body weight) daily [109]. Total lignan content was excreted primarily in faeces (40–83 %) and urine (1.2–5.2 %) in both genders. After 1–7 days dosage with SDG, most of the tissue lignans were accumulated in the liver (48–56 %) in both males and females. Prolonged ^{3}H-SDG dosage demonstrated an increased accumulation in skin and kidneys. Serum concentrations were stable after exposure for 7 days. Gender differences in lignan tissue distribution were observed, with higher concentrations in females in heart and thymus at all time points. This finding suggests the possibility for distinct treatment responses in males and females [109].

In a human study, 6 male and 6 female healthy volunteers ingested a single dose of SDG (1.31 μmol/kg body weight)

[99]. Enterodiol and enterolactone reached their maximum plasma concentrations at 14.8 ± 5.1 h and 19.7 ± 6.2 h, respectively, after ingestion. Enterodiol showed a shorter mean elimination half-life of 4.4 ± 1.3 h than enterolactone (12.6 ± 5.6 h). Enterolactone demonstrated a higher mean area under the curve (1762 ± 1117 nmol/L h) than enterodiol (966 ± 639 nmol/L h). Similarly, enterolactone (35.8 ± 10.6 h) has a longer residence time than enterodiol (20.6 ± 5.9 h). These results indicate that enterolignans accumulate in plasma when SDG is consumed 2–3 times a day before reaching a plateau state. This study also showed that, within 3 days, up to 40 % of the ingested SDG was excreted via urine as enterolignans, with 58 % as enterolactone [99].

The bioavailability and pharmacokinetics of SDG have been measured using a range of dosages and purities of the SDG [110]. Healthy postmenopausal women were given 25, 50, 75, 86 or 172 mg of SDG and purity of 28.8, 43 or 74 % SDG for one week. The sugar moiety of SDG was efficiently hydrolysed by gastrointestinal bacteria to produce secoisolariciresinol. Enterolignans appeared in the plasma after 5–7 h with a plasma elimination half-life of 4.8 h. Serum enterodiol and enterolactone concentrations peaked after 12–24 h and 24–36 h, respectively, and the half-lives were 9.4 h and 13.2 h. This study observed linear dose responses over a relatively wide range of dietary SDG intakes. In addition, the bioavailability of secoisolariciresinol was correlated ($r^2 = 0.835$) with cumulative 5-day lignan excretion. Changes in the SDG purity did not change the pharmacokinetics, and serum enterolignan concentrations reached steady state after one week of daily dosing. This study suggests that SDG is first hydrolysed and then metabolised in a time-dependent sequence in the gastrointestinal tract to secoisolariciresinol, enterodiol and ultimately enterolactone, and these metabolites are efficiently absorbed and predominantly excreted via urine [110].

Therapeutic Benefits in Humans

The effects on cardiovascular function have been investigated using different SDG doses, different purity of the compound, with interspecies differences that influence the pharmacokinetic reactions and length of study period. A better understanding of SDG metabolism is needed to verify the best model and method to determine the potential for improvements in cardiovascular health.

A study on moderately hypercholesterolaemic non-obese men given a low dose of SDG (100 mg, equivalent to 7.5 g of linseed) did not change the body weight and BMI of the subjects at 12 weeks [111]. However, the treatment decreased the ratio of LDL-/HDL-cholesterol and serum cholesterol concentrations. These effects may be mediated by a decrease in the mRNA expression levels of sterol regulatory element-binding protein-1c (SREBP-1c) in the liver. Further, this treatment regimen decreased the activities of the plasma hepatic diseases risk markers, alanine transaminase (ALT) and γ-glutamyl transferase (GGT), but not aspartate transaminase (AST) [111].

Recently, a short-term trial using 40 g dose of golden linseed for 28 days in obese subjects showed no significance difference in body weight, BMI, waist circumference or blood pressure [112]. However, the results obtained demonstrated reduced fasting blood glucose concentrations and the study suggested increased glucose elimination via increased translocation of GLUT4 on the cell membrane and increased basal glucose uptake by redistribution of GLUT1 as plausible mechanisms. Reduction in three indicators of cardiac damage, lactate dehydrogenase (LDH), total creatine kinase (CK) and CK-MB isoform, strongly suggests cardioprotective effects with SDG from golden linseed [112]. Furthermore, the lignans such as SDG were most likely to be responsible for the improved function of the liver (measured as serum activities of ALT and AST) and kidneys (measured as serum creatinine, uric acid and blood urea nitrogen). Furthermore, SDG decreased serum malondialdehyde (MDA) and protein carbonyls and increased glutathione, indicating reversal of the oxidative stress. In addition, increases in antioxidant enzyme activities (superoxide dismutase, catalase and glutathione peroxidase) were observed after 28 days of treatment [112].

Actions in Rodents and Rabbits

SDG and secoisolariciresinol demonstrated strong free radical scavenging activity and antioxidant activity in vitro [113]. The pharmacological responses to SDG and its metabolites have been measured in different animal models including rabbits, rats and mice. Different mechanisms of action leading to anti-atherogenic and cardioprotective effects have been proposed.

SDG treatment in hypercholesterolaemic rabbits showed consistent cholesterol-lowering and anti-atherosclerotic responses [114–116]. Reduction of serum cholesterol, LDL-cholesterol and lipid peroxidation products and an increase in HDL-cholesterol were seen after 8 weeks of treatment with 15 mg/kg body weight of SDG in 1 % cholesterol diet and 40 mg/kg body weight of lignan complex in 0.5 % cholesterol diet in rabbits [114, 115]. These studies suggest the decreases in serum total cholesterol and LDL-cholesterol with lignan complex could be due to the 3-hydroxy-3-methyl glutaric acid and SDG content of the lignan complex [115]. Lower serum and aortic MDA concentrations were associated with reduced development of atherosclerosis in rabbits fed the lignan complex. These effects may be mediated by antioxidant activity of SDG to prevent oxygen radical-induced endothelial cell injury [115].

A different intervention which included 2 months of regular diet following 2 months of a high cholesterol diet with supplementation of 20 mg SDG/kg body weight/day resulted in no reduction in serum lipids. However, prevention of progression of atherosclerosis was seen that may be associated with a reduction of aortic oxidative stress [116].

Lipid homoeostasis was evaluated in diet-induced hypercholesterolaemic female Wistar rats treated with either SDG or secoisolariciresinol at different dosages of 0, 3 or 6 mg SDG/kg body weight/day or 0, 1.6 or 3.2 mg secoisolariciresinol/kg body weight/day, respectively, for 4 weeks [117]. Both SDG and secoisolariciresinol showed dose-dependent reduction in the serum and hepatic cholesterol concentrations. These changes were accompanied by increased hepatic expression of acetyl-CoA acetyltransferase 2 by SDG (3 and 6 mg/kg body weight/day) and decreased hepatic expression of cytochrome P450 7a1 by secoisolariciresinol (3.2 mg/kg body weight/day). No other changes were observed in hepatic expression of lipid controlling targets in rats treated with SDG or secoisolariciresinol [117].

High-carbohydrate (10 % fructose in drinking water) diet-fed male Sprague–Dawley rats as a model of hypertriglyceridaemia were used to measure dose-dependent effects of 0, 3 and 6 mg SDG/kg body weight/day for 2 weeks [117]. In contrast to the hypercholesterolaemic rats, no changes in body and organ weights, serum triglycerides, phospholipids and non-esterified fatty acid concentrations, and hepatic steatosis relative to vehicle control fructose-fed rats were measured at any dose [117]. SDG administration did not change the hepatic expression of PPAR-α or SREBP-1c following 10 % fructose in water supplementation [117].

Acute hyperlipidaemia can be produced in rats with poloxamer-407 (1 ml of 30 %, w/v; intraperitoneally) as it inhibits lipoprotein lipase and elevates the production of triglycerides. Oral treatment with a methanol fraction of linseed lignan concentrate (MF-FLC) in poloxamer-407 hyperlipidaemic rats was evaluated at 0, 15 h and 24 h after injection [118]. The administration of MF-FLC (100 mg/kg) and n-3 fatty acid (1 ml/kg; mainly containing DHA) reduced serum cholesterol, triglycerides and VLDL-cholesterol, whereas HDL-cholesterol was increased [118]. The combination of MF-FLC and n-3 fatty acid acts mainly by blocking cholesterol synthesis.

In rats given 2 % cholesterol for 8 weeks, SDG 20 mg/kg body weight improved lipid profiles after 2 weeks treatment. Dietary supplementation of SDG reduced infarct size, improved myocardial function and enhanced neovascularisation in the infarcted myocardium. These effects were mediated through improved thickness of ventricular wall during diastole and systole as well as improved left ventricular systolic function in the rats treated with SDG. Furthermore, SDG enhanced cardiac function by increasing protein expression of endothelial NOS (eNOS), vascular endothelial growth factor (VEGF) and hemeoxygenase-1 (HO-1) in SDG-treated rats [119].

In high-fat diet-fed mice, SDG supplementation (0.5 and 1.0 % (w/w) for 4 weeks) reduced high-fat diet-induced increases in visceral and liver fat accumulation, and in plasma lipids, insulin and leptin concentrations [120]. These effects of SDG were accompanied by suppressed SREBP-1c mRNA expression in the liver, increased adiponectin mRNA expression in the white adipose tissue and increased CPT1 mRNA expression in the skeletal muscle [120]. Further, the SDG metabolite, enterodiol, induced adipogenesis-related mRNA expression including adiponectin, leptin, GLUT4 and PPAR-γ, and induced PPAR-γ DNA-binding activity in 3T3-L1 adipocytes [120].

Linseed lignans may be able to prevent obesity in rodents. Treatment with 0.02 % SDG (35 %) lignan-enriched linseed powder (LEFP) lowered body weight as compared to high-fat diet control group due to lower visceral fat weight. In addition, lower plasma concentrations of leptin and higher plasma concentrations of adiponectin reflected a low visceral fat weight in treated rats. Supplementation with LEFP for 12 weeks improved systolic blood pressure and serum triglycerides, LDL-cholesterol and HDL-cholesterol concentrations. However, a limitation of this study should be noted as LEFP also contains other bioactive compounds including coumaric acid glucoside and ferulic acid glucoside [121].

Male Wistar rats were pretreated with 500 mg/kg flax lignan concentrate (FLC) for 8 days before isoprenaline (ISO) at a dose of 5.25 mg/kg on day 9 and 8.5 mg/kg on day 10 to induce moderate lesions in the myocardium [122]. The results showed that ISO + FLC rats had lower CK-MB, LDH and AST levels indicating protection against necrotic damage of the myocardial membrane. Inflammation, necrosis and congestion were observed in a smaller area of the heart in FLC + ISO compared to the ISO rats indicating that the rat hearts were partially protected by pretreatment with FLC (500 mg/kg) against isoprenaline-induced cardiotoxicity [122].

Isolation and Therapeutic Effects of Dietary Fibre from Linseed Mucilage

Linseeds contain approximately 8 % of the exopolysaccharide, mucilage [123], that can be easily extracted from the seed coat using water as this allows swelling of cell walls and then extrusion of the content on the surface of the seeds [124]. Basic or acid treatments will also extract the mucilage but these methods lead to higher concentrations of proteins in the mucilage and their subsequent denaturing [125]. Improved techniques have been introduced using

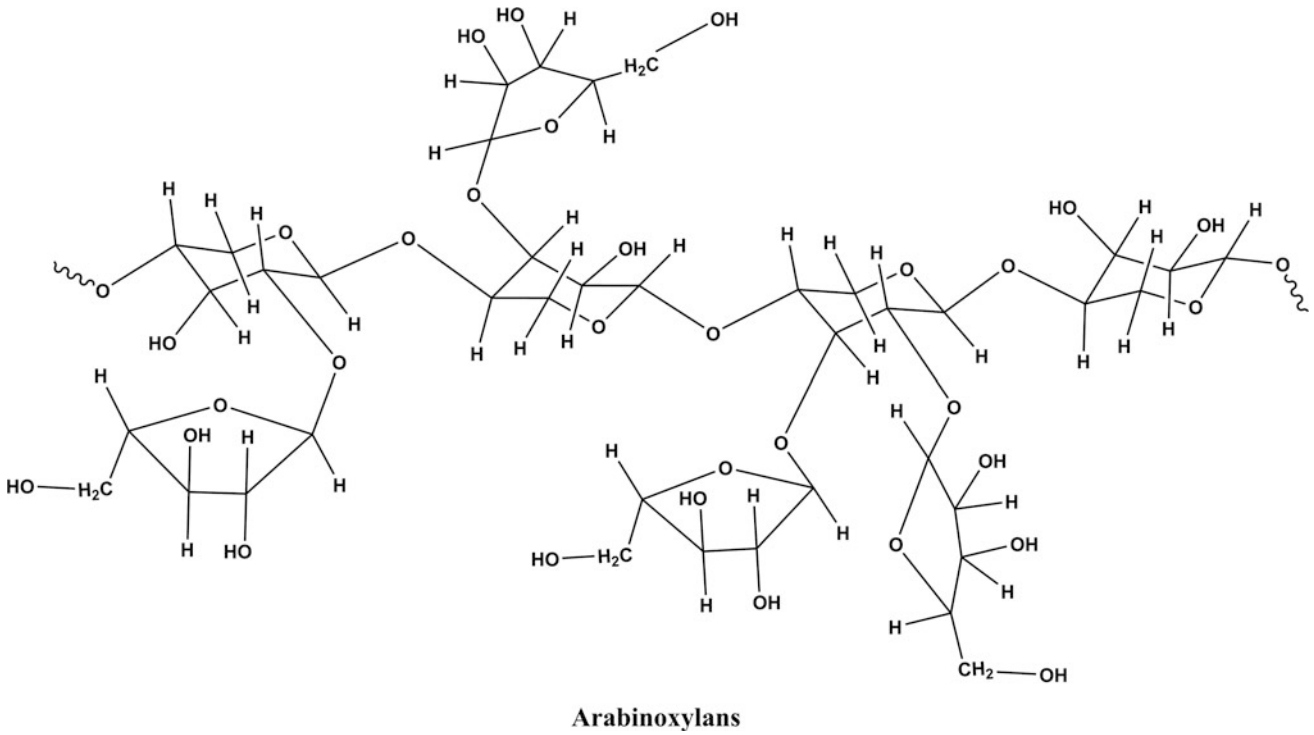

Fig. 13.4 Structure of arabinoxylans

non-classical heating modes such as microwave and ultrasound [126, 127]. However, these procedures with longer treatment times and higher temperatures can lead to degradation of the polysaccharides [127, 128]. The extraction yield of mucilage is also dependent on both the procedure and the variety of the raw material [129]. Linseeds contain approximately 28 % fibre, of which 70–80 % is water-soluble with most of these compounds present in the outermost layer of the seed hull [124]. The kernel of linseeds contains about 20 % fibre [130]. The major constituents of linseed mucilage are neutral and acidic polysaccharides, with the neutral fraction of the linseed mucilage composed mainly of arabinoxylans (Fig. 13.4) [131]. Linseed mucilage is used in the food industry to be incorporated into the daily diet in beverages, dairy products, bakery products and processed foods including thickeners, emulsifiers, stabilisers and fat replacers [130].

Human Studies

Linseed also consists of insoluble (viscous) dietary fibre that was found to play a role in cholesterol regulation. A study comparing three different diets, a low-fibre control diet, a diet with linseed fibre drink and a diet with linseed fibre bread for seven days, showed that a diet with 5 g dietary linseed fibre lowered fasting total cholesterol and LDL-cholesterol by 12 and 15 %, respectively, as compared to control group [132]. Linseed drink increased faecal excretion of energy and fat excretion by 23 and 55 %, respectively. Linseed bread ingestion was less effective than linseed drink due to the effect of differences in food matrix as baked products had less ability to induce viscosity due to processing and storage [132]. These results were consistent with a study showing that consumption of six wheat flour chapattis containing linseed gum (5 g) for three months by type 2 diabetic patients lowered fasting blood glucose, total cholesterol and LDL-cholesterol [133].

An intervention of rye bread enriched with whole linseed (10 %) was tested for 7 days in 11 healthy young men to measure the apparent digestibility of fat and the gastric transit time [49]. Linseed rye bread results in an increase in the faecal fat excretion. The possible mechanisms proposed from the results were inhibition of the pancreatic lipase activity, or binding of fat in the gastrointestinal tract by linseed. Linseed mucilage is proposed to be responsible for these effects as arabinoxylans present in linseed showed faecal bulking effects [49].

In a double-blind randomised crossover 7-h test meal study, 18- to 40-year-old males were given diets containing low dietary fibre, whole linseed, low dose mucilage and high dose mucilage [124]. This study identified the role of these interventions on the changes in plasma metabolic profile including glucose, triglycerides, non-esterified fatty acids and

insulin concentrations during 7 h. High dose mucilage meal reduced the area under the curve for triglycerides and non-esterified fatty acid concentrations during 7 h. High dose mucilage meal also provided greater feeling of satiety with less hunger and food intake compared to low-fibre meal [124]. High dose mucilage-containing meal exhibited a slower increase in ghrelin towards the end of the day indicating a delayed gastric emptying affected by a higher dietary fibre content of the meal. In addition, these effects may be due to the impaired dietary triglyceride absorption from the small intestine and increasing clearance of chylomicron particles and chylomicron remnant uptake, as well as impaired emulsification of lipids [124]. Other mechanisms may involve direct fat-binding and increased viscosity resulting in increased emulsified lipid droplet size and decreased lipolysis due to decreased insulin concentrations. Fermentation of linseed fibre in distal ileum may result in short chain fatty acids which may play a role in reducing the non-esterified fatty acids along with the reduction in the rate of glucose uptake leading to a decreased demand for insulin to match the rate of glucose uptake in peripheral tissues [124]. Another study suggests the plausible mechanism of the involvement of linseed fibre in interference with bile acid metabolism which hinders micelle formation and inhibits re-uptake of bile acids that leads to increased hepatic synthesis of bile acids, thus reducing cholesterol concentrations [132].

Rodent Studies

In male Wistar rats, dietary fibres including extracted linseed fibre and whole linseed fibre were tested for 21 days. Increased faecal fat excretion and reduced body fat and energy digestibility were seen in rats given extracted linseed fibre that in consequence suppressed body weight gain. There results were not seen in the rats that were given dietary fibre from cellulose, whole linseed or ground linseed [134]. The decreased energy availability from linseed fibre could be the reason for the decrease in the body weight. Increased content in caecum and colon was also observed in these rats, indicating increased fermentation with extracted linseed fibre [134].

Conclusion

Linseeds have been used for millennia as food and medicine, but the evidence supporting their use as a functional food for the treatment of obesity is relatively recent. Obesity is a complex disorder, so it is unlikely that a single compound will reverse the multi-organ effects. Linseeds provide ALA as an essential n-3 fatty acid, SDG as a lignan as well as fibre mainly as mucilage. These constituents have multiple biological effects to decrease the changes in obesity, probably by different mechanisms. The combination as linseeds can therefore be accurately described as a functional food. The full potential of linseeds in human obesity needs to be defined, and further studies on molecular mechanisms are also necessary.

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Role of Omega-3 Fatty Acids in Metabolic Syndrome

14

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Abbreviations

AUC	Area under curve
CAVI	Cardio-ankle vascular index
CETP	Cholesteryl ester transfer protein
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
DBP	Diastolic blood pressure
EPA	Eicosapentaenoic acid
EE	Ethyl ester
FBI	Fasting blood insulin
FBG	Fasting blood glucose
FFA	Free fatty acids
FMD	Flow-mediated dilatation
HbA1c	Glycosylated hemoglobin
HDL	High-density lipoprotein
ICAM-1	intracellular adhesion molecule 1
IDL	Intermediate-density lipoprotein
IL	Interleukin
LDL	Low-density lipoprotein
sdLDL	Small-density LDL
PWV	Pulse wave velocity
MetS	Metabolic syndrome
n-3 PUFAs	Long-chain omega-3 polyunsaturated fatty acids
RLP	Remnant lipoprotein particle
SBP	Systolic blood pressure
TG	Triglycerides

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TNF-α	Tumor necrosis factor-α
TNFR	Tumor necrosis factor receptor
TRL	Triglyceride rich lipoprotein
VCAM-1	Vascular cell adhesion molecule 1
VLDL	Very-low-density lipoprotein

Obesity and Metabolic Syndrome

The incidence of the metabolic syndrome (MetS) is increasing at an alarming rate, becoming a major public and clinical problem worldwide. MetS is defined as a cluster of pathophysiological conditions, which include abnormalities in blood pressure, low high density lipoprotein-cholesterol (HDL-ch), high triglycerides (TG), altered glucose tolerance, and increased waist circumference, raising the risk of cardiovascular disease (CVD) and diabetes [1, 2]. Different international organizations have attempted to determine the cutoff for the MetS criteria in adult population, and although there is a consensus in almost all parameters, discrepancies on the cutoff for waist circumference continue [3]. Despite the genetic variations between populations, it is widely accepted that the appearance of MetS is closely related to obesity, inappropriate dietary behaviors, and sedentary lifestyle [1].

The imbalance in the carbohydrate and lipid metabolism in MetS patients is characterized by impaired insulin response, high plasma-free fatty acids (FFA) concentration, hypertriglyceridemia, low levels of HDL-ch, and changes in the composition of both lipoproteins and apolipoproteins; all these alterations work together to deteriorate cardiovascular health and to increase the risk of cardiovascular disease [4].

Initially, in an attempt to restore glucose metabolism, the hyperinsulinemia is the first stage that precedes insulin resistance, and when the insulin signaling is not enough to promote glucose uptake in peripheral tissues, the increment in the glucose production triggers type 2 diabetes mellitus [5]. In a normal state, the insulin secreted by the β-cells of pancreas is able to suppress the release of fatty acids by adipose tissue and to decrease the concentration of lipoprotein lipase in peripheral tissues [4]; however, in an insulin resistance condition, this mechanism fails, promoting an abnormal release of FFA into circulation which in turn favors their flux and accumulation in key energetic organs as the liver [5]. The increment of lipids in the liver may raise the production of TG and the release of triglyceride-rich lipoproteins (TRLs) such as very low density lipoprotein (VLDL), favoring an atherogenic state. Thus, hypertriglyceridemia is associated with the predominance of the atherogenic small-density low density lipoprotein (sdLDL) versus the highly buoyant less atherogenic LDL, as well as with low HDL-ch levels and high levels of LDL-ch [4, 5]. Furthermore, there is a disproportion between the TG synthesis and TG clearance, which favors the increment in VLDL proportion, leading to the augmentation of the apolipoprotein B (apoB) production and of the remnant lipoprotein particle concentration [6].

Additionally, in MetS, there are other factors that are implicated in lipid metabolism disorders as the increment in the synthesis of apolipoprotein C-III (apo C-III) and the cholesteryl ester transfer protein (CETP). The apo C-III is carried through VLDL particles and could disrupt the action of lipoprotein lipase and inhibit the uptake of VLDL remnants by liver receptors [7]. Also, the increased activity of CETP promotes the transfer of TG from VLDL to LDL, resulting in triglyceride-rich LDL, which are the favorite substrate of hepatic lipase, leading to the formation of sdLDL particles [6], which are more likely to be filter into the arterial wall and are more susceptible to oxidation that the highly buoyant LDL particles [4, 7]. Increased levels of VLDL can also promote alterations in the composition of HDL, leading to the formation of sdLDL and increased catabolism of these particles [6].

The metabolic disturbances characterizing the MetS condition perturb the normal endothelial function, increasing the risk of cardiovascular diseases and promoting a vicious cycle in which the high blood pressure, hyperglycemia, hyperinsulinemia, oxidative stress, and inflammation are implicated [8, 9]. Taking into account the fact that endothelium is the major regulator of vascular homeostasis, the endothelial dysfunction is an important factor in the appearance of circulatory pathologies. The alterations in the renin–angiotensin–aldosterone system and the increment of the oxidative stress during hypertension are associated with the impairments of important vasodilator pathways in the endothelium leading to endothelial dysfunction, including alterations in the nitric oxide (NO) cycle [8–10]. Moreover, the increment in the arterial stiffness either in general population and in type 2 diabetic patients is a predictor factor to cardiovascular risk and mortality [11].

In summary, insulin resistance seems to be a major underlying mechanism responsible for the development of MetS, and a number of studies have also suggested that the low-grade chronic inflammatory state associated with obesity and MetS plays a key role in the development of insulin resistance and other metabolic complications [12].

n-3 PUFAs in Obesity and Metabolic Syndrome

The long-chain omega-3 polyunsaturated fatty acids (n-3 PUFAs) are essential nutrients which can be divided according to their natural food source in plant-derived (alpha-linolenic acid, C18:3) and marine-derived (eicosapentaenoic acid (EPA), C20:5 and docosahexaenoic acid (DHA), C22:6). The principal sources of alpha-linolenic acid are the vegetable oil (flaxseeds, canola, soybean), while EPA and DHA are found in fatty fish (salmon, herring, mackerel, and sardines) [13]. Although it has been suggested that alpha-linolenic acid is converted to EPA and DHA, the conversion rate is controversial and apparently modest [14, 15], and the beneficial effects of n-3 PUFAs in cardiovascular health have been principally attributed to the marine-derived EPA and DHA.

In patients with MetS, decreased levels of erythrocyte content of EPA and DHA and increased ratio of n-6/n-3 have been observed, being associated with the development of insulin resistance [16]. Additionally, different studies have observed that high consumption of fish, especially fatty fish rich in n-3 PUFAs, is inversely correlated with MetS [17].

In this context, the consumption and supplementation of n-3 PUFAs for the prevention and amelioration of MetS features have been proposed. In this chapter, we will focus on reviewing randomized, controlled trials that evaluate the effects of supplementation with marine-derived EPA and DHA. We excluded the studies that used functional products enriched with n-3 PUFAs, also trials with population with previous cardiovascular event or heart disease, and studies with subjects under medication that could affect the target metabolic outcomes as lipid-lowering agents, antihypertensive, and hypoglycemic drugs.

Effects of n-3 PUFAs on Weight Loss and Body Composition

Moderate weight loss (5–10 % of body weight) can help to restore insulin sensitivity and greatly reduce MetS features. Several studies have evaluated the effects of n-3 PUFAs supplementation on weight loss, rising to contradictory outcomes (Table 14.1). The majority of trials with n-3 PUFAs supplementation have been performed using a combination of EPA and DHA with or without an energy-restricted regime and with and without exercise.

n-3 PUFAs Trials with Isocaloric Diets

The study by Ebrahimi et al. [18] in Iranian males and females, giving a general dietary advice to both study groups and using a supplementation of 1 g/day of fish oils (containing 180 mg EPA and 120 mg DHA) for 6 months, observed that n-3 PUFAs promoted a reduction in body weight. Moreover, the

Table 14.1 Clinical trials evaluating the effects of n-3 PUFA supplementation on body weight loss and anthropometric parameters

Study design	n-3 PUFAs treatment	Outcomes	References
Randomized, placebo-controlled trial with parallel design in hyperinsulinaemic overweight or obese females (n = 93; age: 21–69 year)	1.3 g/day EPA + 2.9 g/day DHA (EE capsules) for 24 weeks (first 12 weeks with an energy-restricted diet followed by the maintenance period)	↔ Body weight	[28]
Randomized, double-blind, placebo-controlled trial with parallel design in healthy obese females and males (n = 32; age: 18–60 year)	6 × 1 g capsules/day (70 mg EPA + 270 mg DHA per fish tuna oil capsule) for 4 weeks (first 4 weeks with a VLCD of 3000 kJ/day with a meal replacement food followed by the maintenance period)	↔ Body weight, fat mass, fat-free mass, waist and hip circumferences, waist to hip ratio	[29]
Randomized, double-blind, placebo-controlled trial with parallel design in healthy females and males (n = 33; age: 18–60 year)	6 × 1 g capsules/day (70 mg EPA + 270 mg DHA per fish tuna oil capsule) for 12 weeks (with an energy-restricted diet of 5000 kJ for females and 6000 kJ for males)	↔ Body weight, fat mass, fat-free mass, waist and hip circumferences	[30]
Randomized, controlled trial with parallel design in overweight and obese healthy males and females (n = 64; age: 18–60 year)	180 g/week of fatty fish or 1 g/day of fish oil capsule (420 mg EPA + 210 mg DHA) for 12 months (with an energy restriction of 2 MJ and dietary education)	↔ Body weight, fat mass	[24]
Randomized, controlled trial with parallel design in overweight and obese healthy females and males (n = 324; age: 20–40 year)	150 g cod 3 times/week; 150 g salmon 3 times/week or 1.3 g/day of fish oil capsules (EPA + DHA) for 8 weeks (with a personalized energy-restricted diet <30 % kcal)	↓ Body weight and waist circumference (only in males)	[31]

(continued)

Table 14.1 (continued)

Study design	n-3 PUFAs treatment	Outcomes	References
Randomized, double-blind, placebo-controlled trial with parallel design in overweight and obese healthy females and males (n = 36; age: 18–60 year)	6 × 1 g capsules/day (70 mg EPA + 270 mg DHA per fish tuna oil capsule) for 8 weeks (with prior 4-week n-3 PUFAs supplementation followed by a VLCD of 3000 kJ/day with a meal replacement food)	↓ Body weight and BMI (only in females) ↔ Fat mass, fat-free mass, waist and hip circumferences	[27]
Randomized, controlled trial with parallel design in overweight and obese females with MetS (n = 136, age: 30–65 year)	2130 mg/day of n-3 PUFAs (1280 mg EPA + 850 mg DHA) for 12 weeks (calorie restriction of 500–800 kcal/d with or without meal replacement diet)	↔ Body weight, BMI, waist circumference, fat mass, fat-free mass	[25]
Randomized, single-blind, trial with parallel design in obese females and males with MetS (n = 52 [34]; n = 60 [35]; age: 51.9 ± 2.55 year)	1.8-g EPA-rich capsules (>98 % as EE) for 3 months (energy-restricted diet 25 kcal/kg ideal body weight)	↔ BMI and waist circumference	[34, 35]
Randomized, double-blind, placebo-controlled trial in overweight and obese healthy females (n = 77; age: 20–45 year)	1.3-g EPA-rich capsules (80 % as EE) for 10 weeks (energy-restricted diet <30 %)	↓ Waist-to-hip ratio (moderately) ↔ Body weight, fat mass, lean mass, waist and hip circumferences	[36]
Randomized, placebo-controlled trial in severely obese women (n = 20; age: 54.27 ± 5.36 year in n-3 PUFAs group and 49.78 ± 12.35 in placebo group)	2.98 g/day of n-3 PUFAs (EPA + DHA at a ratio of 2:1) for 3 weeks (with a VLCD of 2200 kJ/day and light-to-moderate physical activity 60 min/day)	↓ Body weight, BMI, hip circumferences ↓ Fat oxidation ↔ Waist circumference	[32]
Randomized, placebo-controlled trial with parallel design in overweight and obese females and males, 30 % with MetS (n = 81; age: 45.6 ± 8.3 year in n-3 PUFAs group and 47.0 ± 7.8 year in placebo group)	5 capsules/day (3 g/day of EPA + DHA at a ratio of 5:1 in a 60 % concentration) for 24 weeks (with exercise and dietary counseling)	↔ Body weight, body fat mass, waist circumference	[26]
Randomized, controlled trial with parallel design in obese females and males with MetS (n = 65; age: 47.9 ± 9.98 year)	3 g/day of n-3 PUFAs (180 mg EPA plus 120 mg DHA per capsule) for 90 days (usual diet)	↔ Body weight and waist circumference	[21, 22]
Randomized, double-blind, placebo-controlled, crossover trial in females and males with MetS (n = 29; age: 26–70 year)	2 g/day of n-3 PUFAs (46 % EPA, 38 % DHA) for 12 weeks with 4-week washout period (usual diet)	↔ Body weight	[23]
Randomized, controlled trial with parallel design in overweight and obese females and males with MetS (n = 89; age: 40–70 year)	1-g capsules/day of n-3 PUFAs (180 mg EPA and 120 mg DHA) for 6 months (general dietary advice)	↓ Body weight	[18]
Randomized, placebo-controlled trial with parallel design in females and males with at list one risk factor: mild hypertension, elevated TG or elevated total-ch (n = 65 (67 % males), age: 25–65 year)	6 g/day of tuna fish oil (60 mg EPA + 260 mg DHA per gram) for 12 weeks (usual diet with or without 3 times/week of 45-min exercise)	↓ Fat mass ↔ Body weight	[20]
Randomized, double-blind, placebo-controlled trial with parallel design in postmenopausal women with T2DM, 50 % with hypoglycemic treatment (n = 26; age: 40–60 year)	1.8 g/day of n-3 PUFAs (1.08 g EPA + 0.72 g DHA) for 2 months of run-in period followed by 2-month treatment (dietary counseling)	↓ Fat mass ↓ Adipocyte diameter ↔ Body weight	[19]

BMI body mass index; *DHA* docosahexaenoic acid; *EE* ethyl ester; *EPA* eicosapentaenoic acid; *MetS* metabolic syndrome; *n-3 PUFAs* omega-3 polyunsaturated fatty acids; *T2DM* type 2 diabetes mellitus; *VLCD* very-low-calorie diet

trials by Kabir et al. [19] providing 1.8 g/day of n-3 PUFAs (1.08 g EPA + 0.72 g DHA) found no changes in body weight but a reduction in fat mass and adipocyte diameter. Similarly, the study by Hill et al. [20] evaluated the effects of n-3 PUFAs (6 g/day of tuna fish oil containing 260 mg DHA and 60 mg EPA per gram) for 12 weeks in combination or in the absence of a regular exercise (3 times/week), finding that supplementation with fish oil promoted a decrease in fat mass independently of the exercise effect.

In contrast, a 90-day short-time study conducted in Brazilians with a supplementation of n-3 PUFAs in a dose of 3 g/day (180 mg EPA plus 120 mg DHA per capsule) found

no effects of supplementation in body weight and waist circumference [21, 22]. Similarly, the study by Tousoulis et al. [23] in Caucasic males and females showed that the supplementation of the usual diet with 2 g/day of n-3 PUFAs (46 % EPA and 38 % DHA) during 12 weeks with a washout period of four weeks between treatments did not promote a reduction in body weight.

n-3 PUFAs Trials with Hypocaloric Diets

While some trials have reported that supplementation of hypocaloric diets with n-3 PUFAs may promote additional weight loss, most of the studies have not found body weight-lowering properties for n-3 PUFAs (Table 14.1). Thus, the study by Tapsell et al. [24] found no additional effects on body weight loss in Australian overweight or obese adults consuming fatty fish or fish oil supplementation (180 g fatty fish alone or plus 1 g/day of fish oil capsule containing 420 mg EPA and 210 mg DHA) for 12 months. Additionally, the study by Su et al. [25] in Taiwanese women with a calorie-restricted diet supplemented with 2.13 g/day of n-3 PUFAs showed that the treatment had no additional effects on anthropometry and body composition variables. DeFina et al. [26] using a supplementation of n-3 PUFAs (3 g/day EPA plus DHA at a ratio of 5:1) in conjunction with diet and exercise during 24 weeks observed no effects of supplementation in weight loss and body composition in overweight/obese subjects. Also, the study by Munro and Garg [27] supplementing 6 × 1 g capsules/day containing 70 mg EPA plus 270 mg DHA for 12 weeks in conjunction with an energy-restricted diet found no differences in the body weight and waist circumference losses.

Some trials have addressed the effects of n-3 PUFAs supplementation not only during a weight loss period but also during a weight maintenance phase. Thus, the study by Krebs et al. [28], which lasted 24 weeks and included first five weeks on an energy-restricted diet, followed by a maintenance period in the subsequent weeks, observed no differential effect on weight loss with n-3 PUFAs supplementation (1.3 g/day of EPA plus 2.9 g/day of DHA) in overweight/obese women. In the same way, Munro and Garg [29] reported that supplementation for 14 weeks with n-3 PUFAs (6 × 1 g capsules/day containing 70 mg EPA plus 270 mg DHA) during a very-low-energy-restricted diet (4 weeks), followed by a maintenance period of 10 weeks, did not promote a greater decrease in anthropometric measurements and fat mass in obese subjects.

Interestingly, another trial of the same group reported that a prior supplementation during 4 weeks with n-3 PUFAs (6 × 1 g capsules/day containing 70 mg EPA plus 270 mg DHA) followed by a 4-week very-low-energy-restricted diet promoted a greater reduction in weight than placebo but only in healthy overweight/obese women, suggesting that supplementation with n-3 PUFAs had a time-dependent effect on weight loss, especially in females [30]. Some studies have suggested a sex-based interaction of n-3 PUFAs. In this context, the trial by Thorsdottir et al. [31], performed in overweight or obese adults under a personalized energy-restricted diet during 8 weeks, observed a greater reduction in weight loss and waist circumference only in men, in the groups taking fish (150 g cod or salmon 3 times/week) or fish oil capsules (1.3 g/day EPA plus DHA). The study by Kunesova et al. [32], performed in severely obese women during 3 weeks with a very-low-calorie diet and light-to-moderate physical activity, also showed that the supplementation with n-3 PUFAs (2.98 g/day EPA plus DHA at a ratio of 2:1) promoted a decrease in body weight, body mass index (BMI), and hip circumference.

The potential differential effects of EPA and DHA on weight loss and body composition have not been clearly addressed. Interestingly, the study by Kunesova et al. [32] found a significant negative correlation between BMI change and phospholipid docosahexaenoic acid change, suggesting that docosahexaenoate (22:6n-3) seems to be the active component. In this context, the study by Vasickova et al. [33] has also suggested a possible beneficial effect of DHA intake on body weight reduction in obese children that consumed an extra 300 mg DHA and 42 mg EPA daily for a period of 3 weeks. Regarding EPA, the Japan Obesity and Metabolic Syndrome Study, a randomized, single-blind, parallel design trial with a supplementation of 1.8 g/day of highly purified (>98 %) EPA in combination with a hypocaloric diet (25 kcal/kg ideal body weight) during 3 months, did not find changes in BMI or waist circumference [34, 35]. In concordance with this, the study by Huerta et al. [36] with a supplementation of 1.3 g EPA-rich capsules (80 %) shows no additional effect of body weight on the energy-restricted diet in the supplemented groups, although EPA promoted a moderate decrease in the waist-to-hip ratio.

n-3 PUFAs and Carbohydrate Metabolism in Metabolic Syndrome

Although some evidence in murine models and in overweight or obese healthy subjects has suggested that the supplementation with n-3 PUFAs could promote an improvement in insulin sensitivity [37], most of the trials in subjects with MetS characteristics have observed that supplementation with n-3 PUFAs, at different ratios of EPA: DHA and at different doses (1–4 g), has no significant effect on decreasing glucose metabolism parameters or improving the insulin sensitivity [23, 25, 26, 28, 38–46]. In contrast, the study by Ramel et al. [47], performed in overweight/obese young adults following an energy-restricted diet, detected

that the supplementation with fish oil (EPA + DHA) improved insulin sensitivity independently of changes in body weight, TG, or adiponectin. Table 14.2 summarizes the clinical trials assessing the effects of n-3 PUFAs supplementation on glucose metabolism in subjects with MetS features.

Because some studies have suggested that EPA and DHA have different hemodynamic and metabolic effects, randomized trials evaluating the supplementation of both n-3 PUFAs separately are discussed. In this context, studies using a supplementation of DHA (2–3 g/day) in conjunction with isocaloric diet did not find any effect on glucose parameters in subjects with MetS features [41, 48]. Moreover, the study by Mori et al. [49], which evaluated the differential effect of DHA and EPA (4 g/day) on females and males following their usual diet, revealed that both EPA and DHA promoted

Table 14.2 Clinical trials assessing the effects of n-3 PUFAs supplementation on glucose and lipid metabolism and on inflammation, blood pressure, and endothelial function in subjects with MetS features

Study design	n-3 PUFAs treatment	Outcomes	References
Randomized, placebo-controlled trial in non-diabetic males and females with impaired glucose tolerance, impaired fasting glucose, or MetS (n = 34; age: 48 ± 2.3 years in n-3 group and 53.3 ± 2.2 years in placebo group)	4 g/day of fish oil capsules (1 g containing at least 465 mg EPA + 375 mg DHA as EE) for 12 months (diet: ns)	*Glucose metabolism* ↔ FBG, 2-h glucose, insulin sensitivity, first phase of insulin secretion *Lipid metabolism* ↓ TG ↔ Total-ch, LDL-ch, HDL-ch *Endothelial health* ↑ Capillaries in adipose tissue *Inflammation* ↓ Plasma MCP-1 and adipose tissue gene expression of MCP-1 and CD68 ↓ Decreased adipose tissue crown-like structures ↔ Plasma concentration of IL-6, IL-10, IL-12, TNF-α, resistin, PAI-1, and leptin; adipose tissue expression of TNF-α, IL-1, IL-12, and IL-6	[44]
Randomized, double-blind, placebo-controlled, crossover trial in females and males with MetS without statin treatment (n = 29; age: 44 ± 12 years)	2 g/day of n-3 PUFAs (46 % EPA + 38 % DHA) for 24 weeks with 4-week washout period at week 12 (usual diet)	*Glucose metabolism* ↔ FBG *Lipid metabolism* ↓ TG ↓ Total-ch, LDL-ch ↔ HDL-ch *Endothelial health* ↑ FMD ↓ PWV *Inflammation* ↓ IL-6 ↑ PAI-1	[23]
Randomized, single-blind, placebo-controlled trial with parallel design in females and males with MetS, without antihypertensive drugs (n = 98; age: 50 ± 10 years)	Low and high doses of ALA (2.2 g/day and 6.6 g/day, respectively); low and high doses of fish oil 1.2 g/day (700 mg EPA + 600 mg DHA as TG) and 3.6 g/day (2.1 g/day EPA + 1.5 g/day DHA as TG), respectively Duration: 8 weeks with previous 4-week run-in period (usual diet)	*Glucose metabolism* ↔ FBG, FBI *Lipid metabolism* ↓ TG ↑ LDL-ch ↔ Total-ch, HDL-ch *Blood pressure* ↓ SBP and DBP *Inflammation* ↔ MCP-1, IL-6, sICAM-1	[40]
Randomized, double-blind, placebo-controlled trial with parallel design in females and males without hypertension (n = 84; age: 25–70 years)	4 g/day of fish oil capsules (containing 60 % of n-3 PUFAs with 367 mg EPA + 255 mg DHA per capsule) for 12 weeks (usual diet)	*Glucose metabolism* ↔ FBI, HOMA-IR, HbA1c *Lipid metabolism* ↓ TG, apo B-48 ↔ apo B ↔ Total-ch, LDL-h, FFA ↑ HDL-ch	[42]
Randomized, double-blind, placebo-controlled trial with parallel design in hypertriglyceridemic males with MetS features (n = 34; age: 39–66 years)	7.5 g/day of DHA oil (containing 3 g/day of DHA) for 90 days (usual diet). Test breakfast with a total intake of 850 kcal (3553 kJ)	*Glucose metabolism* ↔ FBG, FBI, HOMA-IR, Matsuda index, glucose, or insulin AUC *Lipid metabolism* ↓TG ↓Postprandial TG ↓ apo CIII ↓ RLP-TG ↓ Diameter of VLDL-ch particles ↓ Ratio of TG:HDL-ch	[41, 55, 58, 63]

(continued)

Table 14.2 (continued)

Study design	n-3 PUFAs treatment	Outcomes	References
		↓ sdLDL, LDL particle size ↓ AUC for small LDL, small HDL, and large VLDL particles ↑ AUC for large LDL, large HDL, and small VLDL particles ↔ Total-ch, HDL-ch, apo A1, apo B, apo E ↓ FFA *Inflammation* ↓ CRP, IL-6 ↑ MMP-2 ↔ NO, IL-1β, IL-2, IL-10, TNF-α, SAA ↔ ICAM-1, VCAM-1, E-selectin	
Randomized, double-blind, placebo-controlled trial with parallel design in abdominally obese men with dyslipidemia (n = 39–48; age: 53.5 ± 9 years)	4 g/day of fish oil (45 % EPA + 39 % DHA as EE) either with atorvastatin placebo or 40 mg/day of atorvastatin. Duration: 3-week run-in period with usual diet following 6-week treatment (usual diet)	*Glucose metabolism* ↔ FBG, HOMA-IR *Lipid metabolism* ↓ TG ↓ VLDL, VLDL production rate ↑ VLDL conversion rate to LDL and IDL ↑ IDL conversion rate to LDL ↔ VLDL apo C-III pool size, VLDL apo C-III kinetic parameters ↓ Production and catabolic rate of apo A-I and apo A-II ↔ Apo A-I, apo A-II, apo B ↔ Apo C-III ↔ Total-ch, LDL-ch ↑ HDL-ch and HDL_2-ch ↔ HDL_3-ch ↔ non-HDL-ch, LDL-ch, RLP-ch ↔ FFA *Inflammation* ↔ CRP, TNF-α, IL-6	[39, 51–53, 57]
Randomized, placebo-controlled trial with parallel design in females and males with at least one risk factor: mild hypertension, elevated TG, or elevated total-ch (n = 65 (67 % males), age: 25–65 years)	6 g/day of tuna fish oil (60 mg EPA + 260 mg DHA per gram) for 12 weeks (usual diet with or without 3 times/week of 45-min exercise)	*Lipid metabolism* ↓ TG ↑ HDL-ch *Endothelial health* ↑ FMD	[20]
Randomized, single-blind with parallel design in normotensive, centrally obese, dyslipidemic, and insulin-resistant females and males (n = 99, age: 18–75 years)	4 g/day of n-3 PUFAs (46 % EPA and 38 % DHA as EE) for 16 weeks (12-week energy-restricted diet followed by 4-week maintenance period)	*Glucose metabolism* ↔ FBG, FBI, HOMA-IR *Lipid metabolism* ↓ TG ↓ Postprandial TG and apo B48 response ↓ VLDL, apo B100, apo B48 ↔ LDL-ch *Blood pressure/endothelial health* ↓ SBP, heart rate ↔ DBP Improved arterial elasticity (measured by C1 and C2) *Inflammation* ↔ Adiponectin	[45, 46]
Randomized, placebo-controlled trial with parallel design in hyperinsulinaemic overweight or obese females (n = 93; age: 21–69 years)	1.3 g/day EPA + 2.9 g/day DHA (EE capsules) for 24 weeks (first 12 weeks with an energy-restricted diet followed by the maintenance period)	*Glucose metabolism* ↔ FBG, FBI, HbA1c, HOMA-IR ↔ Glucose and insulin AUC *Lipid metabolism* ↓ TG ↔ Total-ch, LDL-ch, HDL-ch *Endothelial health* ↔ SBP, DBP *Inflammation* ↑ Adiponectin ↔ TNF-α, IL-6, CRP, leptin	[28]
Randomized, controlled trial with parallel design in females with MetS (n = 136, age: 30–65 years)	2130 mg/day of n-3 PUFAs (1280 mg EPA + 850 mg DHA) for 12 weeks (calorie restriction of 500–800 kcal/d with or without meal replacement diet)	*Glucose metabolism* ↔ FBG, FBI, HOMA-IR *Lipid metabolism* ↔ TG ↔ Total-ch, HDL-ch ↓ LDL-ch (with the meal replacement diet) ↓ MetS severity (with the meal replacement diet)	[25]

(continued)

Table 14.2 (continued)

Study design	n-3 PUFAs treatment	Outcomes	References
Randomized, double-blind, placebo-controlled trial with crossover design in dyslipidemic females and males, some with moderate hypertension or with MetS (n = 20; age: 52 ± 12 years)	8 fish oil capsules/day providing a total of 3.7 g n-3 PUFAs (1.7 g EPA + 1.2 g DHA) for 12 weeks with 2-week washout period at week 6 (usual diet)	*Glucose metabolism* ↔ FBG *Lipid metabolism* ↓ TG ↓ VLDL particles ↑ LDL-ch, sdLDL-ch, large LDL-ch ↔ HDL-ch ↔ FFA *Inflammation* ↔ TNFR1, TNFR2, CRP, TNF-α, IL-6, sICAM-1, sVCAM-1, MCP-1, sE-selectin	[38]
Randomized, double-blind, placebo-controlled trial in hyperlipidemic females and males (LDL-ch: 130–220 mg/dL and TG: 150–400 mg/dL) without lipid-lowering medication (n = 26; age 57 ± 4 years (Placebo); 61 ± 4 years (DHA 1.5); 58 ± 4 years (DHA 3.0))	DHA at 2 different doses: 2.5 g/day of DHA and 1.25 g/day of DHA for 6 weeks of run-in phase stabilization period (National Cholesterol Education Program step I diet with <30 % fat) following by 6-week intervention	*Lipid metabolism* ↓ TG ↑ non-HDL-ch, LDL-ch (only the highest dose) ↔ HDL-ch	[54]
Randomized, double-blind, placebo-controlled trial with parallel design in hyperlipidemic females and males (n = 38; age: 40–69 years)	2 groups supplemented with 4 g/day of n-3 PUFAs: one supplemented with EPA (3.05 g EPA per day with 85 % EPA capsules as EE) and the other with DHA (2.84 g DHA + 0.52 g DPA with 70.7 % DHA capsules as EE) for 2 weeks of familiarization period followed by 7-week treatment (usual diet)	*Lipid metabolism* ↓ TG ↓ VLDL-TG ↔ Total-ch, LDL-ch, HDL-ch *Endothelial health* ↔ SBP, DBP ↑ Systemic arterial compliance No differences between EPA and DHA treatments	[56]
Randomized, double-blind, placebo-controlled trial with parallel design in mildly hyperlipidemic, normotensive men without antihypertensive and lipid-lowering drugs (n = 56, age: 20–65 years)	2 groups supplemented with 4 g/day of n-3 PUFAs: EPA (96 % as EE) and DHA (92 % as EE) for 3-week run-in period followed by 6-week treatment (usual diet)	*Glucose metabolism* ↑ FBI ↔ FBG *Lipid metabolism* ↓ TG ↑ LDL-ch (only DHA) ↑ LDL-ch particle size (only DHA) ↑ HDL_2-ch (only DHA) ↔ Total-ch, HDL-ch (only DHA) *Endothelial health* ↓ DBP, SBP (only DHA) ↓ Ambulatory SBP and SBP (only DHA) ↓ Ambulatory and fasting heart rate (only DHA group) ↑ Vasodilatory response (only DHA) ↓ Constrictor response (only DHA)	[49, 61]
Randomized, placebo-controlled trial with parallel design in overweight and obese females and males, 30 % with MetS (n = 81; age: 45.6 ± 8.3 years in n-3 PUFA group and 47.0 ± 7.8 years in placebo group)	5 capsules/day (3 g/day of EPA + DHA at a ratio of 5:1 in a 60 % concentration) for 24 weeks (with exercise and dietary counseling)	*Glucose metabolism* ↔ FBG, FBI *Lipid metabolism* ↑ LDL-ch ↔ HDL-ch, TG *Endothelial health* ↔ SBP, DBP	[26]
Randomized, crossover trial in overweight or obese women (n = 32) categorized into 2 groups: high-inflammatory status/low-inflammatory status based on serum SA	Fish oil [5 × 1 g capsules/day (1.3 g EPA, 2.9 g DHA)] for 12-week intervention, 4-week washout, and 12-week intervention	*Glucose metabolism* ↔ FBG, FBI *Lipid metabolism* ↓ TG ↔ Total-ch, HDL-ch, LDL-ch *Inflammation* ↓ IL-6, CRP ↔ PAI-1, SAA	[50]
Randomized, controlled dietary intervention in overweight/obese adults (n = 324; age: 20–40 years)	30 % caloric restriction. 150 g cod or 150 g salmon, three times/week or 3 g/day of fish oil capsules (DHA/EPA) for 8 weeks	*Glucose metabolism* ↑ insulin sensitivity	[47]
Randomized, double-blind, placebo-controlled trial with parallel design in overweight/obese females and males (n = 36; age: 18–65 years)	21-day run-in period with 5 mL/d of placebo supplement. 5 ml/d of DHA from microalgae *Crypthecodinium cohnii*, EPA free (2 g/day DHA) for 133 days. During the study period, an isocaloric diet was provided throughout standardized food products (34 % E fat, 51 % E carbohydrate, 15 % E protein)	*Glucose metabolism* ↔ FBG, FBI, HOMA-IR, HbA1c, glucose, and insulin AUC *Lipid metabolism* ↓ TG ↓ VLDL-TG ↓ Particle diameter for VLDL, LDL, HDL ↓ Concentration of large and medium VLDL ↓ IDL ↓ sdLDL	[48]

(continued)

Table 14.2 (continued)

Study design	n-3 PUFAs treatment	Outcomes	References
		↑ Large LDL ↓ Medium HDL ↑ Large HDL ↔ Small HDL ↔ VLDL-ch, LDL-ch, total-ch, HDL-ch ↔ Concentration of small VLDL *Endothelial health* ↔ Ambulatory SBP and DBP *Inflammation* ↑ IL-10 ↔ IL-1β, IL-6, TNF-α, lipopolysaccharide-binding protein	
Randomized, double-blind, placebo-controlled trial with 3-period crossover design in hypertriglyceridemic males (88.5 %) and females (n = 26; age: 21–65 years)	n-3 PUFAs (≈465 mg EPA + ≈375 mg DHA at a ratio of 1.2:1 per gram as EE) at 2 different doses: 0.85 g/day EPA + DHA and 3.4 g/day EPA + DHA. Three 8-week intervention period (placebo; low DHA and high DHA) with 6-week washout period (usual diet)	*Glucose metabolism* ↔ FBG, FBI *Lipid metabolism* ↓ TG (only high dose) ↔ Total-ch, LDL-ch, HDL-ch *Endothelial health* ↓ Heart rate (more at the highest dose) ↓ Mean arterial pressure (only high dose) ↓ Stroke volume (only high dose) ↓ Cardiac output ↔ FMD and reactive hyperemia index *Inflammation* ↔ IL-1β, IL-6, TNF-α	[43, 60]
Randomized, double-blind, placebo-controlled trial with parallel design, followed by open-label period in hyperlipidemic females and males (n = 57 in the double-blind period and n = 42 in open-label period; age: 18–70 years)	*Double-blind period* 4 g/day of n-3 PUFAs (1.6 g free plant sterols + 1.3 g EPA + DHA as EE per day) for 12 weeks (usual diet) with previous 2-week run-in period *Open-label period* 2 g/day of n-3 PUFAs (0.8 g free plant sterols + 0.65 g EPA + DHA as EE per day) for 12 weeks of follow-up period (usual diet)	*Lipid metabolism* ↓ TG ↔ Apo A, apo B ↔ Total-ch, LDL-ch, HDL-ch, Total-ch/HDL-ch ratio ↔ TG and LDL-ch (in the open-label phase from endpoint value of the double-blind period) *Endothelial health* ↓ DBP ↔ SBP	[59]

AUC area under curve; *CAVI* cardio-ankle vascular index; *CRP* C reactive protein; *DBP* diastolic blood pressure; *DHA* docosahexaenoic acid; *EE* ethyl ester; *EPA* eicosapentaenoic acid; *FBI* fasting blood insulin; *FBG* fasting blood glucose; *FFA* free fatty acids; *FMD* flow-mediated dilatation; *HbA1c* glycosylated hemoglobin; *HDL* high-density lipoprotein; *IDL* intermediate-density lipoprotein; *ICAM-1* intracellular adhesion molecule 1; *IL* interleukin; *LDL* low-density lipoprotein; *sdLDL* small-density LDL; *LTB4* leukotriene B4; *PWV* pulse wave velocity; *MCP-1* macrophage chemoattractant protein 1; *MetS* metabolic syndrome; *NO* nitric oxide; *PAI-1* plasminogen activator inhibitor-1; *RLP* remnant lipoprotein particle; *SAA* serum amyloid A; *SBP* systolic blood pressure; *TG* triglycerides; *TNF-α* tumor necrosis factor-α; *TNFR* tumor necrosis factor receptor; *VCAM-1* vascular cell adhesion molecule 1; *VLDL* very-low-density lipoprotein

an increment in fasting insulin levels without significant changes in fasting glucose. The differential effects observed between trials with DHA supplementation could be due to the different n-3 PUFAs source (ethyl ester capsules or algae source), the isocaloric diet, and the dose of DHA used.

In summary, the evidence suggests that supplementation with n-3 PUFAs has none or marginal effects on glucose homeostasis and insulin sensitivity in subjects with MetS features.

N-3 PUFAs and Lipid Metabolism in Metabolic Syndrome

Effects on Triglycerides and Triglyceride-Rich Lipoproteins

Several trials performed in subjects with MetS features, using either different ratios of EPA:DHA or these marine-derived n-3 PUFAs separately, in combination or not with an energy-restricted diet and with a range duration between 6 weeks and 1 year, have evidenced the ability of n-3 PUFAs to decrease TG levels [23, 28, 39, 40, 42–44, 46, 48–56] along with the reduction in the TRL and remnant lipoprotein particle concentration [38, 45, 48, 53, 55, 57, 58] (see Table 14.2 for further information about these trials). Additionally, based on the present data, the detriment in TG blood concentration could promote a drop in both the production and concentration of some apolipoproteins, principally apo B48 and apo CIII [41, 42, 45]. Moreover, the supplementation with n-3 PUFAs has shown to improve the postprandial TG response characteristic of MetS subjects [45, 55]. However, in some few trials, this hypotriglyceridemic effect was not clearly perceived. Thus, Su et al. [25] observed a greater but non-significant reduction in TG levels with n-3 PUFAs supplementation in MetS females with an energy-restricted diet. Moreover, DeFina et al. [26] reported also a lack of additional effect on TG in females and males with n-3 PUFAs supplementation in conjunction with exercise and dietary counseling. However, it is important to mention that this study was conducted mainly in healthy

overweight to obese individuals (<30 % with MetS), which likely masked potential metabolic reduction in triglycerides in those with hypertriglyceridemia [26].

Effects on LDL-ch and Non-HDL-ch Fractions

According to the outcomes of trials summarized in Table 14.2, there is not a consensus about the effects of n-3 PUFAs regarding the LDL-ch fraction. Some studies in dyslipidemic subjects with MetS features have observed that supplementation with n-3 PUFAs could rise LDL-ch concentration [26, 38, 40, 49, 54], while others have found no effect [25, 28, 42–44, 50, 56, 59]. Furthermore, the data suggest that although the overall effect of n-3 PUFAs on LDL-ch remains unclear, the supplementation could decrease the concentration of the proatherogenic sdLDL-ch promoting the formation of the highly buoyant less atherogenic LDL-ch [41, 48, 49, 55]. Moreover, Chan et al. [57] concluded that the decrease in VLDL blood concentration observed in subjects supplemented with n-3 PUFAs was produced by an increase in the conversion rate of VLDL to LDL and intermediate-density lipoprotein (IDL) and by the amelioration in the VLDL production rate. Additionally, it has been proposed that n-3 PUFAs consumption could decrease VLDL particle diameter [48, 55]. The non-HDL-ch fraction was evaluated in one study, observing that in dyslipidemic patients following their usual diet, the supplementation of n-3 PUFAs (EPA + DHA) had no effect on the non-HDL-ch [51, 57].

Effects on HDL-ch

The overall effects of n-3 PUFAs in HDL-ch fraction remain unclear. Thus, some trials have found that the supplementation promoted an increment of HDL-ch [20, 42, 51, 52], while others have observed no relevant effects [23, 25, 26, 28, 38, 40, 43, 44, 49, 50, 54–56, 59]. Additionally, some studies have suggested that there could exist differential effects of n-3 PUFAs on the different HDL-ch subfractions, promoting an increase in HDL2-ch and a decrease in HDL3-ch [49, 52]. Also, Neff et al. [48] observed that n-3 PUFAs are capable of modifying HDL particle concentrations, decreasing medium HDL while increasing large HDL without changes in small HDL (Table 14.2).

N-3 PUFAs and Blood Pressure and Endothelial Health

Regarding the effects of n-3 PUFAs on blood pressure, studies using a supplementation between 1 and 2 g/day of n-3 PUFAs at different ratios of EPA:DHA with duration between 8 and 12 weeks have not found any significant effect on blood pressure [20, 25, 40, 60]. Moreover, the fish oil supplementation between 3 and 4 g/day with different ratios of EPA:DHA has reported contradictory outcomes (see Table 14.2). Thus, the trial by Wong et al. [45] found that 16-week supplementation with n-3 PUFAs promoted a significant reduction in systolic blood pressure (SBP) without additional changes to weight loss in diastolic blood pressure (DBP). In contrast, the study by Dewell et al. [40], which lasted 8 weeks, observed that the supplementation with n-3 PUFAs decreased both and mainly DBP. Also, Skulas-Ray et al. [60] concluded that n-3 PUFAs reduced the median blood pressure, but only at the highest dose tested (3.4 g/day). However, other trials have not observed any significant effect of n-3 PUFAs on blood pressure parameters [26, 28]. Although apparently the n-3 PUFAs (EPA + DHA) have no clear effect on blood pressure in the studies reviewed, the evidence suggests that they could improve cardiovascular health by promoting a decrease in arterial stiffness [23, 46] and by recovering endothelial dysfunction [20, 23, 56, 60].

Additionally, several trials have evaluated the effects of EPA and DHA separately. The study by Nestel et al. [56], performed in hyperlipidemic subjects, found an improvement in systemic arterial compliance without changes in blood pressure and no differences between both n-3 PUFAs (~3 g/day of EPA or DHA). In contrast, the study by Mori et al. [49, 61] in hyperlipidemic men with 4 g/day of DHA observed that supplementation decreased blood pressure and induced beneficial changes in ambulatory blood pressure and heart rate, as well as in vasodilator and constrictor responses, with no effect on EPA supplementation. Additionally, Neff et al. [48], using a supplementation of algal DHA at a dose of 2 g/day, showed no effects on either systolic and diastolic ambulatory blood pressures in dyslipidemic subjects.

The effects of n-3 PUFAs on blood pressure are in accordance with the European Food Safety Authority (EFSA) report. According to the evidence, the effects of n-3 PUFAs on blood pressure have been demonstrated with a dose of >3 g/day of EPA + DHA. Moreover, the response to n-3 PUFAs treatment is conditioned to the initial levels of blood pressure [62]. The population of the trials revised for this chapter includes subjects without and with hypertension, as a characteristic of the MetS; in this sense, the different outcomes found in trials with a supplementation between 3 and 4 g/day could be affected by the proportion of hypertensive and non-hypertensive populations and by the hypertension degree. Furthermore, the effects on endothelial health could be affected also by the diet consumed during the study.

n-3 PUFAs and Inflammatory Markers

The increment in the inflammatory factors and the excessive production of oxidants in the organism during obesity and MetS have been proposed as one of the factors that could be implicated in the increment of insulin resistance and cardiovascular risk [8].

Evidence about the role of n-3 PUFAs in inflammatory markers in subjects with MetS is confusing (see Table 14.2). Some studies have observed no effect on the proinflammatory parameters measured [25, 28, 38, 40, 43, 46], while others have described that dietary supplementation with n-3 PUFAs could help to reduce the chronic inflammatory state associated with obesity by increasing anti-inflammatory molecules, such as adiponectin or interleukin (IL) 10 [28, 48], and by decreasing levels of proinflammatory markers and cytokines, such as C reactive protein or IL-6 [23, 50, 63].

Also, it has been suggested that n-3 PUFAs exert its anti-inflammatory effects at the adipose tissue level, decreasing the production of chemoattractant proteins, the infiltration of proinflammatory macrophages, and the endothelial dysfunction markers [30]. In this sense, Spencer et al. [44] showed that supplementation with EPA + DHA decreased plasma concentration and gene expression of macrophage chemoattractant protein-1 (MCP-1) and CD68, decreasing the formation of adipose tissue crown-like structures. Contrariwise, other studies have observed no effect on plasma levels of MCP-1 and other adhesion molecules as ICAM-1, VCAM-1, and E-selectin [50, 57, 63].

Although some studies suggest that n-3 PUFAs supplementation might counteract the systemic inflammatory state in obesity and MetS, the controversial outcomes of the reviewed trials make still unclear to state the efficacy and necessary dose and type of formula of n-3 PUFAs supplementation to decrease the proinflammatory response. However, some trials suggest that n-3 PUFAs supplementation may attenuate inflammation in key metabolic organs, such as adipose or muscle tissues, and perhaps the importance of n-3 PUFAs could be evident at the tissue level. Larger studies examining these issues are needed.

Conclusions and Future Perspectives

The hypotriglyceridemic benefits of n-3 PUFAs supplementation are strongly supported by most of the trials, and intakes of approximately 3 g EPA + DHA per day are able to decrease fasting TG levels by 25–35 % [64]. Based on this available evidence, the American Heart Association and the Panel on Dietetic Products, Nutrition and Allergies (NDA Panel) of the EFSA have recommended intakes of 2–4 g/day as an effective therapy for hypertriglyceridemia [65] and as an alternative to pharmacological approaches. Regarding the blood pressure-lowering properties of marine n-3 PUFAs, a recent meta-analysis of 70 randomized, controlled trials has concluded that the supplementation of ≥2 g/day of EPA + DHA may reduce both SBP and DBP, with the strongest benefits observed among hypertensive individuals who are not on antihypertensive medication [66]. Therefore, oral supplementation with n-3 PUFAs may constitute an interesting therapeutic option to reduce hypertriglyceridemia and hypertension in patients with MetS. However, the ability of n-3 PUFAs to promote weight loss, insulin sensitivity, and changes in cholesterol metabolism in patients with MetS remains controversial. Thus, further larger clinical trials are needed to better elucidate the efficacy of n-3 PUFAs on these features of MetS.

A rising interest is paid on the bioavailability and effects of different formulations of n-3 PUFAs, including re-esterified TG, ethyl ester (EE), carboxylic acids, and phospholipids (PLs) [67, 68]. In the USA and Europe, an EE n-3 PUFAs formulation containing EPA and DHA (Lovaza®/Omacor®) was approved for the treatment of severe hypertriglyceridemia. More recently, an EE formulation (Vascepa®) containing only EPA, as well as a formulation (Epanova®) containing DHA and EPA as free fatty acids, has been also approved in the USA [69]. All prescription n-3 PUFAs products effectively lower TG, but it has been suggested that carboxylic acids have greater bioavailability than EE forms since they do not require pancreatic enzyme activity for absorption [70, 71]. It should be considered that some trials suggest that products that contain DHA can raise levels of LDL-ch, which is of particular concern in patients with atherosclerosis. Krill oil is characterized by a higher amount of EPA compared to DHA (ratio of 2:1), and EPA is mainly in PL and not in TG form. It has been suggested that the delivery of EPA and DHA to tissues in the PL form is higher than that in TG form [72].

Another important issue is the evidence about the heterogeneity in response to n-3 PUFAs supplementation within population. In this context, it has been proposed that genetic background may influence this differential responsiveness [64]. For example, polymorphisms in the *CD36* gene modulate the ability of fish oil supplements to lower fasting TG and raise HDL-ch [73]. There is also limited but consistent evidence that apoE and tumor necrosis factor-α (TNF-α) genotypes interact with n-3 PUFAs in outcomes relating to both inflammation and blood lipid responses [64]. The rising development of nutrigenetics will allow identifying different genotype-dependent responses to n-3 PUFAs supplementation.

There is a strong evidence that obesity-induced low-grade inflammation plays an important causative link between obesity and its associated diseases such as type 2 diabetes and atherosclerosis. In obesity, the expanding white adipose tissue makes a substantial contribution to the development of obesity-linked inflammation via increased secretion of proinflammatory cytokines, chemokines, and adipokines and the reduction of anti-inflammatory adipokines. The state of chronic low-grade inflammation is powerfully amplified through the infiltration of macrophages into adipose tissue. This dysregulated situation initiated primarily within adipose tissue can affect the function of other metabolic organs,

including liver, muscle, and pancreas. Several trials in rodents and some few in humans have suggested that n-3 PUFAs are able to prevent and/or ameliorate inflammation associated with obesity and MetS by reducing adipose tissue inflammation [74]. During the last decade, Serhan and collaborators discovered that n-3 PUFAs are enzymatically converted into bioactive endogenous lipid mediators such as resolvins (Rv), protectins (PD), and maresins with powerful anti-inflammatory and proresolving properties [75]. In a study using a metabolo-lipidomics approach, Claria et al. [76] reported that human subcutaneous adipose tissue has a range of these specialized proresolving mediators including RvD1, RvD2, PD1, lipoxin (LX) A4, and the monohydroxy biosynthetic pathway markers of RvD1 and PD1 (17-HDHA), RvE1 (18-HEPE), and maresin 1 (14-HDHA). Interestingly, the levels of some of these proresolving lipid mediators such as PD1 and 17-HDHA were reduced in fat from patients with peripheral vascular disease [76]. Importantly, the study by Itariu et al. [74] showed that n-3 PUFAs supplementation to severely obese non-diabetic patients significantly increased the production of some of these proresolving lipid mediators, including RvE1, 17-HDHA, PD1, and RvD1 in visceral adipose tissue in parallel with the reduction of adipose tissue and systemic inflammation and the reduction of circulating TG. Several studies in animal models of obesity and MetS have suggested that some of these lipid mediators play a role in governing the local inflammatory tone in obese fat and that the treatments with these n-3 PUFAs-derived proresolving lipid mediators are capable of resolving the inflammation associated with obesity as well as insulin resistance and hepatic steatosis [77–81]. This is an interesting research field that deserves that these observations be further explored to determine the role of these n-3 PUFAs-derived lipid mediators in the prevention of the progression of MetS features in obese humans.

Evolution of "-omics"—transcriptomics, proteomics, metabolomics, and lipidomics—together with the advances in nutrigenomic and nutrigenetic research will greatly contribute to the deciphering of the molecular networks involved in MetS and their regulation by n-3 PUFAs. The major challenge will be in translating these outcomes into personalized recommendations.

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Balancing Omega-6: Omega-3 Ratios in Oilseeds

15

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Introduction

By definition, oilseeds are those seeds or crops that are mainly grown for oil production. The fatty acid composition and total oil produced vary greatly from crop to crop. This variation mainly results due to the genetic makeup of each crop particularly with respect to the fatty acid biosynthesis pathway and is modulated by various factors such as soil type, climate, maturity of seeds, agricultural practices, and geographical location where the crop is grown [1]. Fats and oils are the essential constituents of human diet, and humans consume on an average 25 kg fats per person per year, of which nearly 80 % are obtained from plant sources [2]. Edible oils and fats of plant origin are lipid mixtures consisting of esters derived from fatty acids (FA) attached to the glycerol backbone. Nearly 98 % of the plant and animal lipids consist of acylglyerols, namely triacyl glycerol (TAG), diacyl glycerol (DAG), and monoacyl glycerol (MAG), which contain three-, two-, and one-ester bond, respectively. Of these, TAG is the most abundant constituent in oils and fats of commercial and dietary importance. In vegetable oils, TAG comprises more than 95 % of the total lipids. Further, it is observed that both physical and chemical properties of oils and fats are greatly influenced by the kind and proportion of the fatty acids on the TAGs.

Fatty acids can be grouped in two classes as saturated and unsaturated, based on the absence or the presence of double bond in the fatty acyl chain, respectively. Unsaturated fatty acids can be further differentiated as monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. Further, depending on the position of the first double bond from the ω end (methyl end) in the fatty acyl chain, MUFAs and PUFAs can be denoted as omega-9 (ω-9; omega "minus" nine; first double bond at the 9th carbon from the methyl end), omega-6 (ω-6; first double bond at the 6th carbon from the methyl end), and omega-3 (ω-3; first double bond at the 3rd carbon from the methyl end) fatty acids. Of these, ω-9 fatty acids are considered nonessential, while ω-6 and ω-3 are considered as essential PUFAs for humans, as they cannot be synthesized in the human body and must be obtained from dietary sources. The predominant fatty acids present in vegetable oils and fats are saturated and unsaturated compounds with straight aliphatic chains having even number of carbon atoms (from 16 to 18) with a single carboxyl group, such as palmitate (16:0), stearate (18:0), oleate (18:1), linoleate (18:2), and α-linolenate (18:3) [3]. A number of other minor fatty acids including a small amount of branched chain, cyclic and odd number straight chain acids, may also be present in some vegetable sources [3–5]. For example, the oil in Brassicaceae (*Brassica napus*, *Arabidopsis thaliana*) is rich in elongated acyl chains ranging from C20 to C24, whereas the oil in Lauraceae family contains shorter acyl chains ranging from C8 to C14. In castor bean (*Ricinus communis*), a hydroxylated fatty acid species (Ricinoleate) is predominantly accumulated. Other families have epoxidated or methylated acyl chains in their seed oils [6]. Further, it is now known that the FAs are not randomly esterified to the hydroxyl groups of the glycerol backbone of TAGs, but are present at a particular position (sn-1, sn-2, or sn-3). Saturated FAs are usually present at the sn-1 and sn-3 positions on the TAGs, while unsaturated FAs occur mainly at sn-2 [7].

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Fatty Acid Composition of Oilseeds

The main sources of oils and fats in human diet are oilseed crop plants, mostly soybean (*Glycine max*), rapeseed (*Brassica napus*), palm (*Elaeis guineensis*), and sunflower (*Helianthus annuus*). These oilseeds together account for about 75 % of the fats and oils of plant origin that are consumed worldwide [8, 9]. Oils from these crops are rich in 18-carbon ω-6 fatty acids. The FA composition of major sources of oils and fats is presented in Table 15.1.

Importance of the Balance of ω-6 and ω-3 Fatty Acids in Human Diet

It has been demonstrated that excess consumption of ω-6 fatty acids leads to the depletion of ω-3 fatty acids in human body tissues, with numerous negative health effects [10–13]. Linoleic acid (LA) and α-linolenic acid (ALA) are the parent essential fatty acids (EFAs) of ω-6 and ω-3 class, respectively, and are physiologically and metabolically distinct. LA and ALA serve as precursors of the longer chain polyunsaturated fatty acids (LC-PUFAs) such as arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Humans and animals can in general metabolize LA to ARA and ALA to EPA and DHA [14]. They are further required in the synthesis of biologically active prostaglandins, thromboxanes, and leukotrienes. Depending on the parent compound from which they are synthesized, these compounds are either involved in the pro- or anti-inflammatory pathways, active during disease conditions. The balance between these mutually antagonistic compounds determines the final outcome of the disease process. EFAs also help to maintain the barrier of the skin and are involved in cholesterol metabolism [15]. While LA is plentiful in human diet and is found in the seeds of most food plants, the sources of ALA are few. In high amount, ALA is only found in the chloroplast of green leafy vegetables, such as spinach and purslane, and in seeds of a few plants such as walnut, linseed/flax, soybean, canola, and hemp.

Several studies have suggested that man evolved on a diet that was much lower in saturated FAs than is today's diet [16]. Moreover, the diet contained small and nearly equal proportion of ω-6 and ω-3 long-chain FAs, having a ratio of about 1:1; whereas, today this ratio has greatly and unfavorably increased to 16–25:1, indicating that modern diets are severely deficient in ω-3 FAs compared to the diet on which humans evolved and their genetic patterns were established [16, 17]. There has been a major shift in the human fat consumption pattern over the past century because of the technological and industrial developments [18].

Table 15.1 Fatty acid composition (% fatty acid range) of various vegetable oils

S. no.	Type of oil/oilseed	Fatty acid composition (% FA)				Ratio	
		Saturated	MUFA	PUFA		Saturated/unsaturated	ω-6/ω-3
			OA (18:1) + others	LA (ω-6)	ALA (ω-3)		
1	Canola	4.00–7.00	56.00–64.00	>19.00 (19.00–21.00)	>9.00 (9.00–11.00)	0.05–0.07	1.91–2.11
2	Castor	1.00	86.00–90.00	>5.00	–	0.01	No ω-3
3	Coconut[a]	85.00–90.00	5.00–8.00	0.00–2.00	–	9.00–17.00	No ω-3
4	Corn[a]	12.00–13.50	19.00–49.00	34.00–62.00	0.90–1.00	0.12–0.22	37.78–62.00
5	Cotton	25.00–27.00	17.00–35.00	42.00–55.00	0.30	0.30–0.42	140.00–183.33
6	Linseed/flax	4.00–9.00	12.00–34.00	17.00–24.00	35.00–65.00	0.06–0.07	0.37–0.49
7	Olive[a]	13.00–15.00	65.00–80.00	4.00–10.00	0.60	0.17–0.19	6.67–16.67
8	Palm[a]	46.00–49.00	38.00–52.00	5.00–11.00	0.30	0.77–1.06	16.67–36.67
9	Peanut	12.00–17.00	52.00–60.00	13.00–27.00	–	0.18–0.20	No ω-3
10	Rapeseed	4.00–6.00	22.00–60.00	14.00	6.00–8.00	0.07–0.1	1.75–2.33
11	Rice bran[a]	17.00–19.00	40.00–50.00	29.00–42.00	0.50–1.00	0.20–0.24	42.00–58.00
12	Safflower	6.00–7.00	13.00–21.00	73.00–79.00	–	0.07	No ω-3
13	Sesame	10.00–14.00	40.00–50.00	35.00–45.00	0.20	0.13–0.15	175.00–225.00
14	Soybean	11.00–14.00	22.00–34.00	43.00–56.00	5.00–11.00	0.14–0.16	5.09–8.60
15	Sunflower	7.00–10.00	14.00–35.00	44.00–75.00	0.50	0.09–0.12	88.00–150.00

[a]Non-seed oil source

Sources White [119], Anonymous [120], Cordain [121]

Linoleic acid (LA, 18:2, ω-6 FA), abundant in vegetable oils, such as soybean, groundnut, corn, sunflower, and safflower (Table 15.1), contains two double bonds, while α-linolenic acid (ALA, 18:3, ω-3 FA) abundant in oilseeds, such as linseed, contains three double bonds and hence is more susceptible to oxidation. Thus, the oils rich in ALA (ω-3 FA) tend to become rancid much more quickly than those containing LA (ω-6 FAs), which are more stable. Hence, the oil industry favored the oils rich in ω-6 FA, because of their longer shelf life and plentiful availability, and disregarded the oils rich in ω-3 FA, which could become rancid in only a couple of weeks and also were not as readily available as the ω-6 rich oils.

Apart from this, aquaculture or fish farming may have also contributed to increasing the ω-6/ω-3 ratios. Marine fishes have been the principal source of ω-3 FAs, particularly in the Western diets. However, in the recent past, the natural fish stocks from water bodies have depleted due to overfishing to meet the increasing demand, climate change, or other factors. Hence, to ensure regular supply of fish, commercial fish farming in tanks or enclosures was initiated. However, as fish obtain the ω-3 FAs by feeding on their primary producers (algae and seaweeds), the commercial fish farms need to be supplemented with the ω-3 FA producing algae or seaweeds. However, due to economic or practical issues, such supplements might not be provided in adequate amounts, negatively affecting the ω-3 FA contents of the aquacultured fish compared to the fish caught from their natural habitats.

Thus, modern agriculture and animal and fish farms, with their emphasis on production, have decreased ω-3 FA contents in many food sources, including green leafy vegetables, animal meats, eggs, and fish [19]. All of these might have contributed to increasing the ratio of ω-6/ω-3 in the present-day diets. High amount of ω-6 FAs in the body leads to the production of pro-inflammatory eicosanoids, which may contribute in the pathogenesis of many diseases, including cardiovascular diseases, cancer, osteoporosis, and inflammatory and autoimmune diseases; whereas increased levels of ω 3 PUFA (a lower ω-6/ω-3 ratio) exert suppressive effects by anti-inflammatory eicosanoid production [10, 20, 21]. Additionally, due to the changing lifestyles, there has been an increase in the intake of trans-FAs, found mainly in products made with hydrogenated vegetable oils and animal products derived from grain-fed livestock. However, in the recent past, the significance of proper ω-6/ω-3 FA ratio in the diet has been realized and studied in detail.

According to the joint recommendation by the Food and Agriculture Organization and World Health Organization (FAO/WHO) committee, the ideal ω-6/ω-3 ratio in diet should be between 5:1 and 10:1. On the other hand, various studies indicate that the optimal ω-6/ω-3 ratio may vary in disease conditions [20] and the therapeutic dose of ω-3 FAs will depend on the degree of severity of disease resulting from the genetic predisposition of an individual. Nevertheless, a lower ω-6/ω-3 ratio is more desirable in reducing the risks of many chronic diseases of high prevalence in Western societies, as well as in the developing countries. Therefore, to promote normal growth and development as well as to maintain good human health, it is essential to rectify the imbalance in the ω-6/ω-3 ratio by restoring the sources of ω-3 FAs in the diet. There are few agricultural sources of high ALA (ω-3 FA), such as linseed, green leafy vegetables, chia, perilla, hemp, and purslane. Linseed, with its high level of ALA and ω-6/ω-3 ratio of 0.3–1.0, can help to restore the ω-6/ω-3 FA balance in the human body [22]. This has made linseed nutritionally a very important crop and has contributed to its popularity in food, feed, and nutraceutical industries.

It is now possible to modify the fatty acid composition of oilseeds using biotechnological tools. The change in the relative abundance of individual fatty acids in seed oil can lead to the production of healthier oil with ideal ω-6/ω-3 ratio. Moreover, with pathway engineering, it might also be possible to produce nutritionally more desirable fatty acids such as EPA or DHA, which are not normally produced by crop plants. For this, it is necessary to understand the fatty acid biosynthetic pathways in target plants, the genes involved, sources, and the spatial and temporal expression of those genes.

Fatty Acid Biosynthesis in Plants

The fatty acid biosynthesis pathway is considered as the primary metabolic pathway, since it is found in every cell of a plant and is essential for its growth. In plants, the major site of the FA synthesis is plastid, which is unlike that in animals and fungi, where the fatty acids are primarily synthesized in the cytosol. The pool of acetyl coenzyme A (acetyl-CoA) present in the plastid serves as the carbon source for fatty acid synthesis [23, 24]. The process involved in the FA biosynthesis can be divided into the following major steps.

Synthesis of Acetyl-CoA Inside Plastid

As mentioned before, acetyl-CoA is the precursor for fatty acid synthesis (Fig. 15.1). However, it cannot be transported across plastidial membrane and needs to be synthesized de novo inside the plastid for FA synthesis to occur [25, 26]. In green tissues, the carbon fixed through photosynthesis is utilized for acetyl-CoA synthesis in chloroplasts, whereas in nongreen tissues such as roots and seeds, the glycolate pathway provides the precursor for FA biosynthesis [27].

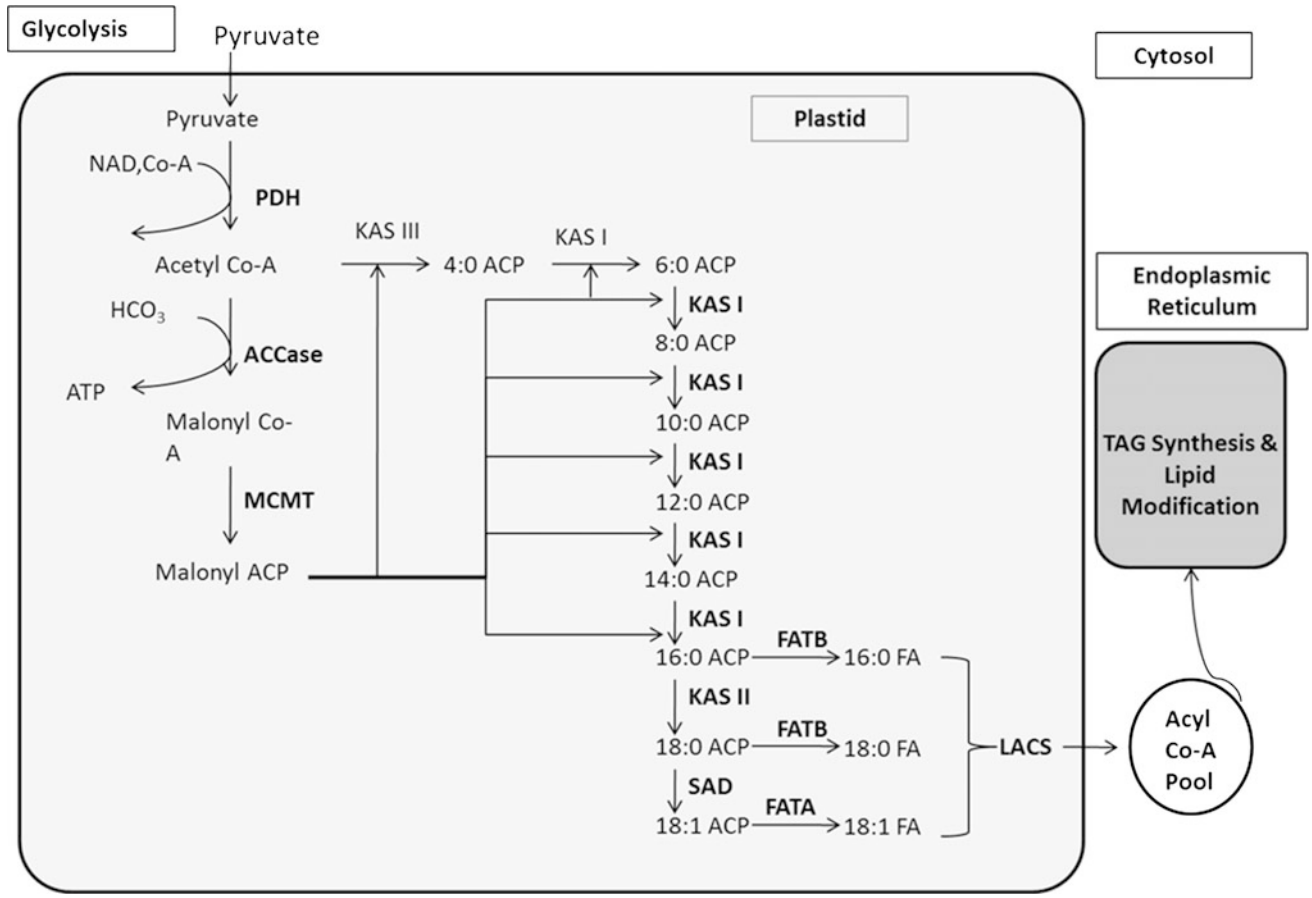

Fig. 15.1 De novo fatty acid biosynthesis pathway

There are four reported mechanisms through which acetyl-CoA pool in plastids is generated: (i) by activation of free acetate in plastid by acetate CoA synthetase (ACS) [28–30], (ii) by conversion of pyruvate via pyruvate dehydrogenase enzyme complex, (iii) from acetylcarnitine by plastidial carnitine acetyl transferase enzyme [31], and (iv) by ATP-citrate lyase (ACL) reaction [32].

In most cases, pyruvate is the direct source of acetyl-CoA and is either transported from cytosol or synthesized from phosphoenolpyruvate (PEP) inside the plastid by plastidial pyruvate kinase. More than 75 % of plastidial pyruvate is used for FA synthesis [33, 34]. Along with this, the metabolites such as glucose-6-phosphate (G6P), acetate, and malate are imported from cytosol for acetyl-CoA synthesis. Utilization of the metabolites varies among species as well as in different tissues [27, 35, 36].

Energy Requirement for Fatty Acid Synthesis

ATP and NADPH are required for fatty acid biosynthesis [37]. Chloroplast and mitochondria are involved in the generation of energy through photophosphorylation and oxidative phosphorylation, respectively [38]. NADPH is generally provided by the oxidative pentose phosphate pathway in plants [39], while malate also provides reducing power in minor amount [35]. PEP serves as an energy-rich metabolite for plastidial metabolism. However, since the PEP produced by glycolytic pathway is not enough, plastid imports PEP from cytosol, through PEP/phosphate translocators, or by plastidic enolase. Plastidic pyruvate kinase catalyzes the conversion of PEP to pyruvate, which acts as a precursor for fatty acid biosynthesis and simultaneously leads to the production of an additional ATP molecule [34]. ATP is imported in chloroplast during night and in nongreen tissues by nucleotide transporter (NTT) [40], and carbon is transported in the form of G6P by G6P/phosphate translocator [41].

Fatty Acid Biosynthesis Pathway in Plants

Formation of Malonyl-CoA and Malonyl Acyl Carrier Protein (ACP)

In the first committed step for de novo fatty acid synthesis, malonyl-CoA is formed from acetyl-CoA by the addition of

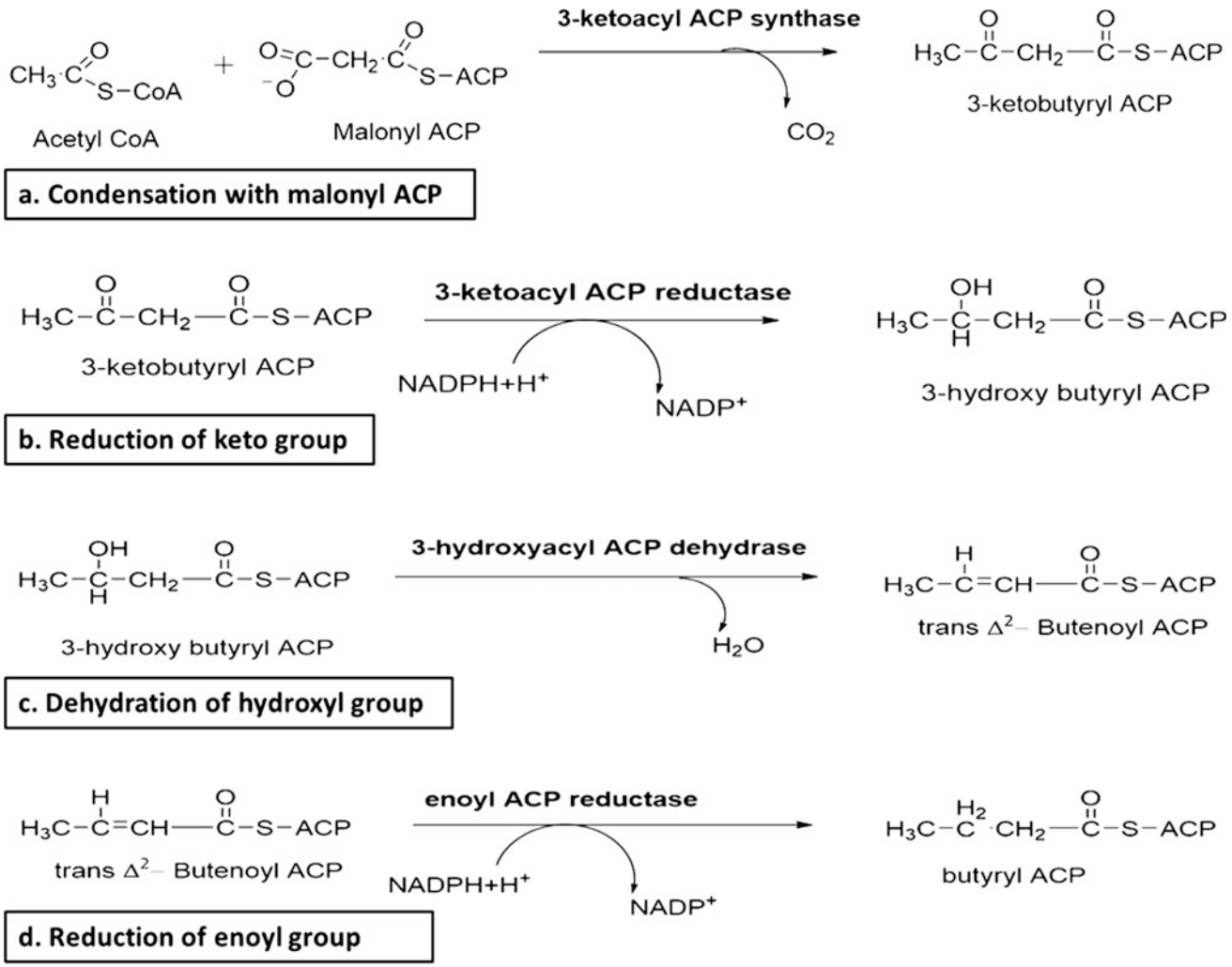

Fig. 15.2 Steps involved in fatty acid elongation

CO_2 using the biotin prosthetic group of the enzyme acetyl-CoA carboxylase (ACCase) and serves as the carbon donor for subsequent elongation reactions of fatty acyl chain [42]. Further, FA synthesis in plastid occurs through a repeated series of condensation, reduction, and dehydration reactions that add two carbon units derived from malonyl-ACP to the elongating FA chain in each cycle. This process is represented schematically in Fig. 15.2 and is catalyzed by an enzyme complex called fatty acid synthase (FAS). FAS is a multi-subunit complex of monofunctional enzymes that are easily dissociable [43]. Overall, nearly 30 enzymatic reactions are required to produce a 16- or 18-carbon FA from acetyl-CoA and malonyl-CoA [44]. Before entering the FA synthesis pathway, the malonyl group of malonyl-CoA is transferred from CoA to a protein cofactor, acyl carrier protein (ACP) with the help of the enzyme malonyl-CoA: ACP transacylase [45]. Further reactions of the pathway involve ACP until the 16- or 18-carbon product is ready for transfer to glycerolipids or for export from the plastid. The ACP plays a significant role in fatty acid synthesis [46]. The ACP is a small acidic protein having molecular weight of about 9 KDa and contains a prosthetic group 4′ phosphopantetheine, which is attached to a conserved serine residue. The growing acyl chain is attached to the prosthetic group in the form of a thioester [44].

Condensation with Malonyl-ACP

Malonyl-ACP undergoes several condensation reactions mediated by a set of at least three separate condensing enzymes, known as 3-ketoacyl-ACP synthase (KAS) to produce an 18-carbon fatty acid. The first condensation of acetyl-CoA and malonyl-ACP to form a four-carbon product is catalyzed by the enzyme KAS III. A second condensing enzyme, KAS I, is responsible for producing fatty acid chain lengths from 6 to 16 carbons. Finally, KAS II is required for elongation of the 16 carbon palmitoyl-ACP to 18 carbon stearoyl-ACP [47]. In addition to these three enzyme-catalyzed reactions, after each condensation step, the

3-ketoacyl-ACP product is reduced, dehydrated, and reduced again by 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydrase, and enoyl-ACP reductase, respectively. As a result, saturated FA that is two carbons longer than at the start of the cycle is obtained (Fig. 15.2a). The steps are described in detail as follows:

Reduction of Keto Group

The keto group of 3-ketobutyryl ACP is reduced by 3-ketoacyl-ACP reductase (KAR) into 3-hydroxybutyryl-ACP. This reaction utilizes NADPH as an electron donor (Fig. 15.2b).

Dehydration of Hydroxyl Group

A water molecule is removed from 3-hydroxybutyryl-ACP by 3-hydroxyacyl-ACP dehydrase enzyme which produces trans-Δ^2butenoyl-ACP (Fig. 15.2c).

Reduction of Enoyl Group

The final step is the reduction of a double bond via enoyl-ACP reductase which forms a saturated fatty acid, and this step also requires NADPH for reduction (Fig. 15.2d).

These three steps lengthen the fatty acid chain by two carbons in each cycle, subsequently producing 16:0-ACP and 18:0-ACP.

Termination of Fatty Acyl Chain Elongation

The elongation of FAs in the plastids is terminated when the acyl group is removed from ACP by either of the two enzyme systems, an acyl-ACP thioesterase or acyltransferases in the plastid. Acyl-ACP thioesterase hydrolyzes the acyl-ACP and releases free FA, whereas acyltransferases transfer the FA from ACP to glycerol-3-phosphate or to monoacylglycerol-3-phosphate. The transport of FA outside plastid is determined on the basis of their release from ACP by either a thioesterase or an acyltransferase. Only when thioesterase acts on acyl-ACP, free FAs can leave the plastid. On the outer membrane of the chloroplast envelope, an acyl-CoA synthetase assembles an acyl-CoA thioester that is then available for acyltransferase reactions to form glycerolipids in the endoplasmic reticulum (ER) [42, 44].

Once the 18:0 ACP is produced, the enzyme, stearoyl Δ9 ACP desaturase (SAD), converts 18:0 ACP to 18:1 ACP. Long-chain acyl groups are converted into free form by the action of acyl-ACP thioesterase enzyme. Two types of acyl-ACP thioesterases, FatA and FatB, are involved in release of free fatty acids. FatA shows substrate specificity toward 18:1-ACP (unsaturated) while 16:0-ACP and 18:0-ACP (saturated) are released by FatB [48, 49]. Both FatA and FatB show high sequence and structural similarity; however, the reason for the difference in their substrate specificities is not yet known.

Desaturation

Desaturation is an important biochemical process in the FA biosynthesis pathway, which is facilitated by the fatty acid desaturases (FADs). These key enzymes convert the saturated FAs with single bond between two carbon atoms (C–C) into unsaturated FAs with double bond (C=C) at specific locations in the fatty acyl chain. Desaturases are classified into two phylogenetically unrelated groups: the membrane-bound FADs and the soluble FADs [50, 51]. Soluble desaturases such as stearoyl-ACP desaturase (SAD or Δ9) are acyl–acyl carrier protein (ACP) desaturases. SAD converts stearic acid (SA), an 18 carbon fatty acid with no double bonds (18:0), to oleic acid (OA, 18:1) by introducing the first double bond at the 9th position from the carboxylic end (α end) of the fatty acyl chain. Membrane-bound desaturases, such as Δ12 desaturase (FAD2) or Δ15 desaturase (FAD3), further introduce double bonds into fatty acids that are either esterified as acyl-CoA or bound to the glycerol moiety of glycerolipids [52]. FAD2 (Δ12 desaturase) converts oleic acid (OA, 18:1) to linolenic acid (LA, 18:2) by introducing a double bond at the 12th position from the α end or 6th position from the ω end (ω-6), while the third double bond is introduced at the 15th position from the α end (or ω-3 position) of LA by FAD3 (Δ15 desaturase) to form ALA (18:3) [53, 54]. Although the FA biosynthesis process is complex and controlled by the action of several genes at different stages, the three desaturases, SAD (Δ9), FAD2 (Δ12), and FAD3 (Δ15), act sequentially and appear to drive the PUFA synthesis pathway in plants.

The Role of Rubisco in Developing (Green) Oilseeds

The conversion of pyruvate to acetyl-CoA by the action of pyruvate dehydrogenase complex leads to the loss of one CO_2 molecule (Fig. 15.1). However, the yield of FAs through conversion of sugars suggests that this loss is minimized. In case of soybean, the efficiency of this conversion is almost 80 %. The efficient conversion of sugars to FAs is due to the increased expression and activity of Rubisco in developing green seeds. However, though Rubisco is active and plays an instrumental role in CO_2 fixation, Calvin cycle is not functional in seeds. The increase in glyceraldehyde-3-phosphate through CO_2 quenching is obtained by Rubisco and non-oxidative steps of pentose phosphate pathway cooperatively. The energy in the form of ATP and reducing power in the form of NADPH are provided mainly by photosynthesis as the flux through tricarboxylic acid cycle (TCA) is reduced in green oilseeds. Figure 15.3 summarizes the role of Rubisco in the increased FA turnover. However, in case of developing sunflower

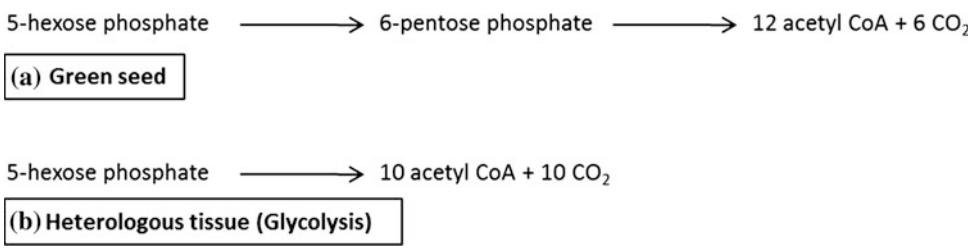

Fig. 15.3 Role of Rubisco

seeds (an example of heterotrophic tissue) in contrast to green seeds, the conversion efficiency of sugar to oils accounts to nearly 50 %. Instead of scavenging the released CO_2 by the activity of pyruvate dehydrogenase during the synthesis of acetyl-CoA for increased FA production, malate from TCA is channeled for FA synthesis in developing sunflower seeds.

Formation of TAGs

Triacylglycerol (TAG) synthesis in plants takes place by either acyl-CoA-dependent or acyl-CoA-independent pathways as summarized in the Fig. 15.4. Synthesis of diacylglycerol (DAG) is instrumental in the biosynthesis of TAG, and its constituent acyl chains determine the ratio of saturated and unsaturated fatty acids. Two pathways exist for the formation of DAG, and the balance in the flux through these two pathways governs whether the ω-6 or ω-3 FA will be incorporated in TAG [55–57]. The formation of TAGs has been explained in the following steps.

Cytoplasmic Fatty Acid (FA) Pool

Palmitate (PA), stearate (SA), and oleate (OA) are the three fatty acids released from the plastid to cytoplasm by the action of acyl-ACP thioesterase and transporter proteins and constitute the nascent FA pool. These fatty acids are activated to form acyl CoAs and can directly be incorporated for DAG synthesis or further modified by incorporating into phosphatidyl choline (PC) by the action of PC:acyl editing cycle. The oleate incorporated into PC by the action of lyso-PC acyltransferase (LPCAT) is subjected to further desaturation leading to the release of LA and ALA into the FA pool [55–57]. The conversion of OA to LA and ALA takes place in the ER membrane and is catalyzed by Δ12 desaturase (FAD2) and Δ15 desaturase (FAD3), respectively. Other FA modifications such as hydroxy-FAs (HFAs) also act on acyl:PC, and synthesis of many unusual FAs is carried out by enzymes that are homologs of FAD2. In case of *R. communis*, 12-hydroxy-9-cis-octadecenoic acid (18:1-OH), also known as Ricinoleate, is the major constituent of FA in TAG and is synthesized by hydroxylase, which uses 18:1: PC as the substrate [55–57].

De Novo Synthesis of DAG

FAs from the cytoplasmic FA pool (containing both nascent and unusual or modified FAs) can be incorporated into DAG by conventional pathway also known as Kennedy pathway (TAG synthesis). In de novo synthesis of DAG, two sequential acylations take place on glycerol-3-phosphate in ER. The first acylation occurs at sn1 position and is catalyzed by acyl-CoA:G3P acyltransferase (GPAT) to form lysophosphatidic acid (LPA). Acyl-CoA:LPA acyltransferase (LPAT) catalyzes the next acylation at sn2 position forming phosphatidic acid. The phosphate group is removed later from PA by PA phosphatase (PAP) to yield DAG [55–57].

Synthesis of PC-Derived DAG

PC in ER is synthesized from the de novo synthesized DAG and choline: DAG choline phosphate (CDP) by choline: DAG choline phosphotransferase (CPT) enzyme and marks the first step in PC-derived DAG synthesis. As mentioned earlier, modification of FA released from plastid takes place while conjugated to PC (PC-mFA). The DAG with modified FA can thus be synthesized by the reversible action of CPT or phospholipase C/D or PA phosphatase on PC-mFA. Alternatively, PC-derived DAG can also be formed by the action of PC: DAG choline phosphotransferase (PDCT), also known as reduced oleate desaturation 1 (ROD1) as identified in *A. thaliana* [58]. In this case, the phosphocholine head group is transferred from PC-mFA to de novo DAG, thus forming PC-18:1 and DAG with mFA at sn2. PDCT-mediated interconversion of PC and DAG is thus an efficient mechanism to increase the mFA-DAG species. Unlike the CPT-mediated pathway, PDCT pathway does not lead to net production of PC [55–57].

TAG Synthesis from DAG

TAG can be synthesized from heterogenous pool of DAG and FAs by the activity of acyl-CoA:DAG acyltransferase (DGAT), which completes the TAG synthesis by Kennedy pathway. Alternatively, TAG can also be synthesized by acyl-CoA-independent pathway by the transfer of acyl chain from PC to DAG by the phospholipid:DAG acyltransferase (PDAT). In *A. thaliana,* it has been shown that the loss-of-function mutation in any one of these had little effect on the total TAG synthesis; however, TAG synthesis was

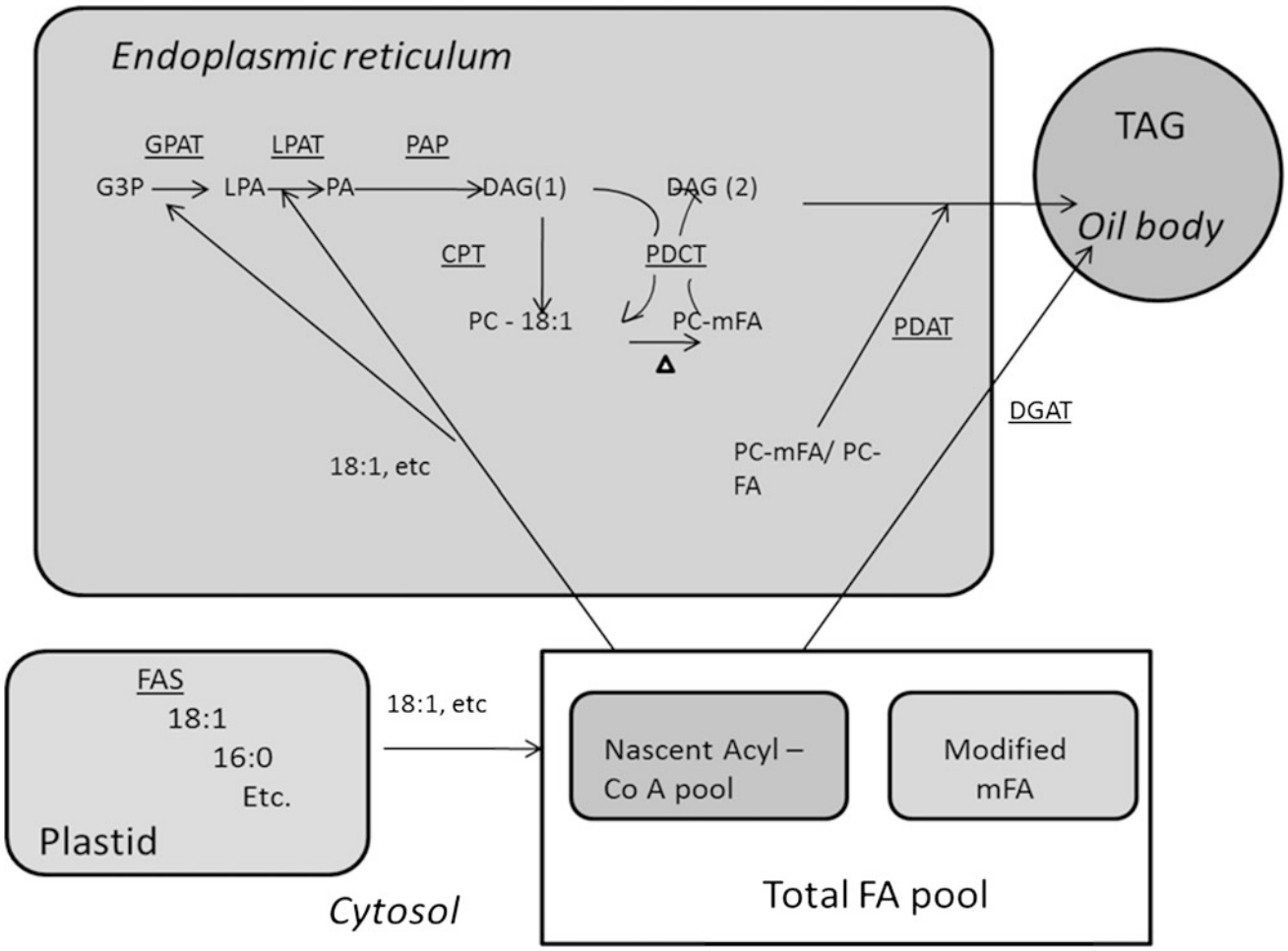

Fig. 15.4 Overview of triacylglycerol biosynthesis

greatly hampered in PDAT-DGAT double mutant. This suggests that both these pathways contribute in majority of TAG synthesis in oilseeds [55–57].

Metabolic Flux of TAG Synthesis

It is known that plants can synthesize TAG by using both de novo DAG and PC-derived DAG; however, the final oil composition depends on the relative fluxes of these DAG/TAG synthesis pathways. The flux of TAG synthesis by Kennedy pathway or acyl-CoA-independent pathway greatly varies from plant to plant, and it may further vary in different tissue types and seed developmental stages. These fluxes need to be considered and thoroughly studied before attempting metabolic engineering of oilseeds for the enhanced quality and/or quantity of oil production. In vivo radioactive or stable isotope labeling experiments can be performed for studying the metabolic fluxes through alternative pathways [55–57, 59].

The plants that contain oils with PC-modified PUFAs direct the synthesis of TAG by increasing the flux of PC-derived DAG production. Soybean, rich in LA, and flax, rich in ALA, both channel FAs to DAG/TAG synthesis through PC [55], whereas the loss-of-function mutation of PDCT in *A. thaliana* leads to ~40 % decrease in oleate desaturation in the total oil, suggesting that like soybean and flax, most of the DAG/TAG synthesis in *A. thaliana* happens through PC-DAG interconversion [58].

TAG Synthesis and Oil Accumulation During Seed Development

The seed reserve components usually consist of seed storage proteins (SSPs), carbohydrates (mostly starch), and/or storage lipids (waxes or TAGs). The relative proportion and tissue localization of these compounds vary greatly depending on the plant species under consideration. As described earlier, TAGs are the esters of glycerol, where

each of the three hydroxyl groups of a glycerol backbone is esterified with fatty acids. Once assembled in the ER, TAGs are transferred and accumulated into subcellular structures called oil bodies or oleosomes. They are spherical organelles comprising a core of TAGs surrounded by a phospholipid monolayer and the protein termed oleosin [60]. These oil bodies serve as an energy source during seed germination until photosynthesis becomes effective and occupy nearly 60–90 % of the cell volume in the cotyledons of mature embryos of many oilseed plants [61–63].

The fatty acid profiles and the total lipid/oil content of seeds vary greatly for different plant species as well as across different seed developmental stages. Overall, seed development can grossly be grouped into early, mid-, and late maturation stages. Early stages include embryo morphogenesis. In the mid-phase of development, the embryo cells undergo a period of cellular expansion and differentiation and simultaneously accumulate storage proteins (nitrogen compounds) and lipids (carbon compounds). During the last stage (late maturation), the embryo becomes metabolically dormant and tolerant to desiccation [64–66].

FA accumulation in early embryo morphogenesis stages is the lowest and gradually increases with the increase in seed dry weight. During the mid-maturation phase, and again at the beginning of the late maturation phase, FA accumulation sharply increases [67, 68]. In *A. thaliana*, it was observed that in the early stages, TAG represented a very small fraction of the total lipid population, but its proportion increased sharply at the onset of mid-maturation stages representing nearly 60 % of the total FA. Further, this proportion increased gradually to reach 93 % at the mature stage [67]. The authors also observed that active FA synthesis and differentiation took place at the mid-maturation stage. Even in case of oilseeds such as rapeseed (mustard) and soybean, the mid-maturation stage was the most rapid phase of TAG assembly and oil/lipid deposition [68, 69].

The gene expression programs related to oil accumulation are activated during different seed maturation stages. Several studies carried out on developing seeds and/or embryos of various plants have shown that the biosynthetic pathways for fatty acids and TAGs are regulated mostly at the transcription level [70, 71]. Microarray analyses in *A. thaliana* have revealed differential timing of activation of the genes involved in storage lipid metabolism during seed maturation [72]. The genes encoding fatty acid synthesis enzymes show a bell-shaped expression pattern at the onset of seed maturation stages, whereas the expression level of several fatty acid-modifying enzymes and oleosins increases later as the seeds reach maturity [6]. O'Hara et al. [71] determined the spatial and temporal expression of fatty acid and lipid biosynthetic genes during embryogenesis in *B. napus* and found that most of the fatty acid and lipid biosynthetic genes were expressed at constant molar ratios but at different absolute levels during embryogenesis. Another study in *B. napus* revealed that the fatty acid biosynthesis-related genes were highly expressed primarily at mid-maturation stage of seed development [73].

In order to study the timing and the site of lipid synthesis within the developing seeds, gene expression of enzymes representing the four key steps of fatty acid synthesis, namely acyl chain elongation, termination, desaturation and TAG synthesis, was analyzed by Venglat et al. [74] in flax. In this comprehensive study based on the abundance of expressed sequence tags (ESTs), it was observed that the acyl chain elongation activity increases during early embryonic stages. The activity of *FatA* and *FatB* genes representing termination of FA chain elongation also appears to peak during early stages of seed development. The ESTs representing DGAT (responsible for FA transfer to glycerol backbone) were found in low quantities in early embryonic stages, but their activity peaked later, during the cotyledon embryonic stage (mid-maturation stage). Desaturation is the key step that results in the desirable omega-3 and omega-6 fatty acids, and it was observed that the number of ESTs representing the FADs 2, 3, 5, and 8 spiked at the mature embryo stages. Similarly, Rajwade et al. [75] also observed bell-shaped expression pattern for most of the Δ9, Δ12, and Δ15 desaturases in developing flaxseeds, where the highest expression was at mid-maturation stage. Further, the expression of putative homologs of Arabidopsis oleosin (proteins associated with oil bodies) 1, 2, and 3 genes in flax was observed at the beginning of the early embryonic stages, with greater levels in mature stage. This coincides with the expression of FADs involved in formation of omega-3 and omega-6 fatty acids in the mid- and late seed-maturing stages.

Biotechnological Approaches to Achieve Balanced Omega-6: Omega-3 Ratio

For normal growth and development as well as to maintain good health, it is essential to have the balanced intake of omega-6 and omega-3 FAs (ideally in 5:1–10:1 ratio). As the commercially available edible oils are rich in omega-6 FAs, we need to tap the oilseeds having favorable omega-6: omega-3 ratios. Alternatively, a commonly consumed oilseed can be modified to have the balanced omega-6: omega-3 ratio. As clear from Table 15.1, linseed has the most favorable omega-6: omega-3 ratio (0.37–0.49), considering the severe deficiency of omega-3 FAs in human diets. Thus, inclusion of linseed oil should be sufficient to improve the omega-6: omega-3 ratio in human diets. However, due to its high ALA (omega-3 FA) content, the linseed oil is not suitable as a cooking or frying oil and needs to be consumed in other ways.

There are few oilseeds that are rich in LA content as indicated in Table 15.1. These oilseeds produce ALA in fairly less quantities. In these oilseeds, the flux of FA biosynthesis can be modified to produce more omega-3 FA, so that the omega-6: omega-3 ratio becomes favorable. Damude et al. [76] employed the fungal bifunctional Δ12/ω-3 desaturase to direct the flux toward higher ALA accumulation in soybean. The LA:ALA (ω-6:ω-3) ratio was determined to be 1:22.3, which was 110-fold greater than the control non-transformed soybean and sevenfold greater than linseed. The ω-3 desaturases can utilize a variety of ω-6 substrates, such as LA, GLA, DGLA, and AA, and convert them to their respective ω-3 products. Such enzymes can be exploited to balance the ratio of ω-6/ω-3 in plants for human consumption. Though such "balanced oils" with more ALA content will not be suitable as as cooking oils, this marks the first step for producing oils rich in nutritionally important fatty acids such as EPA and DHA. The next section deals with engineering of LC-PUFA biosynthetic pathway in plants, and stresses on the enzymes involved in conversion of LA/ALA to EPA and DHA, and the choice of host plant for carrying out this metabolic engineering.

Engineering the Long-chain Polyunsaturated Fatty Acid (LC-PUFA) Pathway in Plants

It has been well-established that LC-PUFAs such as eicosapentaenoic acid (EPA, ω-3), docosahexaenoic acid (DHA, ω-3), and arachidonic acid (ARA, ω-6) play vital roles in human nutrition and brain development [77]. They serve as the precursors for synthesis of a variety of eicosanoids, growth regulators, and hormones. In addition to these functions, they maintain fluidity of biological membranes and cell permeability. Brain, retina, and testis contain high amount of ARA and DHA [78]. EPA furthermore reduces the risks of acquiring a wide range of disorders and diseases such as cardiovascular disorders, obesity, type 2 diabetes [79], rheumatoid arthritis, Crohn's disease, autoimmune disorders, hypercholesterolemia, and cancer [80, 81]. Similarly, DHA and ARA play instrumental roles in infant brain development [82]. Hence, the balanced intake of ω-6 FA and ω-3 FA is vital for proper growth, development, and health.

Higher plants are not capable of synthesizing the LC-PUFAs, as they lack the genes to extend the PUFA pathway. Plants can synthesize up to 18 carbon fatty acids, containing a maximum of three C=C bonds, *viz*. α-linolenic acid (ALA, 18:3, ω-3), γ-linolenic acid (GLA, 18:3, ω-6) and stearidonic acid (SDA, 18:4, ω-3) [9, 83, 84]. Mammals however are not efficient in synthesizing the EFAs such as LA and ALA and depend greatly on the dietary intake of these EFAs from plants [85]. Though humans possess the genes for conversion of LA to ARA and ALA to EPA and DHA, this conversion is not efficient and severely restricted during early childhood and in old age [86]. This deficit in nutrition is often balanced by consumption of fishes such as sardine, mackerel, tuna, and salmon, which are the rich sources of EPA and DHA [87]. However, people on strict vegetarian diet avoid consumption of fish-derived sources of LC-PUFAs. An alternative source is therefore required which fulfills the basic need of LC-PUFAs on a large scale. Plants or microbes rich in EFAs are the most suitable and sustainable machinery for the production of LC-PUFAs through transgenic approaches [77, 88–90].

Enzymes for LC-PUFA Biosynthesis

The enzymes involved in LC-PUFA biosynthesis have been identified from several microbial sources over the last decade. Three desaturation reactions are required for synthesis of DHA from ALA, and these are catalyzed by Δ6, Δ5, and Δ4 desaturases, respectively. The desaturation in these microbes is carried out by microsomal "front-end" desaturases, which are the members of *N*-terminal cytochrome b5-fusion superfamily. The presence of cytochrome b5 domain differentiates these desaturases from Δ12 and Δ15 desaturases of plants [84]. The microbial front-end desaturases' third histidine box contains glutamine instead of histidine (Q-X [2, 3]-H-H]) found in plant desaturases. However, similar to the plant Δ12 and Δ15 desaturases, these enzymes also utilize glycerolipid-linked substrates for their activity [91].

In addition to desaturation, two elongation reactions catalyzed by Δ6 and Δ5 elongases are required for synthesis of DHA from ALA. Each elongation step increases the length of fatty acids by two carbon atoms. Elongation of fatty acids takes place in cytoplasm and requires CoA-linked acyl chains as substrates. The elongases (β-ketoacyl CoA synthase, KCS; β-ketoacyl CoA reductase, KCR; hydroacyl CoA dehydratase and enol CoA reductase) are similar to that of plastidial FASs [92]. Two groups of KCS-condensing enzymes are known: first group comprises ELO-like sequences [93], while the second group comprises fatty acid elongation-1 (FAE1) like plant specific KCS enzymes that are involved in biosynthesis of saturated and monounsaturated fatty acids of more than 22 carbons in length [94].

The elongases are often considered as the regulators of substrate specificity in terms of the length of the acyl chain and the position of double bonds [95]. This elongation step is often rate limiting; however, heterologous expression of KCS alone is enough for the desired activity. Thus, KCS is often referred to as 'elongase' though semantically incorrect [95–97].

LC-PUFA Biosynthesis Pathway

The conventional pathway present in most microbes for synthesizing long-chain ω-6 and ω-3 FAs is represented schematically in Fig. 15.5. The first step of this pathway is catalyzed by Δ6 desaturase, which independently acts on LA and ALA to convert them into GLA and SDA (stearidonic acid), respectively. Subsequently, GLA is converted into DGLA (dihomo γ-linolenic acid) and SDA to ETA (eicosatetraenoic acid) by the action of Δ6 elongase. Further desaturation of DGLA into ARA and ETA into EPA is catalyzed by Δ5 desaturase. Δ17 desaturase links the ω-6 and ω-3 pathways by converting ARA to EPA. The EPA is further acted upon by Δ5 elongase to form DPA (docosapentaenoic acid). Lastly, the final desaturation of DPA to DHA is carried out by Δ4 desaturase [88]. As mentioned earlier, the desaturation reactions require phosphatidylcholine (PC)-linked fatty acid and elongation takes place on CoA-linked fatty acid. This change in carrier molecule of acyl chains from PC to CoA is mediated by acyl-CoA: lysophosphatidylcholine acyltransferase (LPCAT) [98].

The LC-PUFA biosynthesis pathway varies among different groups of organisms. In mammals, Sprecher's pathway is present, where two additional steps are required for the production of DHA from DPA. In this case, DPA is converted into tetracosapentaenoic acid (TPA) by Δ5 elongase and is followed by desaturation to tetracosahexaenoic acid (THA) by Δ6 desaturase. Partial oxidation of THA in peroxisome leads to the formation of DHA [99, 100]. The ARA and EPA act as precursors for producing pro-inflammatory and anti-inflammatory eicosanoids, respectively, during disease conditions, and the balance between these mutually antagonistic pathways determines the final outcome of the disease process. This further stresses the importance of omega-3 FAs in human diet.

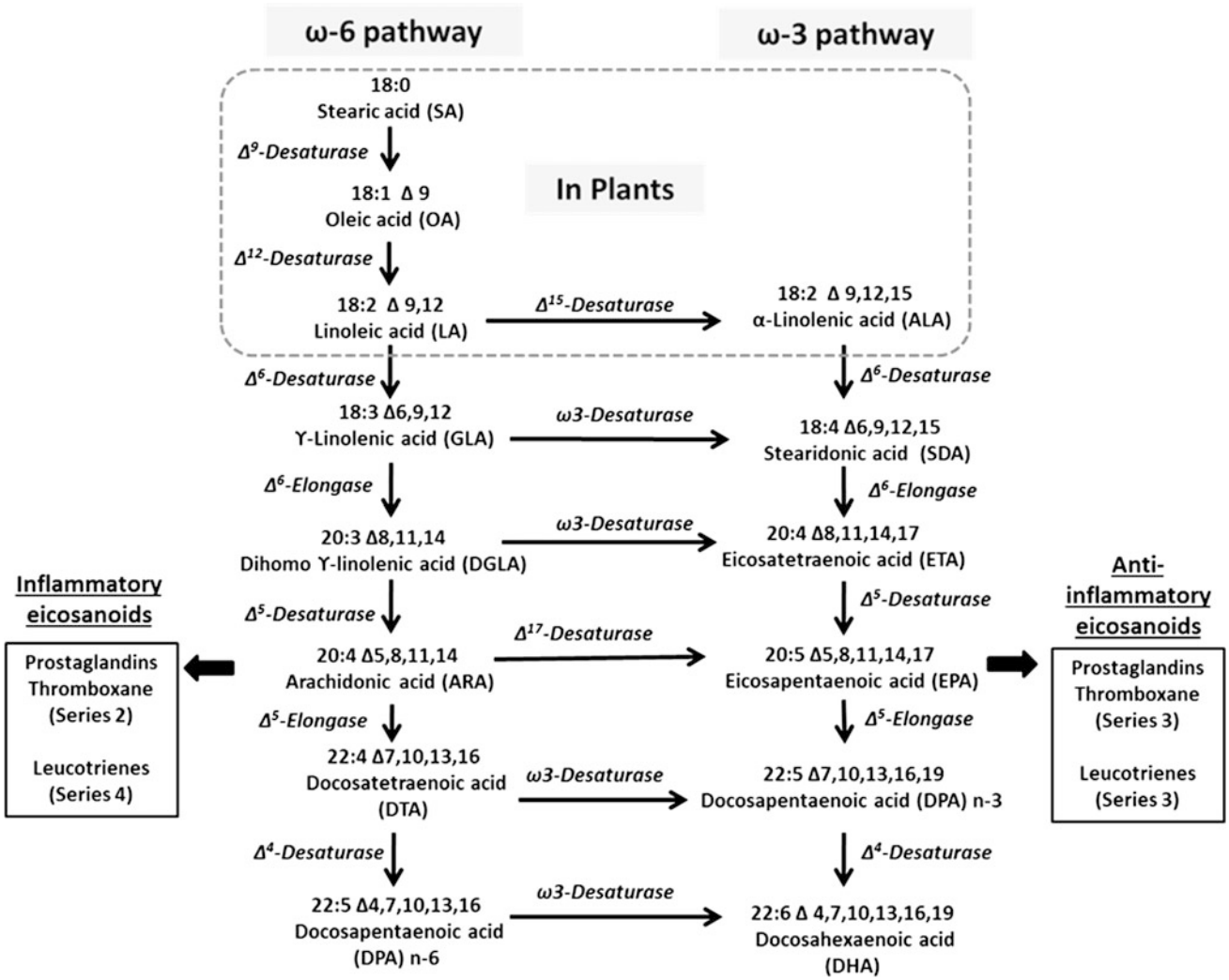

Fig. 15.5 Conventional omega-3 and omega-6 fatty acid biosynthesis pathways

Choice of Oilseeds for Balancing the Omega-6: Omega-3 Ratio

As stated earlier, plant oils are rich in monounsaturated fatty acids such as OA and polyunsaturated fatty acids such as LA and ALA, the latter being precursors of nutritionally important LC-PUFAs. Engineering of LC-PUFA production in oilseeds is an attractive area of research and aims at reducing the dependency on fish oils for EPA and DHA. However, oil rich in PUFAs has a limited scope in terms of the method of consumption. The oils containing high proportion of PUFAs (such as ALA) and LC-PUFAs (such as EPA and DHA) are highly susceptible to oxidation through contact with air or light, heating, and irradiation, which often lead to the formation of off-flavors and odors. Furthermore, oxidation of such oils reduces their nutritional value, and the free radicals released in the process may lead to oxidative stress-mediated health complications. As a result, such oils need to be stabilized by hyper-fortifying with antioxidants [101]. Hence, even if high amounts of EPA and DHA were obtained in a transgenic oilseed, the oil would be unfit for the purpose of cooking (frying or baking), which demands high amounts of monounsaturated fatty acids such as OA. Thus, the method of consumption of such oilseeds is one of the important factors for choosing the oilseed for developing transgenics to produce EPA and DHA [101]. Fatty acid composition of certain oilseeds makes them attractive targets for the choice for metabolic engineering. Oilseeds, such as niger and linseed, which are rich sources of ALA and can produce up to 60 % of plant ω-3 fatty acids, can be engineered for the production of EPA and DHA. Other oilseeds such as soybean (~55 % LA) and sunflower (~75 % LA) that have high LA content can be engineered for increased ALA production and further modified for producing LC-PUFAs. However, the information about carbon flow for TAG synthesis, substrate specificities of the native enzymes involved in FA synthesis and oil production, is critical in determining the plant that can be used for metabolic engineering. As discussed in the next section, we are far from understanding the fluxes involved and much research still needs to be done for accurately engineering the LC-PUFA synthesis in plants.

Heterologous Expression of LC-PUFA Biosynthesis Enzymes in Plant Seeds

LC-PUFA production has been characterized in many microbes including marine microalgae, diatoms, fungi, yeasts, mosses, and bacteria. These organisms provide a large repertoire of genes that can be used to develop transgenic oilseeds with enhanced nutritional quality. The organisms and the LC-PUFA biosynthesis enzymes characterized from them are summarized in Table 15.2. Several attempts have been made to develop transgenic oilseeds that can produce EPA and, to a lesser extent, DHA [90, 102–106]. The highest EPA content in an oilseed crop is observed in soybean in the study carried out by Kinney et al. [104]. EPA consisted of about 19.6 % of the total fatty acids in the seeds with about 77.8 % Δ6 desaturation activity. In transgenic *Camelina sativa,* up to 31 % EPA production and about 11 % DHA production was observed [106]. Other reports on linseed and *Arabidopsis* show reduced EPA and DHA production; however, these studies can help us to understand and address the bottlenecks to successfully develop a transgenic oilseed producing LC-PUFAs.

Promoters for Ectopic Gene Expression

The success of ectopic production of LC-PUFAs in oilseeds greatly depends on the coordinated expression of several genes of the LC-PUFA biosynthetic pathway. The choice of promoters for expression of multiple genes in the vector is as important as the choice made for genes for LC-PUFA synthesis. Most studies often use the CaMV35S promoter for the expression of heterologous genes in plants. However, the CaMV35S promoter is a strong, tissue non-specific and constitutive promoter. As the fatty acid biosynthesis pathway is precisely regulated spatially and temporally to produce a desired composition of FAs in oilseeds, such constitutive and tissue non-specific expression of the heterologous genes might in fact show unexpected effects or undesired phenotypes. To circumvent such problems, many seed-specific promoters that have been discovered previously can be used in altering the oil quality through transgenic approaches [107].

As linseed is the richest agricultural source of ALA, which is the last ω-3 FA in the FA biosynthetic pathway that plants can normally synthesize, it is an attractive target for metabolic engineering of LC-PUFA biosynthesis. We have attempted to explain how suitable promoters can be employed for LC-PUFA synthesis using linseed as a model plant. In linseed, the maximum flux toward biosynthesis of fatty acids, their modification, and subsequent incorporation in TAG takes place during 11–30 days after anthesis [108]. Oil accumulation fairly remains constant after this stage. The promoters that are seed specific and active during these stages of seed development are highly desired for developing transgenic linseed. Table 15.3 summarizes the activity of different promoters in different tissue types of linseed [109]. The CaMV35S promoter shows increased expression of the reporter gene (GUS) in leaves as compared to that of seeds. Both Napin and KCS promoters show reduced expression of GUS in seeds as well as in leaves and roots. The promoters evaluated by Drexler et al. [109], viz. Dc3, LeB4, and USP,

Table 15.2 The LC-PUFA biosynthesis enzymes characterized from various organisms

S. no.	Enzyme	Organisms	Reference
1	Δ6 desaturase	*Physcomitrella patens*	Girke et al. [122]
		Mortierella alpina	Sakuradani et al. [123]
		Thamnidium elegans	Wang et al. [124]
		Rhizopus stolonifer	Wan et al. [125]
2	Δ6 elongase	*Physcomitrella patens*	Zank et al. [126]
		Mortierella alpina	Parker-Barnes et al. [96]
3	Δ5 desaturase	*Pavlova salina*	Zhou et al. [127]
		Pythium irregulare	Hong et al. [128]
4	Δ5 elongase	*Phaeodactylum tricornutum*	Jiang et al. [129]
5	Δ4 desaturase	*Thraustochytrium sp.*	Qiu et al. [130]
		Euglena gracilis	Pollak et al. [131]
		Pavlova lutheri	Pereira et al. [132]
6	Δ17 desaturase	*Saprolegnia diclina*	Xue et al. [133]

show the highest expression of GUS in seeds as compared to roots and leaves. Other promoters that control the expression of the genes involved in fatty acid synthesis in linseed have also been studied. The linseed SAD2 promoter, which normally controls the expression of stearoyl-ACP desaturase, shows constitutive and tissue non-specific expression [110]. The SAD1 promoter alternatively shows expression only in developing seeds and roots [110]. The promoter of Δ15 desaturase from linseed (LuFAD3A) shows strong seed-specific expression; however, the activity of the reporter gene is absent in the seed coat. Another strong seed-specific promoter Conlinin1 [111] shows the highest expression of the reporter gene at 15 DAA, which reduces gradually. Given this information about the activity of promoters in linseed, LuFAD3A, Conlinin1, DC3, LeB4, and USP may serve as ideal promoters to drive the LC-PUFA biosynthesis in transgenic oilseeds.

Potential Hurdles in Achieving Balanced ω-6/ω-3 Ratio

Though many efforts have been made to produce transgenic oilseeds rich in LC-PUFAs, much is left to obtain fish-like FA composition. Two main reasons associated with the reduced EPA and DHA production and the probable solutions are described below.

Substrate Dichotomy Bottleneck

Substrate dichotomy is the major bottleneck associated with the conversion of LA and ALA into nutritionally important LC-PUFAs such as ARA, EPA, and DHA. As discussed before, desaturases utilize glycerolipid (PC)-linked acyl substrates, whereas elongases act on acyl-CoA. The release of this modified FA back to the cytoplasmic pool of acyl-CoA is not efficient, and thus, the production of EPA and DHA is stalled at Δ6 desaturation, at the beginning of the process. The transfer of acyl chains from PC to CoA and vice versa is carried out by lysophosphatidylcholine acyltransferase (LPCAT) (Fig. 15.6). Yeast and animal LPCAT genes [98] catalyze the transfer of acyl chains from CoA to PC, but the reverse reaction is not seen [112, 113]. The possible solutions for this bottleneck are identification and isolation of an efficient acyltransferase, which possesses this bifunctional activity, the conversion of PC to CoA and back.

Another solution for this problem is the use of desaturases that act on the substrates linked to CoA, so that no other acyltransferases are required, since both desaturase and

Table 15.3 Expression profiles of GUS under the effect of different promoters tested in linseed

S. No.	Promoter	Tissue of expression	Activity during seed development	Maximum activity	Source	Reference
1	CaMV35S	Seed, leaves, roots	Throughout	NA	Cauliflower mosaic virus	Drexler et al. [109]
2	Napin	Seed, leaves, roots	17-30DAA	NA	*Brassica napus*	Drexler et al. [109]
3	KCS	Seed, leaves, roots	NA	14DAA	*Brassica napus*	Drexler et al. [109]
4	Dc3	Seeds	NA	NA	*Daucus carota*	Drexler et al. [109]
5	LeB4	Seeds	NA	11DAA	*Vicia faba*	Drexler et al. [109]
6	USP	Seeds	NA	5-6DAA	*Vicia faba*	Drexler et al. [109]
7	SAD1	Seeds, roots	4-50DAA	14-28DAA	*Linum usitatissimum*	Jain et al. [110]
8	SAD2	Leaves, apices, stem, roots, flower buds, flowers, seeds	4-50DAA	14-28DAA	*Linum usitatissimum*	Jain et al. [110]
9	LuFAD3	Seeds	10-20DAA	20 DAA	*Linum usitatissimum*	Qiu et al. [134]
10	Conlinin1	Seeds	15-25DAA	15 DAA	*Linum usitatissimum*	Qiu et al. [134]

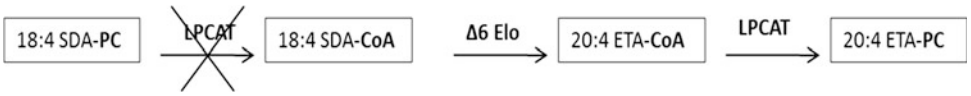

Fig. 15.6 Substrate dichotomy bottleneck involved in conversion of SDA to ETA

elongase will utilize CoA-linked FA substrates. Δ6 desaturases from *Mantoniella squamata* [114] and *Ostreococcus tauri* [115] have such specificity toward CoA-linked substrates. The efficiency of desaturation can be further increased by incorporation of superior desaturases that can act on both 18:3 and 20:4 and serve as a bifunctional Δ6/Δ5 desaturase [116].

Reduced Metabolic Flux for PC-Independent Acyl Modifications

Studies in linseed have revealed that the major flux for DAG synthesis is PC derived. A PDCT-like enzyme is involved in this process. TAG synthesis by acyl-CoA-independent pathway makes use of PDAT to transfer the third acyl chain to the glycerol molecule. As desaturation of 18:3–18:4 and its transfer from PC to either DAG or TAG can take place simultaneously, it gives little room for the elongases to act [117, 118]. It has been shown that metabolic engineering of LC-PUFA production in plants by "acyl-CoA-dependent pathway" leads to higher accumulation of ω-3 LC-PUFA in the oils [114]. Here, the desaturases act on acyl-CoA substrates; however, the TAG synthesis by PDAT is still functional. This may lead to the loss of precursors of EPA and DHA as LA/ALA can still be taken up for TAG synthesis by PDAT. Hence, changes in the flux of DAG and TAG synthesis by blocking the activities of PDAT and PDCT and simultaneous overexpression of LPCAT might lead to the increased EPA and DHA production in linseed. Another approach to increase the content of EPA and DHA in TAG is to overexpress LC-PUFA-specific PDAT.

Conclusions and Future Prospects

Humans have evolved on a diet that was much richer in ω-3 fatty acids than today's diet. With the emphasis on higher production, and longer shelf life of ω-6 fatty acid containing oils, the ω-6: ω-3 ratio has highly skewed in modern times to up to 25:1. As ω-6 FAs are the precursors for pro-inflammatory eicosanoids, their high amounts in the body can contribute to pathogenesis of many diseases, including cardiovascular diseases, cancer, and osteoporosis , whereas increased levels of ω-3 FAs exert suppressive effects by their anti-inflammatory eicosanoid production. Hence, balancing the ω-6: ω-3 ratio is vital. The easiest approach to achieve this would be consumption of oils rich in ω-3 FAs, such as linseed oil. Alternatively, the fatty acid biosynthetic pathway in plants producing high ω-6 FAs can be altered by biotechnological means, so that they produce higher proportion of ω-3 FAs. However, this is very challenging, as the fatty acid biosynthesis is a highly complex pathway modulated by several genes expressing at several precise seed developmental stages and tissues or organelles. Nevertheless, there have been a handful of successful examples of altering the pathways to balance the ω-6: ω-3 ratio in few oilseeds. These factors indicate that altering the ω-6: ω-3 ratio might not be possible or successful in all oilseeds. However, a better understanding of the intricacies of the fatty acid biosynthesis in the target oilseeds could help in modulating the desired genes for successfully balancing the ω-6: ω-3 ratio in them.

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Omega-3 Fatty Acids and Diabetic Complications

16

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Introduction

Diabetes mellitus (DM)—one of the most common diseases—known to mankind from the oldest time, as reported in Egyptian manuscript about 3000 years ago, has become a serious global problem today. However, the clear-cut distinction between type 1 and type 2 DM was made only in 1936 [1]. DM is characterized by elevated blood glucose levels in the body due to either impaired response of cells to insulin in type 2 diabetes or its insufficient synthesis in the body in type 1 diabetes [2]. When cells do not utilize glucose in adequate quantities, it accumulates in the blood leading to hyperglycemia. Diabetes is also associated with accelerated atherosclerotic disease affecting arteries that supply the heart, brain, and lower extremities. All forms of diabetes are characterized by hyperglycemia which in turn leads to complications viz. nephropathy, cardiomyopathy, retinopathy, neuropathy, and loss of RBC deformability which result due to macro- and micro-angiopathy [3].

Liver plays a central role in lipid metabolism including that of de novo synthesis of fatty acids. The liver also modifies fatty acid structure through metabolic pathways that include desaturation, elongation, mono-oxidation, and peroxisomal oxidation (chain shortening). These pathways are particularly critical for the generation of end products of polyunsaturated fatty acid (PUFA) synthesis. Thus, starting with alpha-linolenic acid (ALA, 18:2, n-3) liver synthesizes two major omega-3 fatty acids viz. eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3) which are important and essential for membrane structure–function relationships. Arachidonic acid (20:4, n-6) and DHA are the main C20–22 PUFAs present in membranes of all tissues including that of RBC membrane [4]. It has been reported that in diabetes, the elongase and desaturase activities decrease significantly which result in membrane lipid deformability [5]. The ensuing membrane lipid deformability could also contribute to diabetic complications [6]. It also has been widely reported that there is a deficiency in essential fatty acid metabolism in both human and animal diabetes [7, 8].

The dietary sources of omega-3 fatty acids include fish and seafood which are rich in EPA and DHA. Oily fish such as salmon, trout, sardines, anchovies, mackerel, and herring are the best sources. The other omega-3 fatty acid, ALA, is found in plant-based foods such as flaxseeds, flax oil, walnuts, canola oil, and soybean.

The dietary n-3 polyunsaturated fatty acids (PUFAs) have been associated with various important functions such as anti-inflammatory effects, improving endothelial function, controlling the blood pressure, and reducing hypertriglyceridemia and insulin insensitivity. According to some epidemiologic studies, a lower prevalence of Type 2DM was found in populations consuming large amounts of seafood products, which are rich in n-3 PUFAs [9].

The link between omega-3 fatty acids and diabetes and its complications has not been clear so far. Some studies have revealed protective effects with appropriate intakes of omega-3 fatty acid, while others have found no association at all; some researchers have even hinted that high omega-3 intakes might augment risk of diabetes. Numerous in vitro and in vivo studies have been carried out in respect of omega-3 fatty acids and diabetic complications. The present review tries to summarize the reported effects and role of omega-3 and omega-6 PUFAs in nutrition and metabolism on diabetic complications viz. nephropathy, cardiomyopathy, retinopathy, neuropathy, and loss of RBC deformability.

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Omega-3 and Diabetic Nephropathy

Diabetes is the leading cause of end-stage renal disease. The complex metabolic, vascular, and inflammatory perturbations that characterize DM often lead to progressive albuminuria, renal injury, and dysfunction: Diabetic renal nephropathy (DN) and the observed complications seem to be related to the altered membrane fatty acid composition [10, 11].

In type 1 diabetes, dietary n-3 LCPUFAs appear inversely associated with the degree, but not with the incidence of albuminuria [12]. In experimental settings and in epidemiologic studies, intake of omega-6 fatty acids was associated with reduced albuminuria. Apparently, PUFAs do not seem to attenuate glomerular dysfunction. However, the presently available evidence is insufficient to rule out such an effect. The authors suggest that further research is necessary to establish the potential of PUFA consumption and supplementation in DN [13].

In double-blind placebo-controlled trial, it was found that 12 weeks of fish oil supplement had no beneficial effect on vascular endothelial function, but improved renal function. Thus, serum creatinine level was significantly lower in fish oil-treated type 2 DM patients [14].

In meta-analysis, in chronic kidney disease, it was found that the use of n-3 long-chain PUFA (LCPUFA) reduced urinary protein excretion, but there was no decline in the glomerular filtration rate (GFR). However, in view of small number of participants in trials, different methods of assessing proteinuria and GFR, the authors suggest that large, high-quality trials are warranted for reliable clinical outcomes [15].

In animal studies, it was found that in streptozotocin–diabetic rats, twenty four hour urinary albumin excretion was significantly increased compared to the non-diabetic control; gamma linolenic acid (GLA) treatment significantly reduced albuminuria. Intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), fibronectin (FN) mRNA, and protein expression levels were significantly higher in DM kidneys, and these increases were significantly lowered by GLA treatment. Under *in vitro* conditions, in the presence of high glucose concentration, GLA significantly inhibited increases in MCP-1 mRNA expression and protein levels in mesangial and tubular epithelial cells; ICAM-1 and FN expression showed a pattern similar to the expression of MCP-1. Thus, GLA attenuated inflammation by two mechanisms: by inhibiting enhanced MCP-1 and ICAM-1 expression as well as by preventing the accumulation of extracellular matrix (ECM) in diabetic nephropathy [16].

Eicosapentaenoic acid (EPA) has been reported to have beneficial effects on the progression of various renal diseases including diabetic nephropathy. In KKA(y)/Ta mice, EPA improved type 2 diabetic nephropathy possibly by attenuation of metabolic abnormalities and inhibition of renal inflammation, oxidative stress, and TGF beta expression [17]. Monocyte chemoattractant protein-1 (MCP-1) regulating macrophage recruitment protein is known to be up-regulated in patients with diabetic nephropathy. In type 2 diabetic KKA(y)/Ta mice, EPA ameliorated diabetic nephropathy which would suggest that the observed down-regulation of MCP-1 is critically involved in the beneficial effect of EPA, probably in concert with improvement of other clinical parameters [18].

Omega-3 and Diabetic Retinopathy

The potential pathogenic mechanisms that may predispose to diabetic retinopathy include the following: platelet dysfunction, altered eicosanoid production, increased blood viscosity in association with impaired cell deformability, and pathologic leukocyte/endothelium interaction. Omega-3 fatty acids exert several important biological effects on these factors [19].

The vasodegenerative phase of diabetic retinopathy is characterized by retinal vascular degeneration as also by the inadequate vascular repair due to compromised bone marrow-derived endothelial progenitor cells (EPCs). It is proposed that in diabetes, n-3 PUFA deficiency results in activation of acid sphingomyelinase (ASM), the central enzyme of sphingolipid metabolism; ASM represents a molecular metabolic link connecting the initial damage in the retina and the dysfunction of EPCs [20]. ASM is an important early responder in inflammatory cytokine signaling. The endothelial caveolae-associated ASM is believed to be an essential component in mediating inflammation and vascular pathology in in vivo and in vitro models of diabetic retinopathy. Human retinal endothelial cells (HREC), as against glial and epithelial cells, express the plasma membrane form of ASM that overlaps with caveolin-1. Treatment of HREC with DHA specifically reduced expression of the caveolae-associated ASM, prevented tumor necrosis factor-α (TNFα)-induced increase in the ceramide-to-sphingomyelin ratio in the caveolae, and inhibited cytokine-induced inflammatory signaling. ASM is expressed in both vascular and neuroretina. Interestingly, however, only vascular ASM is specifically increased in the retinas of animal models at the vasodegenerative phase of diabetic retinopathy [20, 21].

In type 2 diabetes animal model, DHA-rich diet prevented diabetes-induced increase in the number of retinal acellular capillaries and significantly enhanced the life span of type 2 diabetic animals by blocking the up-regulation of ASM and other inflammatory markers in diabetic retina. DHA-rich diet also normalized the numbers of circulating EPCs, improved EPC colony formation, and prevented the

increase in ASM activity in EPCs [20]. The absence of ASM in ASM(-/-) mice or inhibition of ASM activity by DHA prevents acellular capillary formation [21].

Oxidative stress and inflammation play a significant role in the pathobiology of diabetic retinopathy. It is suggested that increased consumption of PUFAs may prevent or postpone the occurrence of diabetic retinopathy. In STZ–diabetic rats with retinopathy, changes in serum glutathione peroxidase, brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), and interleukin 6 (IL-6) reverted to near control by ALA treatment, especially in ALA + STZ group. The observations lend support to the concept that both oxidative stress and inflammation are involved in DR, and ALA treatment is of benefit in its prevention [22].

Diabetes increases oxidative stress, nitrotyrosine concentrations, and apoptosis in the retina. As a consequence, the total thickness of retinas decreases significantly compared to that in the control rats. In particular, in the diabetic rats the thickness of the outer and inner nuclear layers was reduced significantly, and loss of ganglion cell layer (GCL) is evident. Administration of insulin and DHA, and lutein alone or in combination with insulin could prevent these retinal changes. In the diabetic rats the electroretinogram showed impairment of b-wave amplitude and latency time. DHA and lutein prevented all these changes even under hyperglycemic conditions. Lutein and DHA were able to normalize all the diabetes-induced biochemical, histological, and functional modifications. In view of the observed beneficial effects relating to the cell death mechanisms, further studies to evaluate the use of insulin and DHA, and lutein as potential adjuvant therapies to help prevent vision loss in diabetic patients, are desirable [23].

In db/db mice, n-3 PUFA diet significantly preserved retinal function to levels similar to those observed in non-diabetic control mice on normal chow. Conversely, retinal function gradually deteriorated on-6 PUFA-rich diet. Also, in the n-3 PUFA-fed mice there was an enhanced ability to respond to glucose challenge. Interestingly, the protection of visual function was independent of cytoprotective or anti-inflammatory effects of n-3 PUFAs.

In T2DM mice, dietary n-3 PUFA preserved retinal function which is consistent with the notion that with dyslipidemia it negatively impacts retinal function. Keeping in mind the beneficial effects of dietary n-3 PUFAs on visual function, the authors suggest that increasing n-3 PUFA intake in diabetic patients may slow the progression of vision loss in T2DM [24].

The n-3 LCPUFAs influence retinal cell gene expression, cellular differentiation, and cellular survival. DHA is a major structural fatty acid of retinal photoreceptor outer segment membranes and hence can influence the function of the photoreceptor membrane. DHA activates a number of nuclear hormone receptors which in turn operate as transcription factors for molecules that modulate oxidation–reduction sensitive and proinflammatory processes. DHA can also affect the retinal cell signaling mechanisms involved in phototransduction. Thus, DHA may play a major role in signaling cascades by enhancing activation of membrane-bound retinal proteins and may also be involved in rhodopsin regeneration. Evidently, insufficiency of DHA is associated with alterations in retinal function [25].

Hammes et al. maintained STZ–diabetic rats on fish oil (750 mg Maxepa, 5 times per week for six months), containing 14 % eicosapentaenoic acid (EPA) and 10 % docosahexaenoic acid. The treatment resulted in a twofold increase of EPA in total fatty acids and a reduction in the thromboxane 2/3 ratio from 600 (untreated diabetic rats) to 50 (treated diabetic rats). However, despite these biochemical changes, diabetes-associated pericyte loss remained unaffected, and the formation of acellular, occluded capillaries was increased by 75 %. Based on these observations, the authors concluded that dietary fish oil supplementation may be harmful for the diabetic microvasculature in the retina. These results are in contradiction with the beneficial effects cited above [19]. However, it may be pointed out that in the studies by Hammes et al., the doses of DHA and EPA were several times higher and also for a longer duration (20 weeks). It is therefore possible that the undesirable adverse effects ensued due to over dosages and treatment for prolonged period. As against this in the former studies, the authors treated the STZ–diabetic rats with 13.3 mg DHA/kg body weight for a period of 12 weeks [23].

Lipid composition of retinal membranes reflected the dietary manipulation. In STZ–diabetic rats, diabetes amplified some fatty acid changes consistent with reduced desaturase activity that was evident. Diabetes produced significant reduction in rod function (−33 %) only in the absence of fish oil, whereas cone responses (−46 %) and inner retinal oscillatory potentials (−47 %) showed either no effect of diet or a partial diet effect with a significant diabetes effect. A diet balanced in long-chain PUFAs modifies retinal lipid membranes in diabetes and prevents rod dysfunction. Dietary modification was not found in the cone or glial response, but a partial improvement was evident in the oscillatory potentials (OPs), most likely secondary to the larger photoreceptor output [6].

Omega-3 and Diabetic Cardiomyopathy

The worldwide increasing prevalence of T2DM poses an immense public health hazard leading to a variety of complications, such as cardiovascular diseases, nephropathy, and neuropathy [11].

Omega-3 polyunsaturated fatty acids (PUFAs) offer protection against cardiovascular disease which is one of the

major causes of death in patients with DM by virtue of their antihyperlipidemic, antihypertensive, anti-inflammatory, and other properties. Omega-6 PUFAs are also cardioprotective [12].

In type 2 diabetic patients, without or with autonomic neuropathy and normal healthy subjects, BP and ECG were monitored during a 24 h period and during a 2 hr hyperglycemic clamp. Delta QTc during the night was blunted in diabetics and delta LF/HF was decreased in patients with autonomic neuropathy. In hyperglycemia, QTc and LF/HF increased significantly in normal healthy subjects while in patients without autonomic neuropathy only LF/HF increased. A 6 month treatment with n-3 PUFA partially restored delta LF/HF and delta QTc only in patients without autonomic neuropathy [26].

Studies on heart rate variability (HRV) and n-3 PUFA have been performed in several populations such as patients with ischemic heart disease, diabetes mellitus, and chronic renal failure, and in healthy subjects. These studies have demonstrated a positive association between cellular content of n-3 PUFA and HRV. Also, supplementation with n-3 PUFA increased HRV and thereby decreased the risk of arrhythmic events and provided protection against sudden cardiac death (SCD) [27].

Administration of formulation omega-3 with Fenugreek terpenenes (Om3/terp) considerably inhibited key enzymes related to diabetes. Thus, α-amylase activity decreased by 46 and 52 %, and maltase activity decreased by 37 and 35 %, respectively, in pancreas and plasma. Additionally, this supplement helped protect the β-cells of the experimental animals from death and damage. Interestingly, the formulation of Om3/terp also modulated key enzyme related to hypertension and angiotensin-converting enzyme (ACE) by 37 % in plasma and kidney. Administration of fenugreek essential oil to surviving diabetic rats improved starch and glucose oral tolerance additively. Om3/terp also significantly decreased the levels of glucose, triglyceride (TG), total cholesterol (TC), and LDL cholesterol (LDL-C) in the plasma and liver of diabetic rats and increased the HDL cholesterol (HDL-C) level, which helps in maintaining the homeostasis of blood lipids. Taken together, the findings suggest that this formulation of Om3/terp exhibits attractive properties and can, therefore, be considered for future application in the development of anti-diabetic, anti-hypertensive, and hypolipidemic foods [28].

It has been suggested that omega-3 FA treatment partially blocks the development of experimental diabetic cardiomyopathy possibly by affecting sarcoplasmic reticulum calcium transport activity [29]. Diabetic cardiomyopathy has also been associated with a decrease in Na^+, K^+-ATPase activity and expression as well as alterations in membrane lipid composition. Diabetes significantly decreased activities of alpha 1 (α1) and alpha 2 (α2) isoforms and mRNA levels of α2 and beta 1(β1) isoforms. At the protein level, α1-isoform increased, while both α2- and β1-isoforms decreased. Changes in fatty acid content of the membrane were consistent with the inhibition of desaturase activity; supplementation with fish oil produced an increase in the incorporation of EPA in the membrane. Supplementation with fish oil also increased the level of β1-isoforms and restored the activity of the α2-isoenzyme without significant changes in the level of α1- and α2-isoforms. Based on these studies, the authors suggest that fish oil therapy may be effective in preventing some of the adverse consequences of diabetic cardiomyopathy [30].

In patients with type 2 DM and cardiovascular autonomic neuropathy, combined treatment with n-3 PUFA, benfotiamine, and α-lipoic acid resulted in significant positive changes in TC, TG, and LDL and HDL cholesterol levels. However, the efficacy of this treatment was not related to the improved compensation of DM, but was due to the direct influence of pharmacological agents on the metabolic rate studied [31].

Diets higher in fish and omega-3 LCPUFA may reduce cardiovascular risk in diabetes by inhibiting platelet aggregation, improving lipid profiles, and reducing cardiovascular mortality. Fish and omega-3 LCPUFA can be recommended to people with diabetes and included into a diabetes management program [32].

In STZ–diabetic rat model, the myocardial levels of matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of matrix metalloproteinase-4 (TIMP-4) changed. There was reduction in troponin (Tnl) and alpha-actinin protein levels. The diabetes-induced alterations in MMP-2 and TIMP-4 contribute to myocardial contractile dysfunction by targeting TnI and alpha-actinin. Treatment with sodium selenate or with omega-3 fish oil with vitamin E had improved these parameters [33]. Doxycycline, a MMP-2 inhibitor, or an antioxidant selenium treatment in vivo prevented diabetes-induced cardiac dysfunction significantly [34]. The author suggests that antioxidants and MMP inhibitors both could regulate MMP function, but may also utilize different mechanisms of action in cardiomyocytes, particularly related to beta-AR signaling pathway [34].

There is also evidence to suggest that besides other cardiomyopathies the anti-inflammatory action of omega-3 PUFAs may have beneficial effects on chronic chagasic cardiomyopathy due to improved control of the inflammatory response. The authors predict that patients will have lower inflammatory markers and an improved metabolic and anthropometric profile [35].

Omega-3 and Diabetic Neuropathy

Diabetic neuropathy is a degenerative complication of diabetes accompanied by an alteration of nerve conduction velocity (NCV) and Na^+, K^+-ATPase activity.

In STZ–diabetic rats, supplementation with DHA only partially prevented the decrease in NCV and did not affect nerve blood flow (NBF). On the other hand, supplementation with GLA–lipoic acid (GLA-LA) conjugate was more effective than supplementation with DHA alone in preventing experimental diabetic neuropathy. The difference could be due in part to an antioxidant protective effect of LA on GLA [7].

A deficiency in essential fatty acid metabolism has been widely reported in both human and animal diabetes. Supplementation with fish oil was less effective on diabetic neuropathy than n-6 fatty acids. The partial effect of n-3 fatty acids might be attributed to the presence of EPA which competes with arachidonic acid and thereby amplifies the diabetes-induced decrease in this fatty acid in serum and tissues. Fish oil supplementation changed the fatty acid content of sciatic nerve membranes, decreasing C18:2(n-6) fatty acids and preventing the decreases in arachidonic acids and oleic acid C18:1(n-9) fatty acids [8].

Whether supplementation with DHA alone could prevent neuropathy in STZ-induced diabetes was determined in separate experiments. Eight weeks of diabetes induced significant decreases in NCV, NBF, and sciatic nerve and erythrocyte Na^+, K^+-ATPase activities. Na^+, K^+-ATPase activity was significantly lower in sciatic nerve membranes of diabetic rats and was significantly restored in diabetic animals that received fish oil supplementation. Diabetes induced a specific decrease of α1- and α3-isoform activity and protein expression in sciatic nerve membranes. Fish oil supplementation restored partial activity and expression to varying degrees depending on the isoenzyme. These effects were associated with a significant beneficial effect on NCV. This study indicates that fish oil has beneficial effects on diabetes-induced alterations in sciatic nerve Na^+, K^+-ATPase activity and function [36].

Liposomes containing DHA phospholipids, at a dose of 60 mg/kg, were given daily to diabetic rats by gavage. DHA phospholipids totally prevented the decrease in NCV and NBF; DHA phospholipids also prevented the decrease in Na^+, K^+-ATPase activity in the RBCs but not in the sciatic nerve. The levels of DHA in the sciatic nerve membranes correlated with NCV. The results demonstrate a protective effect of daily doses of DHA in experimental diabetic neuropathy. Thus, treatment with DHA phospholipids could provide a suitable approach for evaluation in clinical trials [34]. It is also reported that highly purified ethyl esterification product of natural EPA (EPA-E) has significant beneficial effects on diabetic neuropathy and serum lipids as well as other diabetic complications such as nephropathy and macroangiopathy [37].

Omega-3 and Red Blood Cell Deformability

The content of PUFA is known to affect membrane fluidity and cell signaling. Patients with insulin resistance display a pattern of higher proportion of long-chain saturated fatty acids, mainly palmitic, stearic, and arachidic acids. Decreased levels of erythrocyte membrane DHA in end-stage renal diseases with type 2 diabetes patient group suggest that there may be reduced endogenous synthesis of DHA due to the decreased desaturase and elongase activities [5, 38].

Omega-3 fatty acids increased red blood cell (RBC) deformability and decreased plasma viscosity. Also, there is a drop in red cell aggregation after treatment with omega-3 capsule. Thus, there is growing evidence to suggest that omega-3 FA can delay atherogenesis [39]. The omega-3 FA gets incorporated into RBC phospholipids at the expense of C18:2 omega-6 FA. At the same time, the total unsaturation index of phospholipids increases. This in turn brings about increase in membrane fluidity which is responsible for the increased erythrocyte deformability. The observed changes have been attributed to incorporation of omega-3 FA in erythrocyte phosphatidylcholine (PC) [40, 41]. Daily supplementation of 3 g of n-3 FA (EPA and DHA) increased unsaturation of PC and phosphatidylethanolamine (PE). At the same time, there was slight decrease in PC and PE content, but the content of sphingomyelin (SPM) increased. This supplementation caused a 42 % decrease in plasma triacylglycerol levels. However, the membrane fluidity was unchanged [42].

In subjects supplemented with fish oil, the levels of n-3 FA increased significantly in erythrocytes. This was mainly at the expense of linolenic (18:n6) and oleic acid (18:n1); the relative amount of arachidonic acid was unchanged [43]. The total phospholipid/CHL ratio was unchanged while the PC + SPM/PE increased which is consistent with the observations of Popp-Snijders et al. [40].

Conclusion

Supplementation with functional foods which are rich in omega-3 fatty acids (e.g., fish oils among others) may be a novel strategy to reduce insulin insensitivity, dyslipidemia, hypertension, retinopathy and pro-inflammatory state, improved renal function as well as improved RBC deformability [44].

The forgoing results suggest that n-3 PUFA supplementation in diet presents many benefits in T2DM management mainly in terms of diabetic complications. However, the treatment is less effective with respect to glucose control, inflammation and oxidative stress. Nonetheless, n-3 PUFA supplementation may be a reasonable therapeutic strategy in individuals with T2DM to decrease the risk of complications.

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Omega-3 Fatty Acids and Mitochondrial Functions

17

Surendra S. Katyare and A.V. Mali

Introduction

Mitochondria are small membrane-bound intracellular organelles present in the eukaryotic cells that provide energy for cellular activities through a series of reactions known as oxidative phosphorylation (oxphos). Consequently, mitochondria have long been recognized as the major site of bioenergetic pathways [1, 2]. Mitochondria generate most of the cell's supply of energy in the form of adenosine triphosphate (ATP). Hence, these organelles are commonly described as "The Powerhouse of the Cell." [3]. Besides supplying cellular energy, mitochondria are involved in other tasks such as cell signaling, cellular differentiation, cell death as well as maintaining the control of the cell cycle and cell growth [4]. About 3000 genes are required to make a mitochondrion. Out of these, only about 3 % of the genes (100 of the 3000) are allocated for synthesizing ATP. More than 95 % (2900 of 3000) genes are involved with other functions related to the specialized tasks of the differentiated cell. In each cell type, a set of particular genes is expressed by selective transcription in order to meet specialized needs. Thus, the mitochondrion is custom-made to meet the needs of the given cell/tissues. Some cell types have only a few mitochondria. For example, platelets have only two to six mitochondria. Red blood cells do not contain mitochondria, whereas liver cells have more than two thousand mitochondria [5].Mitochondria are the only cellular organelles known to have their own DNA (mtDNA), distinct from the nuclear DNA (nDNA). Thus, two genomes are involved in making the mitochondria functional. mtDNA codes for just 37 proteins out of 3000, and the remaining are coded by the nDNA. In particular, it has been demonstrated that the mitochondrial dehydrogenases and cytochrome c + c1 are coded by the nuclear genes, whereas crucial peptides of cytochromes aa3 and b as well as that of NADH dehydrogenase complex and ATPase are coded by mitochondrial DNA [6].

The resultant proteins are transported to the mitochondria. Defects in nDNA can be inherited from either parent, but defects in the genes of the mtDNA are maternally inherited [7–9].

A number of studies have identified a set of different disorders affecting mitochondrial function and/or structure that are collectively termed mitochondrial diseases (MDs). MDs generally refer to the group of disorders that are attributable to malfunctioning mitochondria or energy metabolism. This results in lack of cellular energy and in the accumulation of by-products that impair or destroy the cell.

Mitochondria are not well protected and are easily damaged by toxins, infections, allergens, and stress, but one of the prominent factors affecting mitochondria is eating too much food leading to too many "empty calories." When the food is metabolized/oxidized, the fall out is the formation of free radicals which have damaging effect. The free radicals damage mitochondria and produce fatigue, metabolic burnout, and all the complications of aging.

Nutrition plays a crucial role in the multidisciplinary management of mitochondrial diseases. Diet and nutrition have the ability to influence mitochondrial function, and in turn the effects of mitochondria-associated diseases [10]. The role of the diet in prevention of mitochondrial damage and pathogenesis of hypertension, coronary artery disease, type 2 diabetes, and atherosclerosis has been extensively studied in the past few decades [11]. One of the promising nutritional components which may play crucial role in the management of MDs is omega-3 polyunsaturated fatty acids (PUFAs). Body cannot synthesize PUFAs, i.e., omega-3 (n-3 PUFA) and omega-6 (n-6 PUFA).

Thus, these are essential fatty acids and must be obtained from the diet. Beneficial effects of n-3 PUFA are probably mediated by their antiarrhythmic, lipid lowering,

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antithrombotic, and anti-inflammatory properties [11]. The precursor of omega-3 fatty acids synthesis is α-linolenic acid (ALA), whereas that of omega-6 fatty acids is linoleic acid (LA). For the omega-3 fatty acids, ALA is converted to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Based on animal studies, it was concluded that the omega-3 PUFAs, i.e., ALA and especially EPA and DHA, have some positive effects on functional parameters of mitochondria. Thus, various mitochondrial dysfunction-related pathological conditions such as neurodegenerative diseases: Parkinson's disease (PD), Alzheimer's disease (AD), aging, cardiovascular diseases, diabetes, and ROS-induced damages have been attributed to deficiency of EPA and DHA. Supplementation with omega-3 PUFAs has been shown to provide protection from the above-mentioned mitochondrial dysfunctions. These aspects are reviewed in the present Chapter.

Neurodegenerative Diseases

A number of evidences suggest that mitochondrial dysfunction plays an important role in the pathogenesis of neurodegenerative diseases. Mitochondrial dysfunction represents a common early pathological event in brain aging and in neurodegenerative diseases, e.g., in AD, PD, Huntington's disease (HD), as well as in ischemic stroke [12]. Impairment of mitochondrial energy coupling efficiency and function is mainly related to alterations in mitochondrial content, amount of respiratory enzymes, or changes in enzyme activities. These alterations lead to oxidative stress, opening of mitochondrial permeability transition pore (MPTP), and enhanced apoptosis [13]. Studies of the brain in AD and other dementias, Down syndrome, stroke, PD, multiple sclerosis, amyotrophic lateral sclerosis (ALS), HD, Friedreich's ataxia, aging, and constitutive disorders demonstrate impairments of the mitochondrial citric acid cycle and enzymes of OXPHOS. Imaging or metabolic assays frequently reveal energy insufficiency and depleted energy reserve in brain tissue in situ [14].

The cells and cellular organelles in the brain are very rich in polyunsaturated omega-3 fatty acids. A simultaneous deficiency of LA and ALA seriously creates the problems in fatty acid composition of all the organs, including the brain [15]. In vivo and ex vivo experiments using animal models of aging and AD, PD, and HD showed improvement in mitochondrial function after treatment with n-3 PUFAs, especially with DHA. Thus, PUFAs seem to be particularly beneficial in animals treated with mitochondria targeting toxins. More recent studies have shown that n-3 PUFAs block apoptotic neuronal cell death and are strongly neuroprotective in acute and chronic neurodegeneration. It was found that ALA deficiency alters the course of brain development, perturbed the composition of brain cell membranes, neurones, oligodendrocytes, and astrocytes as well as subcellular component such as myelin, nerve endings (synaptosomes), and mitochondria [16]. In one study, Afshordel et al. investigated the effects of orally administered long chain n-3 PUFAs on mitochondrial function and processing of the amyloid precursor protein (APP) in brains of young (3 months old) and aged (24 months old) NMRI mice. The mice were supplemented with 1.6 ml/Kg fish oil (FO) *p.o.* for 21 days, and neuroprotective properties were assessed ex vivo in dissociated brain cells (DBC) and in isolated mitochondria. It was found that DHA levels were significantly lower in blood and brains of aged mice which were compensated by FO administration. Isolated DBC and mitochondria from aged mice showed significantly lower ATP levels and reduced activity of complexes I + II and IV of the mitochondrial respiratory chain. FO restored the age-related decrease in respiration and improved ATP production. Moreover, FO increased the levels of anti-apoptotic Bcl-2 protein. Cell membrane fractions isolated from the brain of aged mice exhibited lower membrane fluidity, which was partially improved by FO treatment [17]. One recent clinical study explored the effect of dietary n-3 PUFAs intake on lipid peroxidation in patients with mild cognitive impairment (MCI) in which the plasma lipid hydroperoxide (LPO) levels in 67 MCI patients were compared with those of 134 healthy elderly controls. The results revealed that LPO levels were significantly higher in the MCI group than in the control group; in the MCI group, inverse correlation was found between DHA and EPA intake and LPO level. LPO levels decreased significantly with the increase in DHA and EPA intake. In summary, the findings suggest that DHA and EPA can play a role in alleviating oxidative stress and reducing the risk of neurodegenerative diseases [18]. By contrast, in a transgenic PD mouse model, DHA showed adverse effects and it is not clear whether a diet high or low in PUFA might provide neuroprotective effects in PD. Post-treatment with PUFA, conflicting results were obtained in ischemic animal models but intravenously administered DHA provided neuroprotective effect after acute occlusion of the middle cerebral artery [15]. In summary, there is a lack of direct evidence concerning the role of omega-3 PUFA in the treatment of PD and epidemiological evidence suggests that n-3 PUFA may offer limited neuroprotection. Current evidences indicate that the beneficial effects of omega-3 PUFA are related more to the limitation of the progression of cognitive decline and that n-3 PUFA may have limited efficacy after onset of AD symptoms [19]. Majority of preclinical data indicate some beneficial effects of n-3 PUFA in neurodegenerative diseases, whereas most controlled clinical trials did not meet the expectations.

Because of the high half-life of DHA in the human brain, clinical studies may have to be initiated much earlier and have to last much longer to be more efficacious [12].

Cardiovascular Diseases

Implications of oxidative stress (OS)/mitochondrial dysfunction (MDF) in the pathogenesis of cardiovascular diseases (CVDs) have been investigated extensively [20]. MDF such as mitochondrial aberrations, superoxide dismutase 2 (SOD 2) upregulation, and mtDNA mutations in atherosclerotic plaques in the pathogenesis of CVDs seems to be well established [21–24]. Earlier studies on patients with chronic heart disease revealed decreased electron transport chain (ETC) complex activity and identified a nonpathological point mutation in the mtDNA-encoding cytochrome b gene [25, 26]. The key event in the onset of heart failure is believed to be impaired blood flow, both to and within the heart. Impaired heart flow leads to reduced oxygen availability at the ETC of cardiac mitochondria. Oxygen is the final electron acceptor in the ETC, and thus, a deficit of oxygen leads to the accumulation of electrons at ETC complexes [27]. This accumulation of electrons induces ROS production, which serves as a stress response to signal that oxygen availability is low [28]. However, ROS production leads to mtDNA damage and mutations. In some cases, mtDNA loss and loss of mitochondrial mass along with frequent mtDNA mutations are observed in heart failure which resulted in an impaired ETC activity [29].

Recent evidences have shown that supplementation with n-3 PUFA showed dramatic alterations of mitochondrial phospholipid membranes and favorable changes in mitochondrial function. Panasiuk et al. investigated the influence of dietary n-3 PUFAs administered to rats on functional parameters of hearts mitochondria after isoproterenol-induced injury. They found that isoproterenol-induced heart injury leads to decreased rate of succinate oxidation in isolated mitochondria; administration of omega-3 PUFAs significantly restored the respiration rate of mitochondria. Also, n-3 PUFAs decreased mitochondria swelling by 60 % in nominally calcium-free medium. The results thus emphasize that n-3 PUFAs improve the altered functions of the heart mitochondria evoked by isoproterenol-induced injury [30]. In another study, n-3 PUFAs conferred protection against myocardial injury after ischemia–reperfusion. The authors concluded that interfibrillar mitochondria (IFM) and subsarcolemmal mitochondria (SSM) do not differ in their sensitivity to Ca^{2+}-induced swelling. Dietary omega-3 PUFA protected both mitochondrial fractions against Ca^{2+}-evoked swelling; the protective effect appeared to be more pronounced for the IFM fraction [31]. More recent studies have also shown that supplementation with DHA decreased the susceptibility of cardiac mitochondria to undergo permeability transition, a catastrophic event which often leads to cell death. This finding provides a potential mechanism for the cardioprotective effect of DHA. Relative Ca^{2+} intolerance, increased superoxide formation, and reduced efficiency in the management of reactive oxygen species (ROS) are the important mitochondrial factors that are evident in senescence and predispose the myocardium to be more susceptible to ischemic injury. Age-associated mitochondrial membrane changes include increase in cholesterol, phosphatidylcholine (PC), n-6 PUFA, 4-hydroxy-2-nonenal, and membrane rigidity with decrease in n-3 PUFA and diphosphatidylglycerol (DPG). It was observed that diet rich in n-3 PUFA reversed the age-associated membrane n-3:n-6 PUFA imbalance, and dysfunctional Ca^{2+} metabolism, which resulted in increased efficiency of mitochondrial energy coupling and improved tolerance to ischemia and reperfusion [32].

In animal studies, it was reported that after DHA supplementation the DHA content in mitochondrial phospholipids increased and membrane viscosity decreased. Further, DHA lowered Ca^{2+}-induced mitochondrial swelling, an index of permeability transition, in heart failure animals. Heart failure increased hydrogen peroxide-induced mitochondrial permeability transition compared to sham, which was partially attenuated in IFM by treatment with DHA [33]. In another animal study it was shown that treatment with DHA or EPA normalized Ca^{2+}-induced MPT in cardiomyopathic hamsters but did not prolong survival nor did it improve cardiac function [34]. Khairallah et al. reported that dietary supplementation with DHA but not EPA strongly altered mitochondrial phospholipid fatty acid composition and delayed Ca^{2+}-induced MPTP opening. [35] It has also been reported that supplementation with n-3 PUFA increases membrane phospholipid DHA and depletes arachidonic acid (AA) and can increase cardiolipin, a tetra-acyl phospholipid that is unique to mitochondria and essential for optimal mitochondrial function. In summary, there is mounting evidence that dietary n-3 PUFA, particularly DHA, has profound effects on mitochondrial membrane phospholipid composition and mitochondrial function. Interestingly, other n-3 PUFAs that modify membrane composition to a lesser extent have considerably less effect on mitochondria and do not appear to directly protect the heart [36].

Diabetes

The hallmark characteristic of type 2 diabetes is the elevated level of blood glucose caused either by insulin deficiency or insulin resistance [37]. Despite the fact that diabetes arises from a complex set of factors including

genetic predisposition and lifestyle, mitochondrial dysfunction has also been identified as one of the aspects in the pathogenesis of this disease [27]. Down regulation of complex I and/or IV in diabetic patients indicate that mitochondrial dysfunction plays a prominent part in diabetes [38]. Additionally, mitochondrial structural injury and impaired function contribute to diabetic heart disease [20]. It is believed that mitochondrial functional defects play a key role in the development of insulin resistance. Upon aging, spontaneous mtDNA mutations get accumulated and cause defects in β-oxidation [39]. Though ATP is required for the transport of glucose and insulin, ATP-generating ability of mitochondria may be impaired by such mutations, which in turn leads to a deficit of ATP. Defects in mitochondrial β-oxidation are expected to result in increased fatty acid levels which in turn contribute to fatty acid-induced insulin resistance, which is perhaps the most well-supported example of mitochondrial involvement in type 2 diabetes [40]. These data support the hypothesis that insulin resistance arises from defects in mitochondrial fatty acid oxidation, which in turn lead to an increase in intracellular fatty acid metabolites that disrupt insulin signaling [41]. It is also well established that mitochondrial function is required for normal glucose-stimulated insulin secretion from pancreatic β cells. In addition, maternally inherited defects in mitochondrial DNA that disrupt mitochondrial function are known to cause an insulin-deficient form of diabetes resembling type 1 diabetes. These changes were accompanied by decrease in both mitochondrial oxidative activity and mitochondrial ATP synthesis.

Many researchers have investigated the influence of dietary n-3 PUFAs on functional parameters of mitochondria under diabetic conditions. In one study, it was found the n-3 PUFAs reduced swelling of heart mitochondria of streptozotocin-induced diabetic rat. They also confirmed changes in fatty acid composition of cell membranes in hearts under diabetic conditions with n-3 PUFAs influence. It was concluded that n-3 PUFA have a positive effect on functional parameters of mitochondria due to stabilization of cell membranes of the heart in diabetic animals [42]. Another study indicated that the ROS generation and mitochondrial apoptosis were involved in hyperglycemia-induced tubular injury and EPA had a beneficial effect by suppressing ROS generation and mitochondrial apoptosis, partly through augmentation of an HIF-1α response in diabetic kidney disease [43]. It was reported that long-term supplementation of diabetic patients with n-3 PUFA could positively influence the mitochondrial energy metabolism of their brain, myocardium, and pancreas and protect these organs from some later complications of diabetes mellitus [11].

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Omega-3 Fatty Acids and Alzheimer's Disease

18

Santosh D. Devkar and Surendra S. Katyare

Introduction

The brain is the most complex organ in vertebrate body comprising primarily of two types of cells: neurons and glia. There are several types of glial cells which perform a number of critical functions. These include structural support, metabolic support, insulation and guidance of development. Neurons are considered to be the most important cells in the brain due to their ability to send signals to specific target cells over long distances [1]. Neuronal axon—the thin protoplasmic fibre that extends from the cell body—plays an important role in signal transmission to target cells over long distances. The signal transmission is achieved with the help of an action potential in which polarization and depolarization play a key role [2]. It is estimated that the cerebral cortex contains 15–33 billion neurons, each connected by synapses to several thousand other neurons [3, 4].

Role of Omega-3 in Brain

The omega-3 polyunsaturated fatty acid (omega-3 PUFA), decosahexaenoic acid (22:6, n-3, DHA), plays an important role in maintaining the properties of membrane core. In addition to this, DHA also plays other important roles. Thus, DHA esterified in phospholipids maintains the hydrophobic core, conforms high degree of flexibility to the membrane and direct interaction with membrane proteins. This property is important for neurotransmission, speed of transduction and formation of lipid rafts [5–7]. Unesterified DHA plays an important role in the regulation of gene expression and ion channel activities and it can also be metabolized to neuroprotective metabolites [8–10]. Recently, it has been suggested that DHA can influence neurogenesis and also phospolipid synthesis and turnover [11, 12]. Deficiency of n-3 fatty acids results in decreased cell size of neurons in hippocampus, hypothalamus and parietal cortex. Also, the complexity of dendritic arborizations of cortical neurons is decreased [13]. Consistent with these observations is the report that DHA enhanced neurite outgrowth of hippocampus and cortical neurons and clonal pheochromocytoma (PC12) cells in culture [12–16]. Also, there is evidence to show that DHA may influence the brain development through the effects on gene expression, monoaminergic neurotransmission or protection against apoptotic cell death. Growth of neurite processes from the cell body is a critical step in neuronal development and involves a large increase in cell membrane surface area [12].

Neurodegenerative Diseases

Neurodegeneration denotes progressive structural and functional loss including neuronal death. Neurodegeneration is responsible for occurrence of many neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), Parkinson's, Huntington's and Alzheimer's disease (AD). Neurodegenerative diseases are caused by several factors. These include genetic mutation, membrane damage, mitochondrial dysfunction, impairment of axonal transport and protein misfolding [17–22]. More recently, it is noted that there is an alarming increase in the incidence of AD.

Alzheimer's Disease

Most common features of this neurodegenerative disorder are loss of short-term memory, difficulties in planning or solving problems, completing familiar tasks, confusion with time or place, trouble in understanding visual images and spatial relationships, new problems with words in speaking

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or writing, misplacing things, losing the ability to retrace steps and decreased or poor judgment. The cause(s) and progression of the disease are not well understood. However, the presence of plaques and neurofibrillary tangles (NFT) in the brain is a well-recognized feature [23–26].

In AD, there is a progressive loss of nerve cells and of connectivity leading to progressive shrinkage of brain size. Post-mortem/autopsy has always shown the presence of plaques and NFT. Plaques (also designated as amyloid plaques) are formed from the protein called beta-amyloid (β AP) and are present between the dying neuronal cells. The NFT are present within the neurons and are formed from the disintegration and hyper-phosphorylation of another protein called Tau [23–26].

For reducing the intensity and severity, AD patients are treated with two main types of medications: cholinesterase inhibitors: Donepezil, Tacrine; NMDA receptor antagonist: Memantine. However, these drugs slow down the progress of the disease but have no curative effect. Also, these drugs have undesirable side effects such as headache, nausea, dizziness and loss of appetite [27].

Omega-3 and AD

As cited above, DHA plays an important role in neurogenesis, dendritic arborization and maintaining structural and functional characteristics of the neurons and thus of the brain function [5–7, 11, 12]. In the light of this, investigations have been carried out to find the usefulness of omega-3 fatty acids for the management of AD using animal model systems as well as in human patients. The findings of these investigations are briefly summarized below.

In experimental animals, it has been shown that diets deficient in omega-3 PUFA result in substantial disturbances in neural function; in most circumstances, these can be restored by supplementation of omega-3 PUFA in the diet. Based on these studies, it has been suggested that omega-3 PUFA may prove to be important in the management of neurological disorder such as depression and schizophrenia [28].

Lim et al. evaluated the effect of dietary DHA on amyloid plaques formation using APPswe (Tg2576) transgenic mouse model of AD. The animals were maintained on low-DHA (0 %) or high-DHA (0.6 %) diet. It was found that in aged mice high-DHA diet significantly lowered the β-amyloid by around 70 % compared to low-DHA or control diet groups. Also, the levels of β-amyloid protein 42 (βAP42) decreased. Image analysis of brain sections revealed that the overall plaque burden in the brain decreased by 40–50 % with a maximum effect being seen in the hippocampus and parietal cortex. DHA modulated amyloid precursor protein (APP) processing by decreasing α- and β C terminal fragments and full length APP; activity of beta secretase (beta site APP cleaving enzyme, BACE1), apolipoprotein E (ApoE) and transthyretin gene expression was unchanged. Based on these observations, the authors suggested that dietary DHA could provide protection against β AP production, plaque formation and its downstream toxicity [29].

Calon et al. [30] reported that in APPswe (Tg2576) mice over-expressing human AD gene the (NMDA) receptor subunits NR2A and NR2B decreased in cortex and hippocampus. Also NR1 subunit in hippocampus and calmodulin-dependent protein kinase (CaMKII) activity decreased in the cortex of APPswe mice. These effects of dietary deficiency of n-3 PUFA greatly amplified these effects in APPswe mice compared to non-transgenic mice. The n-3 PUFA depletion potentiated caspase activation in the Tg2576 mice. Dietary supplementation with DHA partly protected from overexpression of NMDA receptor subunits but fully prevented CaMKII decrease. The results thus emphasize the underlying role of omega-3 PUFA in cognition and AD risk.

In transgenic mouse model, DHA decreased amyloid, oxidative damage and synaptic and cognitive deficits. DHA and curcumin had similar protective effect. The author suggests that both DHA and curcumin may exert general anti-ageing benefits [31]. DHA is the principal omega-3 fatty acid in the brain. Animal studies have shown that perinatal deficiency of DHA leads to deficit in neuronal arborization and in synaptic pathology such as deficits in serotonin and in dopamine neurotransmission. Deficiency of DHA during brain development results in impairment in cognitive and behavioural performance, since DHA plays an important role in neurogenesis, neurotransmission and protection against oxidative stress [16]. It has been suggested that low intake of omega-3 FA, especially DHA, may be associated with cognitive decline in elderly, particularly in AD. Fotuhi et al. [32] reported that long chain omega 3 fatty acid may slow down cognitive decline in elderly without dementia, but may not prevent the dementia including AD. DHA seems to be an important regulator of brain glucose uptake by affecting the activity of some glucose transporter [33].

Neurocognitive deficits and elevated behavioural indices of anxiety, aggression and depression are associated with perinatal brain DHA deficiencies. Children/adolescent born preterm exhibit neurocognitive deficits with respect to attention and attention-deficits hyperactivity disorder (ADHD) and schizophrenia [16]. The DHA levels were found to be low in the serum and brains of AD patient possibly due to low intake and/or PUFA oxidation. Epidemiological studies suggested that the increased intake of omega-3 PUFA, especially DHA, reduces the risk of AD (30). Freund et al. [34] reported that the level of total and phosphorylated Tau protein in the plasma of AD patients were high. DHA supplementation decreased these levels. It is possible that in their studies DHA might have provided protection to degenerating neurons thereby preventing the

release of total and phosphorylated Tau in CSF [35]. However, since no data were provided for mnemonical tests, it is difficult to conclude as to whether treatment with DHA *actually* decreased the levels of Tau in the neurons.

Conclusion

The role of n-3 fatty acids in brain development and brain ageing is emerging as a field of considerable public health importance and of intense scientific inquiry. While DHA is important for human brain development, it also fulfils the essential roles including neurotransmission, neurogenesis and protection from oxidative stress which are important throughout the lifespan as also for maximizing cognitive potential in development and minimizing its loss with ageing. From the reported data and animal studies, it can be concluded that dietary DHA supplementation may provide protection from AD-related abnormalities. However, the data from human studies are insufficient to draw a definitive conclusion. Nevertheless, it may be safely recommended that supplementation with DHA can prove to be useful for prophylactic, if not therapeutic application in AD. More detailed investigations along these lines will widen our perspectives and understanding.

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Prevention of Oxidative Stress by Omega-3 Fatty Acids in the Brain

19

Akhlaq A. Farooqui and Tahira Farooqui

Introduction

Neural membranes contain glycerophospholipids, which contain a saturated fatty acid at the sn-1 position of glycerol moiety, whereas the sn-2 position bears a polyunsaturated fatty acid (arachidonic acid, ARA, 20:4, n-6) or docosahexaenoic acid, DHA (22:6, n-3). ARA belongs to the omega-6 family whereas DHA falls under the omega-3 family of essential fatty acids, respectively. Vegetable and fish oils are the most common sources of omega-6 and omega-3 fatty acids, respectively. Fatty acids belonging to omega-6 and omega-3 family not only provide neural membranes with their physical characteristics, such as acyl chain order and fluidity, stability, phase behavior, elastic compressibility, ion permeability, fusion, rapid flip-flop and packing [1], but also function as signaling molecules by supplying lipid mediators, which are formed by the oxidation process. An appropriate ratio of ARA to DHA in the brain promotes development, ameliorates cognitive functions, and provides protection against neurological diseases by enhancing repairing processes [1]. In addition, ARA and DHA also stimulate gene expression, boost synaptogenesis, neurogenesis, and induce and prevent oxidative stress, a process that results from an unbalance between prooxidant and antioxidant systems in the brain. Neurological oxidative stress is either induced by the failure of cell antioxidant (buffering) mechanisms or overproduction of reactive oxygen species (ROS), which are oxygen-based radicals formed in most mammalian cells through the activities of enzymes involved in the mitochondrial electron transport chain, epoxygenase (EPOX), lipoxygenase (LOX) or cyclooxygenase (COX), NAD(P)H oxidases or uncoupled nitric oxide synthase (NOS), and peroxidases, among others [2, 3]. They are normal by-products of healthy cellular metabolic processes and are known to play physiologically useful roles in cell signaling; for example, as part of the immunity-oriented "oxidative burst" [4]. Low levels of oxidative stress are needed for cell functions. The role of oxygen in cell survival is linked to its high redox potential, which makes it an excellent oxidizing agent capable of accepting electrons easily from reduced substrates. High levels of oxidative stress are central disruptor of neural cell homeostasis. It is well known that brain represents only ~2 % of the total body mass and yet accounts for more than 25 % glucose and 20 % of the total consumption of oxygen [5]. In addition to high oxygen utilization, two more reasons make the brain most susceptible to oxidative damage. First is its modest antioxidant defense mechanism and second is the presence of high levels of lipids, which account for 60–65 % of brain dry weight. Thus, the brain is particularly vulnerable to oxidative stress. The major sources of ROS in brain are uncontrolled ARA cascade, mitochondrial respiratory chain, xanthine/xanthine oxidase, myeloperoxidase, and activation of NADPH oxidase (Fig. 19.1).

Long-term consumption of Western diet, which is enriched in saturated fats, ARA, cholesterol, and high simple sugars results in the incorporation of ARA in neural membrane phospholipids (phosphatidylinositol and phosphatidylcholine). ARA is released and oxidized by phospholipase A_2 and cyclooxygenases-2 (PLA_2 and COX-2) respectively [6]. Nonenzymic oxidation of arachidonic acid results in the generation of high levels of ROS, increased expression of inflammatory cytokines (TNF-α, IL1β, and IL-6), and elevated levels of n-6 fatty acid-derived proinflammatory lipid mediators (eicosanoids and platelet-activating factor) throughout the body including brain. Nonenzymic peroxidation of ARA is a radical-initiated and autocatalytic process. Free radicals are molecules that contain an unpaired electron in their outer orbit. Free radicals are associated with many neurochemical activities of cells such as signal transduction, gene transcription, and regulation

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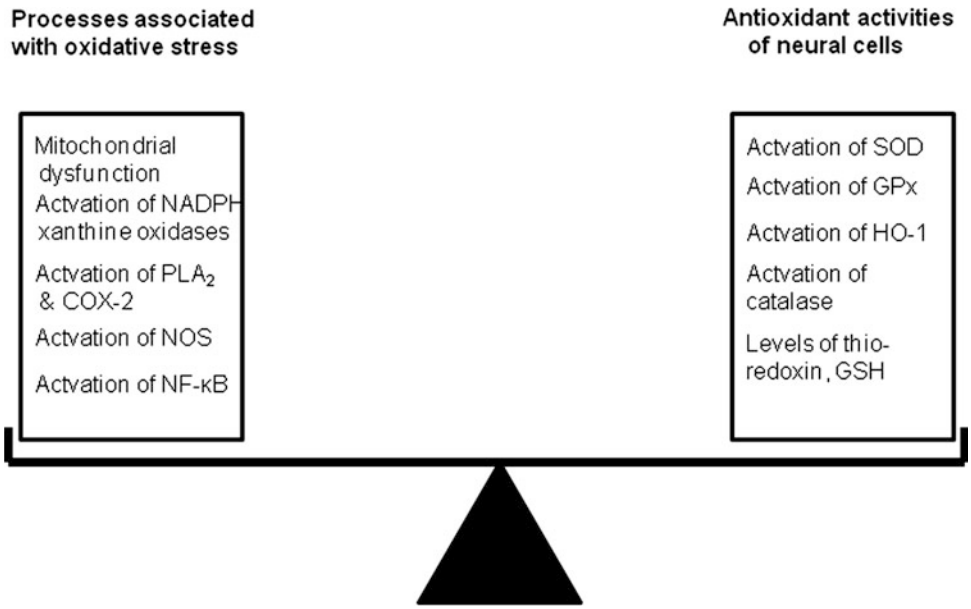

Fig. 19.1 Factors contributing to oxidative and antioxidant activities in the brain

of soluble guanylate cyclase activity. Humans are constantly exposed to free radicals not only generated from the man-made environment, but also from natural resources such as radon, cosmic radiation, as well as cellular metabolisms (respiratory burst, enzyme reactions). The most common reported cellular free radicals are hydroxyl ($OH^{\cdot}$), superoxide ($O_2^{\cdot-}$), and nitric monoxide ($NO^{\cdot}$) [1, 2]. ARA is highly susceptible to radical-mediated oxidation due to the abstraction of the bis-allylic methylene hydrogen by other radical species. The resulting conjugated pentadienyl fatty acid radical ($L^{\cdot}$) can further react with oxygen to give a dienyl peroxyl radical ($LOO^{\cdot}$). This intermediate abstracts a radical from another lipid generating a lipid hydroperoxide (LOOH) and a secondary radical. The lipid hydroperoxide (LOOH) undergoes rearrangement and cleavage reactions that result in the formation of lipoxidation end products. This process generates several products, such as 4-hydroxynonenal, malondialdehyde, acrolein, isoprostane, isofuran, and isoketal (Fig. 19.2) along with excessive production of ROS. High levels of ROS not only cause more lipid peroxidation and oxidation of neural membrane protein and DNA, but also produce impairment in normal neural cell functions leading to neural cell death [7]. Modifications of neural membrane protein and DNA were originally considered solely as markers of oxidative insult. However, recently the modifications of proteins and DNA by lipid peroxidation products are recognized as a new mechanism of cell signaling with relevance to redox homeostasis, adaptive response, and inflammatory resolution [8]. Among the neural cells, astrocytes are most resistant to ROS attack than neurons because they contain higher levels of reduced glutathione (GSH) content than other neural cells [9]. During scavenging of ROS, the reduced form of GSH is converted into oxidized form of glutathione (GSSG). Thus, high levels of ROS generation promote disruption of redox signaling in neural cells [9].

The main impact of high levels of n-6 fatty acid consumption in Western diet is the generation of high levels of superoxide in the mitochondria [1]. Superoxide is a relatively unstable intermediate and in large part is converted to hydrogen peroxide in the mitochondria by superoxide dismutase. The newly formed hydrogen peroxide undergoes a Haber–Weiss or Fenton reaction, generating a highly reactive hydroxyl radical, which can oxidize mitochondrial proteins, DNA, and lipids and amplify the effects of the superoxide-initiated oxidative stress [10, 11]. Molecular mechanisms associated with nuclear and mitochondrial DNA (mtDNA) are not fully understood. However, it is suggested that promoter regions with high guanine–cytosine contents in DNA are specifically vulnerable to oxidative damage. Oxidative damage to promoter regions of DNA may cause changes in conformation leading to loss of DNA affinity for transcription factors [12]. Furthermore, oxidative damage to mitochondrial DNA (mtDNA) may result in downregulation of genes related to respiratory chain leading to impairment in energy metabolism [13, 14]. In addition, telomeres, regions of repetitive nucleotide sequences at each end of a chromosome, are highly susceptible to oxidative stress because of their high content of guanines. Increase in intracellular ROS levels is associated with acceleration in the rate of telomere shortening. Oxidative stress-mediated progressive shortening of telomeres leads to senescence, apoptotic cell death, or the oncogenic transformation of somatic cells in various tissues [15].

As stated above, ARA is released by the action of PLA_2 on neural membrane phospholipids. It is oxidized by COXs and LOXs into proinflammatory eicosanoids (prostaglandins

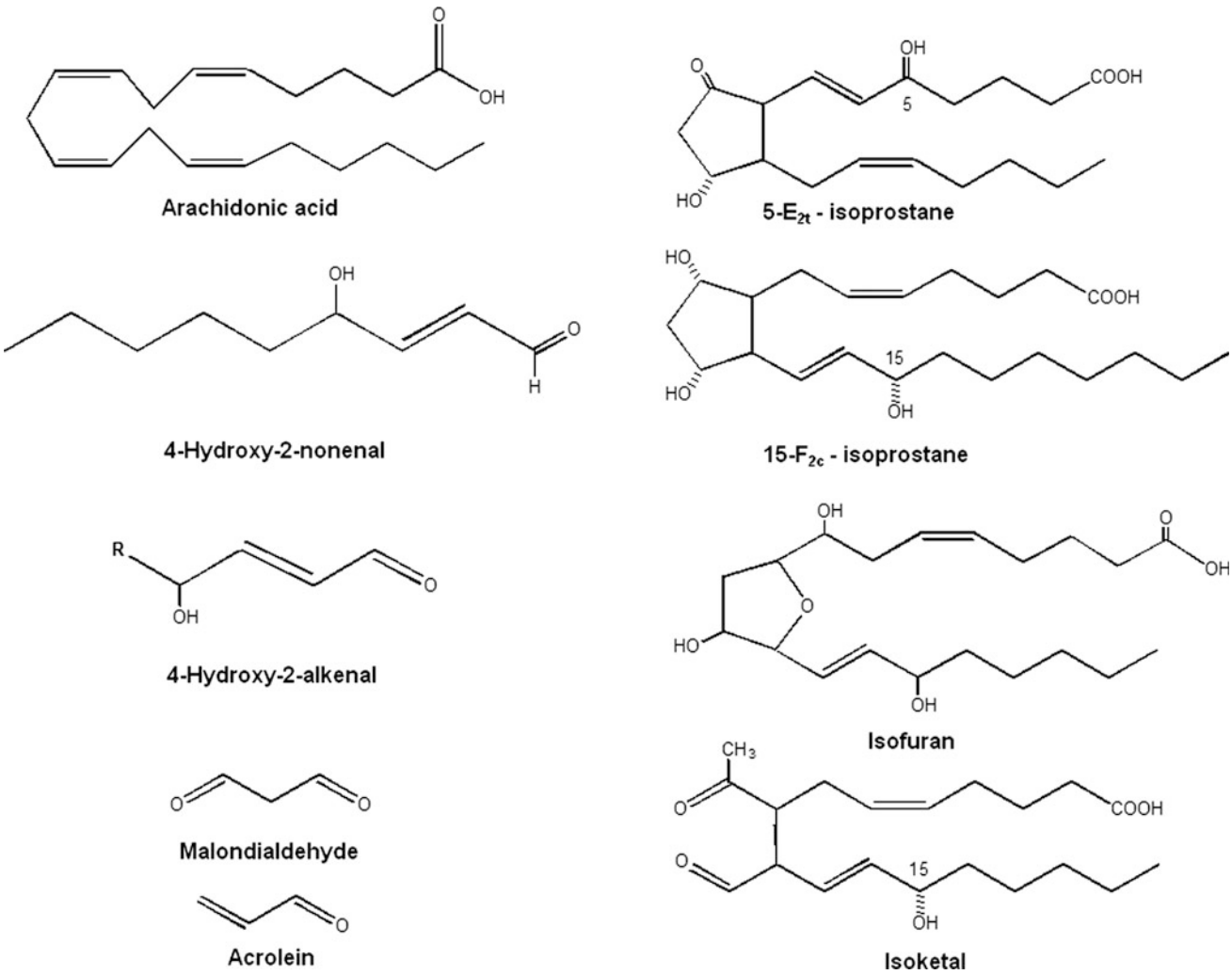

Fig. 19.2 Chemical structures of arachidonic acid-derived lipid mediators in the brain

and leukotrienes) or nonenzymically oxidized into lipid mediators of arachidonic acid metabolism (Fig. 19.2) along with production of ROS. The generation of high ROS activates redox-sensitive transcription factor (NF-κB) that results in numerous downstream effects, including the increased expression of proinflammatory cytokines leading to further increase in ROS production (Fig. 19.3). Production of ROS is also promoted by RAGE receptors, which are activated by advanced glycation products (AGEs). AGEs are generated by an irreversible reaction through the nonenzymatic, long-term glycosylation of proteins. They are strongly resistant to proteolytic processes and induce protein cross-linking. They inhibit the physiological functions of many proteins and receptors promoting the transformation of soluble proteins into insoluble proteins deposits, as well as activating the microglia through specific ligands for cell surface receptors (Fig. 19.3). The complex interplay between inflammatory mediators and markers for oxidative stress caused by the long-term consumption of Western diet has been proposed to regulate the progression of chronic neurodegeneration in neurodegenerative diseases [15, 16]. In addition, nitric oxide (NO) synthesis occurs in mitochondria from the breakdown of arginine into citrulline by a family of NADPH-dependent enzymes called mitochondrial nitric oxide synthases (mtNOS) [17]. Once formed, NO inhibits respiration by binding to heme groups in the proteins of the electron transport chain, including cytochrome c oxidase [18, 19]. Thus, NO is a known inhibitor of the respiratory chain. NO competes directly with O_2 at complex IV, reversibly retarding the formation of this complex and inducing ROS generation [20]. During high oxidative stress, NO reacts rapidly with excess superoxide to form peroxynitrite (Fig. 19.3), a powerful oxidizing and nitrating agent, which irreversibly inhibits multiple complexes of the respiratory chain, as well as dismutase enzymes. These processes not only lead to elevation in oxidative stress and increase in

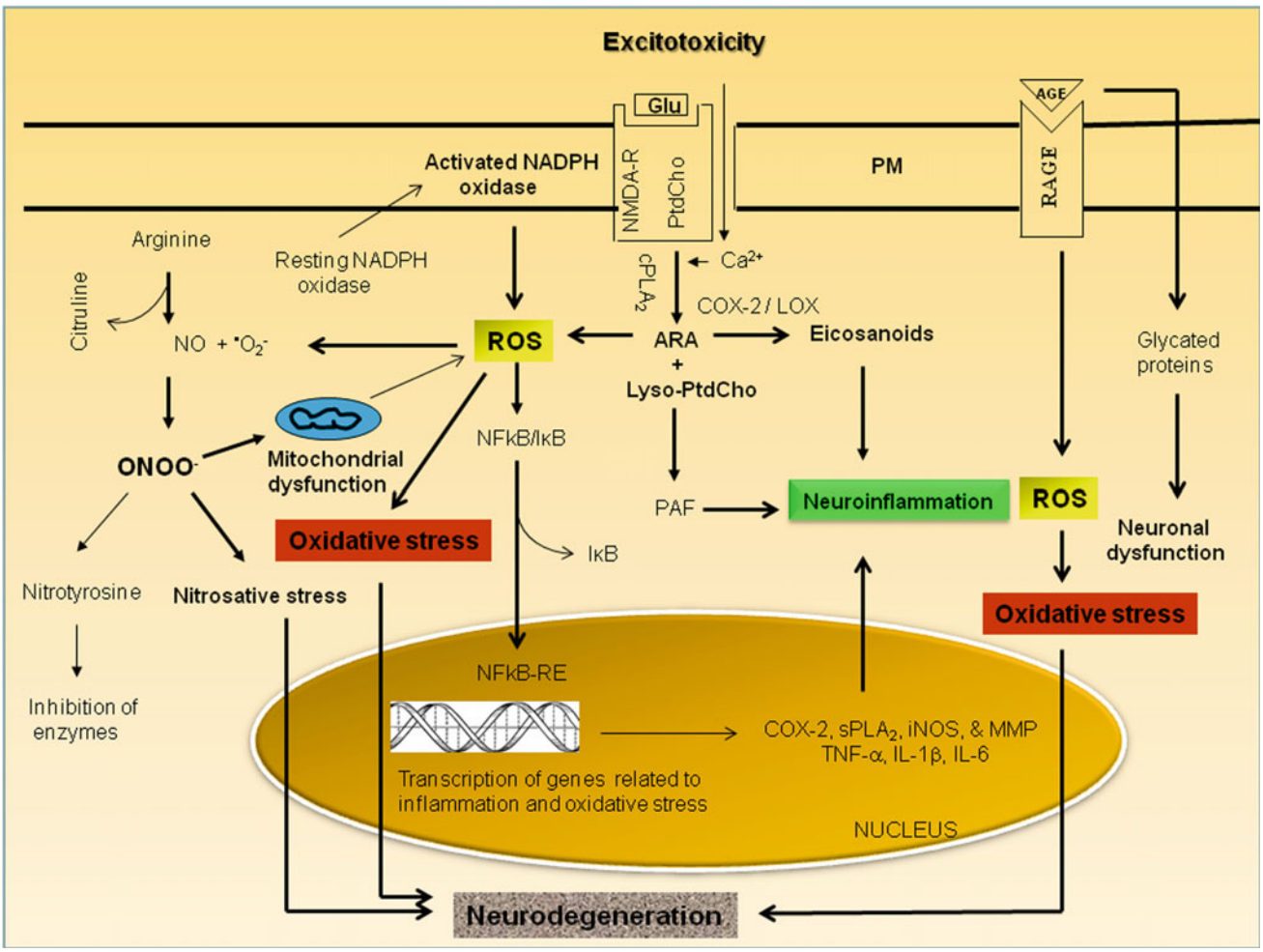

Fig. 19.3 Signal transduction processes contributing to oxidative stress and neuroinflammation in the brain plasma membrane (PM), phosphatidylcholine (PtdCho), arachidonic acid (ARA), lysophosphatidylcholine (lyso-PtdCho), platelet-activating factor (PAF), glutamate (Glu), N-methyl-D-aspartate receptors (NMDA-R), cytosolic phospholipase A_2 (cPLA$_2$), cyclooxygenase-2 (COX-2), lipoxygenase (LOX), reactive oxygen species (ROS), advanced glycation end products (AGEs), receptors for advanced glycation end products (AGEs), nuclear factor-kappa B (NF-κB), nuclear factor-kappa B response element (NF-κB-RE), tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), interleukin-6 (IL-6), nitric oxide (NO), and peroxynitrite ($ONOO^-$)

mitochondrial inner membrane potential ($\Delta\Psi_m$) [21], but also induce alterations in calcium homeostasis [22]. Collective evidence suggests that oxidative stress and inflammation caused by distinct biochemical cascades are processes, which are closely intertwined and generally function in parallel, particularly in the brain, an organ especially prone to oxidative stress.

Enzymic and Nonenzymic Lipid Mediators of ARA-Derived Metabolism and Their Antioxidant and Anti-inflammatory Effects

Stimulation of glutamatergic, serotonergic, cholinergic, or dopaminergic receptors by their agonists in the brain results in the activation of Ca^{2+}-dependent PLA_2 and release of arachidonic acid. The released ARA is oxidized by COXs, LOXs, and EPOXs resulting in the formation of prostaglandins (PGs), leukotriene (LTs), lipoxins (LXs), and thromboxanes (TXs), as well as hydroxyl-eicosatetraenoic acid, epoxyeicosatetraenoic acids, and dihydroxy-eicosatrienoic acids [23, 24]. Most arachidonic acid-derived lipid mediators produce prooxidant, prothrombotic, proaggregatory, and proinflammatory effects. However, arachidonic acid-derived LXs produce anti-inflammatory effects. PGs, LTs, and TXs interact with their receptors and produce potent effects on neuroinflammation, vasodilation, vasoconstriction, apoptosis, and immune responses [23, 24]. The expression of COX-2 is increased during acute inflammation and the ingestion of aspirin leads to acetylation of COX-2, which blocks PG formation [25]. Acetylated COX-2 is catalytically active. It transforms ARA into 15(R)-HETE rather

than PGs [26]. 15(R)-HETE is utilized by 5-LOX for transforming into 15(R)-LXA_4. Aspirin-triggered 15-epi-LXs are ~twofold more potent than 15(S)-LXs [27].

ROS also attack on lipid hydroperoxides. This attack results in the synthesis of isoprostanes (IsoPs) via β-cleavage of the peroxyl acid and subsequent molecular rearrangement. IsoP contains D-, E-, and F-ringed structures similar to cyclooxygenase-generated prostaglandins, except that their hydrocarbon chains are in the *cis* position in relation to the pentane ring as opposed to the *trans* position observed in prostaglandins [24, 28, 29]. The formation of IsoP is used as a "gold standard" to quantify cumulative oxidative stress in neurological diseases. Neurochemical activities of IsoPs are poorly understood [24, 30–32]. However, they may form covalent adducts with cysteine residues in proteins through Michael-type addition reactions [33].

Enzymic and Nonenzymic Lipid Mediators of Omega-3 Fatty Acids

Phospholipids containing DHA account for as much as 15–25 mol% of the lipids of the gray matter in the human brain. It is estimated that 50 % of the weight of a neuronal plasma membrane is composed of DHA [34]. Among phospholipids, DHA is highly enriched in ethanolamine plasmalogen and phosphatidylserine in human brain. Small amount of eicosapentaenoic acid (EPA, 20:5 n-3), another omega-3 fatty acid, is also present in neural membrane phospholipids. From neural membrane phospholipids, DHA is released by the action of plasmalogen-selective and phosphatidylserine-selective PLA_2s. In neural membranes, DHA and EPA play important roles in modulation of membrane fluidity and synaptic plasticity. DHA also protects neurons from cytotoxicity caused by high levels of nitric oxide (NO) and calcium (Ca^{2+}) influx. In addition, DHA-deficient mice exhibit a range of neurocognitive impairments, including problems with learning and memory [35]. Furthermore, DHA has been reported to protect the neural cells from oxidative stress by increasing glutathione reductase activity and decreasing the accumulation of oxidized proteins [36, 37], lipid peroxide, and reactive oxygen species (ROS) [38]. In the murine microglial cell line BV2, DHA reduces proinflammatory molecule synthesis, migration, and lipid droplet formation and induces heme oxygenase-1 expression, by mechanisms involving alteration of lipid raft composition and modulation of PtdIns 3-kinase/Akt, ERK, and NF-κB-mediated signaling [39, 40]. DHA is also known to retard caspase cascade [36] through the regulation of the PtdIns 3K/Akt cascade [41]. Omega-3 fatty acids are not only natural ligands of nuclear receptors associated with the regulation of gene expression (PPAR, hepatic nuclear factor, liver X receptors, and retinoid X receptors), but also involved in modulation of transcription factors, such as sterol regulatory element-binding protein and carbohydrate response element-binding protein [42]. Furthermore, omega-3 fatty acids also modulate genes involved in insulin sensitivity (PPARγ), glucose transport (GLUT-2/GLUT-4), and insulin receptor signaling (IRS-1/IRS-2). In addition, omega-3 fatty acids also increase adiponectin, an anti-inflammatory and insulin-sensitizing adipokine, and induce AMPK phosphorylation, a fuel-sensing enzyme and a gatekeeper of the energy balance [15]. All these mechanisms are interconnected and closely associated with modulation of carbohydrate and lipid metabolism involved in modulation of oxidative stress and inflammation in the brain. Collective evidence suggests that in the brain, DHA is involved in development, neurogenesis, memory formation, excitable membrane function, photoreceptor cell biogenesis and function, and neuronal signaling [43].

Enzymic and Nonenzymic Lipid Mediators of EPA Metabolism and Their Antioxidant and Anti-inflammatory Effects

Like ARA, EPA also undergoes enzymic and nonenzymic oxidation leading to the production of a variety of lipid mediators [24]. The enzymic oxidation of EPA results in the formation of E-series resolvins (RvE_1 and RvE_2), 3-series PGs, and 5-series LTs [44]. These metabolites produce potent anti-inflammation/pro-resolution effects in vivo [45]. They bind to ChemR23 and BLT receptors (seven-membrane spanning G protein-coupled receptors), which are expressed and located on dendritic cells and monocytes [46]. RvE_1 has been reported to suppress the activation of NF-κB and tumor necrosis factor-alpha (TNF-α) by interacting with human polymorphonuclear leukocyte (PMN) [47]. RvE_1 also produces anti-inflammatory effects by decreasing neutrophil infiltration, paw edema, and proinflammatory cytokine expression [48]. EPA is also oxidized by COX and LOX enzymes. This oxidation results in the generation of 3 series of prostaglandins and thromboxanes and 5 series of leukotrienes (Fig. 19.4). These eicosanoids have different biological properties than the corresponding analogs generated by the oxidation of ARA. Thus, TXA_3 is less active than TXA_2 in aggregating platelets and constricting blood vessels [44].

Antioxidant effects of EPA involve nonenzymic oxidation of EPA. The nonenzymic oxidation of EPA results in the synthesis of cyclopentenone-isoprostanes (A_3/J_3-IsoPs) [49]. These metabolites modulate the Keap1-Nrf2-ARE pathway (Fig. 19.4). Nrf2 transcription factor is a member of the leucine zipper transcription factor family [50, 51]. It is present in the cytoplasm as a complex with Kelch-like ECH-associated protein 1 (Keap1). Interactions of A_3/J_3-IsoP with Keap1 not only results in the dissociation of Nrf2 from Keap1, but also in translocation of Nrf2 into the

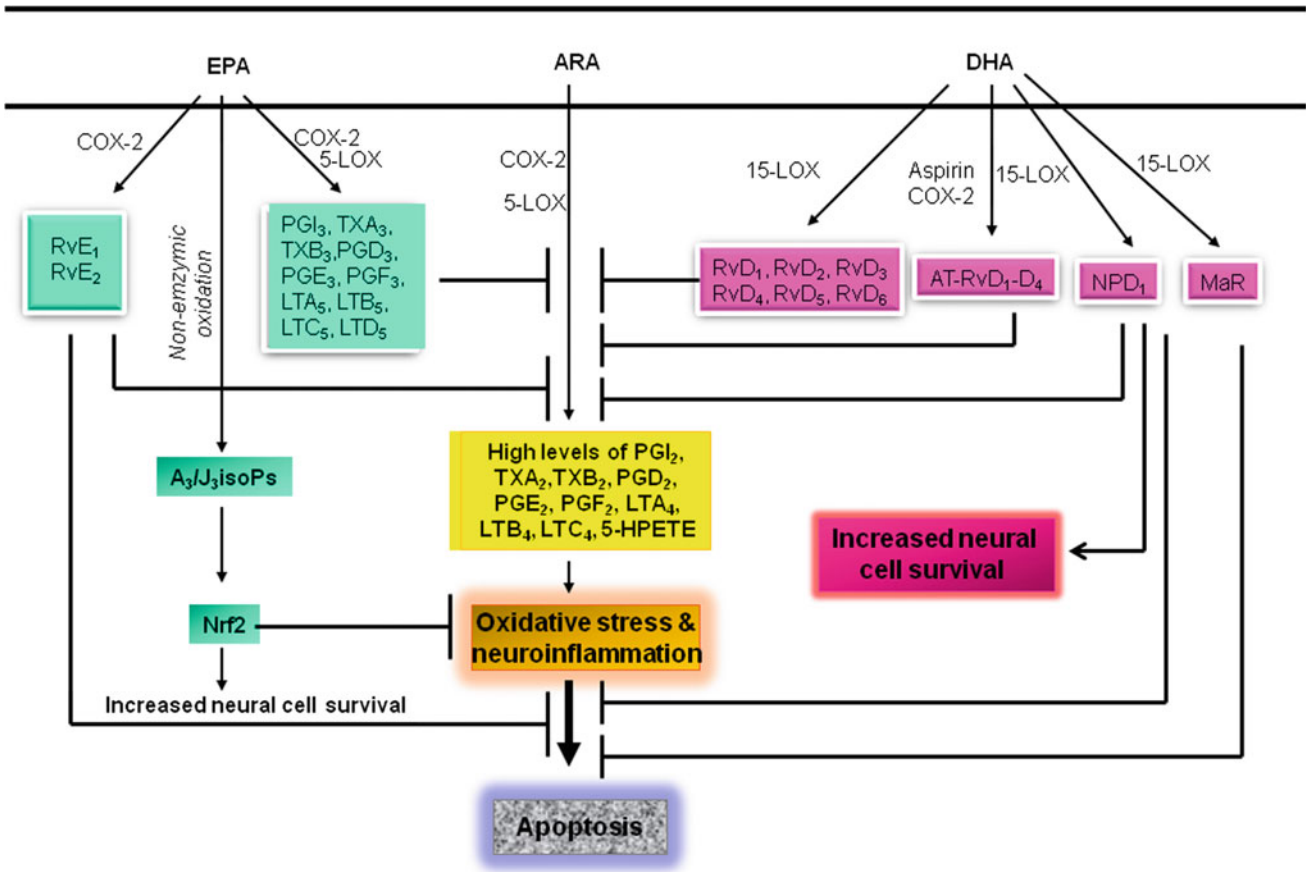

Fig. 19.4 Generation of ARA-, EPA-, and DHA-derived lipid mediators and their neurochemical effects on arachidonic acid (ARA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), cyclooxygenase-2 (COX-2), 5-lipoxygenase (5_LOX), and 15-lipoxygenase (15-LOX); ARA-derived eicosanoids are PGE_2, PGI_2, LTB_4, LTC_4, and TX_2; EPA-derived eicosanoids are PGI_3, PGE_3, LTB_5, LTC_5, LTE_5, and TXA_3; and DHA-derived metabolites are D-series resolvins (D-series Rvs) and neuroprotectin D_1 (NPD_1). In the presence of aspirin, a new set of lipid mediators called aspirin-triggered resolvins and protectins are synthesized

nucleus. In the nucleus, Nrf2 heterodimerizes with the small Maf proteins and binds to specific response elements termed antioxidant or electrophilic response elements (AREs) to coordinate expression of cytoprotective genes, such as genes associated with antioxidant- detoxifying proteins, genes involved in cellular rescue pathways against oxidative injury, inflammation/immunity, and apoptosis. ARE-modulated genes include glutathione peroxidases, glutathione S-transferase, heme oxygenase, NAD(P)H: quinone oxidoreductase 1 (NQO1), and proteasome subunit beta type-5 [52, 53]. Collective evidence suggests that the major function of Nrf2 is to activate the cellular antioxidant response by inducing the transcription of a wide array of genes that are able to combat the harmful effects of oxidative stress. Because of this reason, Nrf2 is traditionally been regarded as the cell's major antioxidant mechanism and Nrf2 is a major regulator of cell survival [53].

Enzymic, Nonenzymic Lipid Mediators of DHA Metabolism and Their Antioxidant and Anti-inflammatory Effects

The enzymically derived lipid mediators of DHA metabolism include D-series resolvins, neuroprotectins (NDPs), and maresins (MaR) (Figs. 19.4 and 19.5). These lipid mediators are collectively known as docosanoids [24]. They not only downregulate proinflammatory cytokines but also produce antioxidant, anti-inflammatory, antithrombotic, antiarrhythmic, hypolipidemic, and vasodilatory effects [1, 43, 54–57]. Resolvin Ds are synthesized from DHA through the action of 15-lipoxygenase (15-LOX). This reaction results in the formation of 17S-hydroperoxy-DHA [55, 58, 59]. Enzymic peroxidation and hydrolysis transform 17S-hydroperoxy-DHA into neuroprotectin D_1 (NPD_1). D-series resolvins (Rvs) and their aspirin-triggered epimers are also synthesized

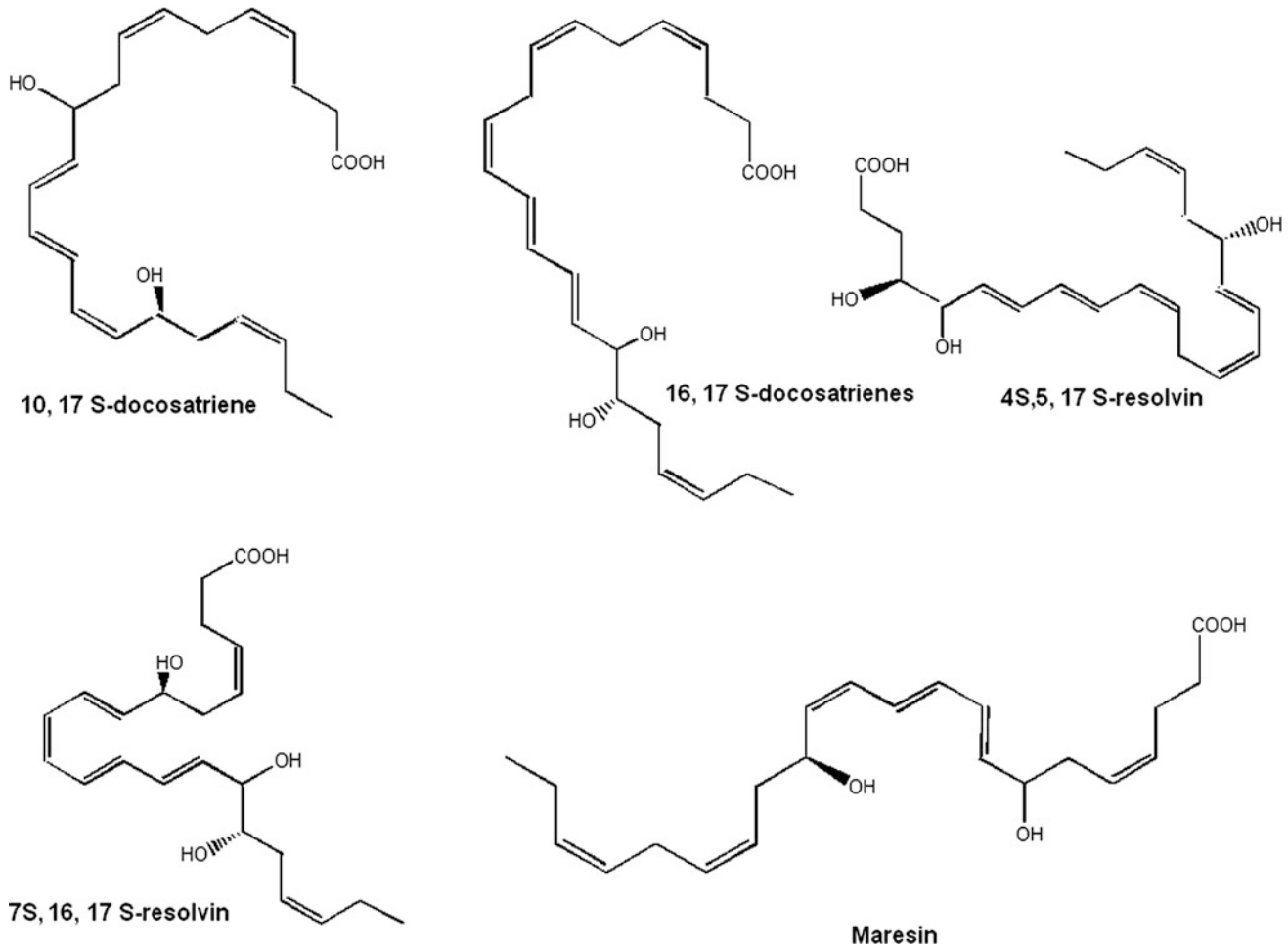

Fig. 19.5 Chemical structures of DHA-derived lipid mediators

from DHA through a pathway with sequential oxygenations, initiated by 15-LOX or aspirin-acetylated COX-2, respectively [27, 54]. Finally, MaRs are synthesized by 14-lipoxygenation of DHA. This process produces 14*S*-hydroperoxydocosa-4*Z*,7*Z*,10*Z*,12*E*,16*Z*,19*Z*-hexaenoic acid (14*S*-HpDHA), which undergoes further conversion via 13 (14)-epoxidation, leading to the synthesis of 7,14-dihydroxy-docosa-4*Z*,8*Z*,10,12,16*Z*,19*Z*-hexaenoic acid (MaR1) [60]. These DHA-derived metabolites provide powerful anti-inflammatory and immunomodulatory roles by reducing the migration of neutrophils and the release of pro-inflammatory cytokines [55, 57]. These metabolites also protect against oxidative damage that is associated with a localized inflammatory response by blocking the migration of PMN to injured tissues, thereby retarding the oxidative stress that stems from PMN activation [55, 57]. In microglia cells, resolvins block the production of pro-inflammatory cytokines such as TNF-α and ILβ1. DHA also reduces pro-inflammatory mediators such as prostaglandin E_2, thromboxanes, and leukotrienes [61]. In brain and retina, the generation of NPD_1 results in the expression of antiapoptotic protein and downregulation of caspase-3 activation and decrease oxidative stress [62]. NPD_1 also promotes Akt translocation and activation and interacts with PPAR-gamma family of ligand-activated nuclear receptors, which may be involved in various aspects of oxidative stress and neuroinflammation [24, 63, 64]. The occurrence of NPD_1 receptors in brain has not been reported, but based on pharmacological studies, it is suggested that NPD1 receptors may occur in neural tissues [43, 54, 62]. Collective evidence suggests that NPD_1 not only retards apoptosis and modulates neuroinflammatory signaling, but also promotes the cellular homeostasis, and restoration of brain damage through its antioxidant, anti-inflammatory, and antiapoptotic effects. This is tempting to speculate that the generation of DHA-derived resolvins and NPD_1 and synthesis of

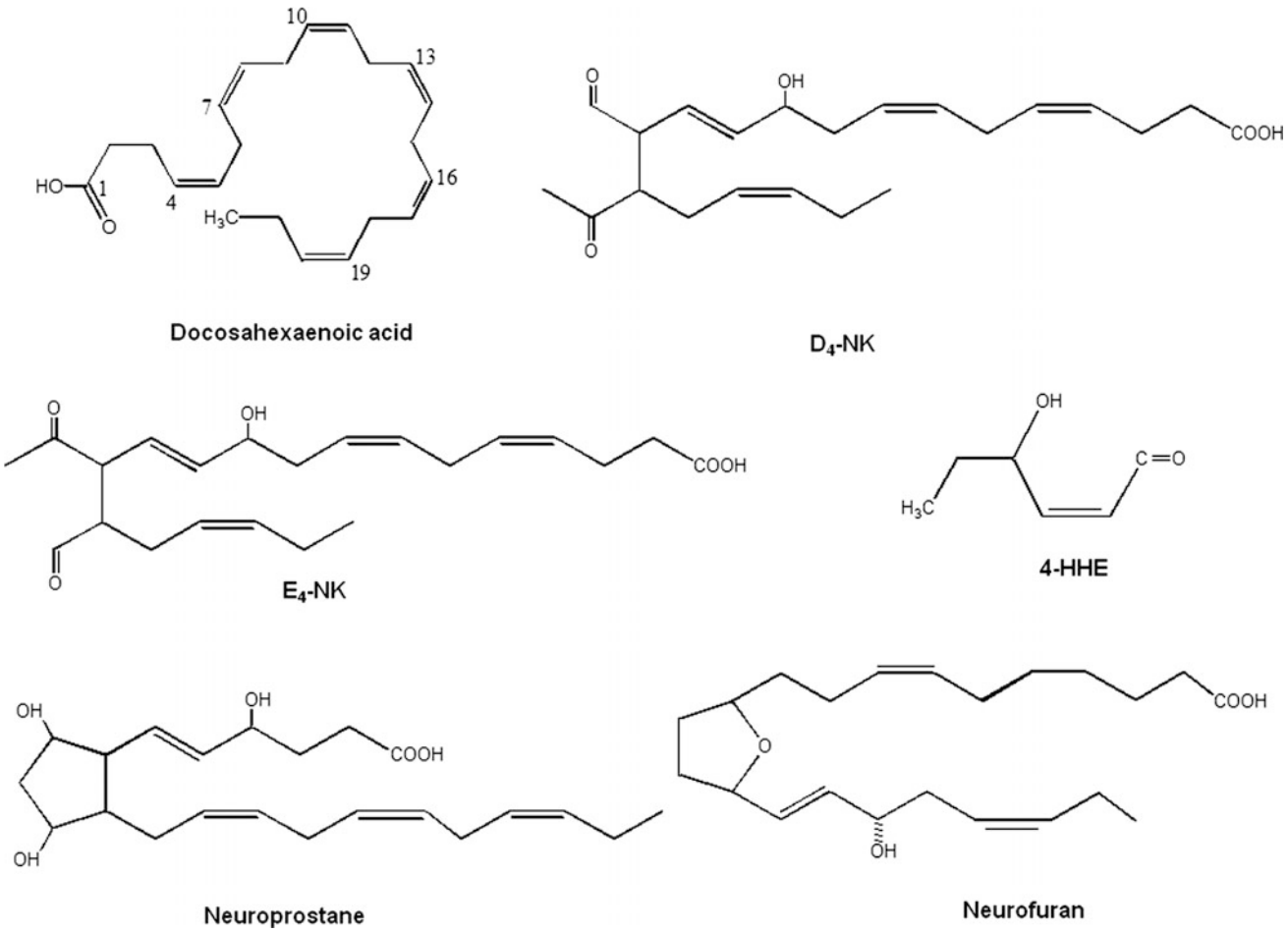

Fig. 19.6 Chemical structures of DHA-derived nonenzymic lipid mediators

ARA-derived lipoxins may be internal neuroprotective mechanisms that may protect from brain damage caused by neurotraumatic and neurodegenerative diseases [24, 58, 59].

The nonenzymic lipid mediators of DHA metabolism include 4-hydroxyhexanal (4-HHE), neuroprostanes (NPs), neuroketals (NKs), and neurofurans (NFs) (Fig. 19.6). Among these lipid mediators, NKs and NFs promote prooxidant and proinflammatory effects, but NPs possess anti-inflammatory properties and also inhibit proteasome activity [65, 66]. ARA- and DHA-derived nonenzymic lipid mediators act synergistically to modulate induction and regulation of neuroinflammation and oxidative stress by controlling the duration and magnitude as well as the return of the injury site to homeostasis in the process of catabasis (the decline of the disease state) [55]. An important function of ARA-, EPA-, and DHA-derived lipid mediators is their involvement in signal transduction network, which conveys the message of extracellular signals from the cell surface to the nucleus to induce a biological response at the gene level. Levels of ARA, EPA, and DHA in diet may partially modulate the levels of their lipid mediators in neural and non-neural tissues. Thus, levels of ARA, EPA, and DHA and their lipid mediators not only modulate the onset of neuroinflammation and oxidative stress by coupling lipid metabolism with neural membrane lipid organization, but also cooperate with the action of lipid-dependent enzymes to execute appropriate downstream actions and responses [24].

Prevention of Oxidative Stress and Neuroinflammation by EPA and DHA

It is well known that in Western diet, the ratio between ARA and DHA is about 20:1 [1]. In contrast, Paleolithic diet on which humans have lived and survived thousands of years contained the ratio between ARA and DHA of 1:1 [67, 68]. Due to the presence of high levels of ARA, long-term consumption of Western diet results in the generation of high levels of ARA-derived enzymic (PGs, LTs, and TXs) and nonenzymic (4-HNE, IsoP, MDA, etc.) lipid mediators, which promote neuroinflammation and oxidative stress, along with apoptotic cell death [1, 15, 69]. As stated above,

oxidative stress and neuroinflammation are closely intertwined processes that generally function in parallel, particularly in the brain, an organ especially prone to both oxidative stress and neuroinflammation. These processes are also closely associated with the pathogenesis of neurotraumatic, neurodegenerative, and neuropsychiatric diseases [16]. In addition, consumption of Western diet induces obesity, which correlates with reduction in focal gray matter volume and enlargement in white matter in the frontal lobe [70], altering learning, memory, and executive function in humans [71] and cognitive deficits in a rodent model [72]. Furthermore, long-term consumption of Western diet also decreases levels of BDNF in the hippocampus leading to impairment in neurogenesis [69]. Consumption of the Western diet also mediates significant decrease in Nrf2 DNA-binding activity, reduces Nrf2 responsive pathway proteins (heme oxygenase-1 and NAD(P)H dehydrogenase, quinone 1), and decreases the expression of Nrf2 protein expression [73, 74] as depressive symptoms. Collective evidence suggests that long-term consumption of Western diet increases the chances of brain damage through oxidative processes along with changes in neural cell homeostasis leading to metabolic abnormalities, behavioral disturbances, and motor and cognitive impairments. Increase in fish consumption or inclusion of fish oil in the diet not only decreases levels of ARA-derived enzymic and nonenzymic lipid mediators, but also generates DHA- and EPA-derived lipid mediators (D-series Rvs, E-series Rvs, NPD_1, and MaRs) [24]. These omega-3 fatty acid-derived lipid mediators produce antioxidant and anti-inflammatory effects and retard the onset and pathogenesis of neurotraumatic, neurodegenerative, and neuropsychiatric diseases [1, 16]. It is also proposed that consumption of omega-3 fatty acid-containing diet counteracts signals that respond to oxidative stress and set in motion brain repair and neuroplasticity responses needed for achieving neural cell homeostasis [1].

Conclusion

Consumption of high levels of ARA produces high levels of ARA-derived enzymic and nonenzymic lipid mediators along with generation of ROS, which react with DNA, proteins, and lipids and play important roles in the pathogenesis of neurotraumatic, neurodegenerative, and neuropsychiatric disease. Consumption of DHA- and EPA-containing food (fishes or fish oil) results in the production of resolvins and protectins/neuroprotectins. These lipid mediators regulate immune systems by modulating signal transduction processes associated with oxidative stress, neuroinflammation, and neurodegeneration. EPA-derived resolvins (i.e., RvE_1 and RvE_2) and DHA-derived resolvins (RvD_1 and RvD_2) produce potent antioxidants, anti-inflammatory, and pro-resolution effects. They inhibit oxidative stress, retard excessive inflammation, and promote resolution by enhancing clearance of apoptotic cells and debris from inflamed brain tissue. These properties result in the beneficial effects of EPA and DHA in human health and neurotraumatic and neurodegenerative diseases.

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Role of Omega-3 Fatty Acids for Eye Health

20

Tassos Georgiou and Ekatherine Prokopiou

Introduction

Over the last few decades, there is an increasing interest in the role of omega-3 polyunsaturated fatty acids (PUFAs) and chronic inflammation. Numerous evidence exist from pre-clinical and clinical studies which prove the effectiveness of omega-3 PUFAs against heart disease, cancer, diabetes, neurological and autoimmune diseases [1]. This chapter will mainly focus on the role of omega-3 PUFAs in maintaining or improving the vision of different eye pathologies (either investigated in vivo or in a clinical setting), including age-related macular degeneration, macular dystrophies and severe dry eyes. In particular, emphasis will be given on the anti-inflammatory effect of omega-3 PUFAs. Also, some observational results from our patients are presented and future directions regarding how to benefit from the omega-3 PUFAs are briefly discussed.

Polyunsaturated Fatty Acids

Currently, several studies have been focusing on the therapeutic role of omega-3 PUFAs, which are considered anti-inflammatory molecules. The resolution of inflammation is an active process primarily driven by a new family of mediators, termed resolvins, derived from the omega-3 PUFAs, eicosapentaenoic acid (EPA, C20:5ω-3) and docosahexaenoic acid (DHA, C22:6ω-3) [2]. These PUFAs are highly concentrated in the brain and retina and have an important role in the neuronal development and damage repair [3]. DHA is abundantly expressed in the photoreceptors, and vital retinal functions depend on its existence [4].

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Among the major mediators of the inflammatory response is the generation of pro-inflammatory eicosanoids generated from the omega-6 PUFA, arachidonic acid (AA, C20:4ω-6). These include pro-inflammatory prostaglandins (e.g. PGE_2) and leukotrienes (e.g. LTB_4), which can act as mediators for leucocyte chemotaxis and inflammatory cytokine production. The balance between the pro- and anti-inflammatory molecules plays a key role in the disease progression and the resolution of an inflammatory response.

Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly. It is estimated that 200 million people suffer worldwide and the number of these individuals is going to be increased up to 50 % by 2040 [5, 6]. The main symptom of early AMD is blurring of central vision which results in difficulty in reading and recognising faces. There are two different types of AMD: the dry form which is the most common one and occurs in 9 in 10 cases, and the wet form (choroid neovascularisation, CNV) which occurs in about 1 in 10 cases. In dry AMD, the retinal pigment epithelium (RPE) cells of the macula, which are crucial for the function of the rods and cones, will gradually degenerate. In wet AMD, in addition to the RPE cells' degeneration, newly formed blood vessels grow from the choroid, break through Bruch's membrane and migrate into the macula part of the retina. These vessels are immature and leak fluid within the retina resulting in scarring of the macula and damage of the rods and cones [7, 8].

Pathogenesis of Age-Related Macular Degeneration

Currently, there is no definite cause for the pathogenesis of AMD, but several different aetiologies. Ageing is one of the most common contributing factors of AMD, due to the

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accumulation of oxidised lipoproteins and free radicals in the retina and choroid. This in turn results in oxidative stress and a decrease in the number of RPE cells and photoreceptors [9, 10]. In addition to oxidative stress, natural age advancement also leads to immunosenescence which is the gradual deterioration of the immune system, specifically the T-cell population [11, 12].

Genetic predisposition, as with multiple pathologies, plays a role in the development of AMD; although there are several genetic associations, the most studied ones are some polymorphic links, in particular related to inflammatory genes, such as the *complement factor H* (*CFH*) and some complement components (e.g. *C3* and *C2*) [13, 14]. The *CFH* gene which controls the activation of the complement system through the alternative pathway was found to be associated with an increased risk of developing AMD [15, 16]. Environmental factors, including smoking, sunlight exposure, high-fat diet, obesity and diabetes, are all associated with the development and progression of AMD [17].

Para-Inflammation in Age-Related Macular Degeneration

Furthermore, a tissue adaptive response, recently described as para-inflammation, where the innate immune system mount a low-grade inflammatory response in order to restore tissue homoeostasis, has been implicated in the pathogenesis of AMD [18, 19]. In particular, chronic inflammatory infiltrates, such as macrophages, lymphocytes and mast cells, have been detected in the choroid of AMD eye donors [20, 21]. Inflammatory-related proteins, including C-reactive protein (CRP) [22–25], interleukin-6 (IL-6) [25, 26] and tumour necrosis factor-α (TNF-α) [25, 27], have been associated with AMD; however, the results from different groups are inconsistent. The fact that systemic inflammatory markers are not strongly related to AMD might suggest that local low-grade inflammation is more likely to be involved in the pathogenesis of AMD. In addition, resident retinal microglia were found to be activated in the outer nuclear layer, in regions of ongoing photoreceptor cell death, in patients with AMD, retinitis pigmentosa and late-onset retinal degeneration [28]. The microglia cell activation is an indication of an immune response to ocular injury or inflammation, as well as retinal degeneration. During normal ageing and also in pathological conditions, such as AMD, there is an observed accumulation of microglia in the subretinal space, localised in the areas of RPE cell death. Apart from microglia activation, complement activation is also involved in ageing and in both forms of AMD. It is suggested that the damage of RPE cells and photoreceptors in AMD may, at least in part, be caused directly by complement activation at the retinal/choroidal interface [29, 30]. Evidence demonstrated that a local complement regulatory system exists in the eye, by the detection of complement components, such as the C3, membrane cofactor protein (MCP), decay-acceleration factor (DAF), membrane inhibitor of reactive lysis (CD59) and cell surface regulator of complement (Crry) [31, 32].

Treatment Options for Age-Related Macular Degeneration

With regards to the wet AMD, treatment options are based on anti-VEGF (vascular endothelial growth factor) therapies which aim to attenuate angiogenesis and vascular permeability. However, this type of targeted therapy does not lead to complete vascular or disease regression [33]. To assess the long-term outcomes, Rofagha et al. investigated the effect of intensive ranibizumab therapy in exudative AMD patients 7–8 years after initiation of the treatment. One-third of the patients demonstrated good visual outcomes 7 years post-treatment, whereas another third had poor outcomes. Almost half of the eyes were stable, whereas one-third declined by 15 letters or more and 37 % ended blind despite numerous injections [34]. These results may indicate that even a long-term therapeutic regime does not reduce the risk for substantial visual decline.

In contrast, for the dry form of AMD, there are no current guidelines for the first-line treatment, although several anti-oxidants, vitamins and zinc may reduce its progression according to the Age-related Eye Disease Study (AREDS). This study was a major clinical trial sponsored by the National Eye Institute which was designed to evaluate the effect of high doses of vitamin C (500 mg), vitamin E (400 international units), β-carotene (15 mg), zinc (80 mg) and copper (2 mg) on the progression of AMD and cataract [35].

Following the AREDS, an additional study was performed, the AREDS2, which was a multi-centre five-year randomised trial, designed to examine the effects of oral supplementation of macular xanthophylls (10 mg lutein and 2 mg zeaxanthin) and/or omega-3 PUFAs (650 mg EPA and 350 mg DHA) on the progression to advanced AMD. Overall, there was no additional benefit from adding the omega-3 PUFAs or a mixture of lutein and zeaxanthin to the formulation. Although the addition of omega-3 to the AREDS formulation was not proven beneficial, it is believed that higher doses of EPA and DHA may have a desirable effect.

To further examine the association of omega-3 dietary intake (from fish sources) with incidents of late-stage AMD (both neovascular and geographic atrophy), SanGiovanni et al. estimated nutrient and food intake from a validated food frequency questionnaire in AREDS participants. The data obtained indicated that people who were consuming the

highest levels of EPA and EPA + DHA had a 50 % reduced likelihood in disease progression (from bilateral drusen to central geographic atrophy) [36]. This shows a clear correlation between the dietary lipid intake and the development of AMD into a more severe clinical presentation.

Therefore, the inconclusive results from the clinical studies led to further investigations in order to examine the possible mechanisms of action of the omega-3 PUFAs and to assess any positive outcomes with regards to the disease progression.

Current Research in Age-Related Macular Degeneration

Numerous in vitro studies demonstrated that treatment of endothelial cells with omega-3 PUFAs effectively inhibited pro-inflammatory responses through modulation of nuclear factor-κB (NF-κB), TNF-α and interleukin-1β (IL-1β)-induced cell adhesion molecule (CAM) expression [37–40].

Dry Age-Related Macular Degeneration

Furthermore, several animal studies focused on the dietary supplementation of omega-3 PUFAs in murine models for macular degeneration. In particular, Tuo et al. reported the therapeutic effect of a high omega-3 diet in the *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ double knockout model, which develops focal retinal lesions with certain features of AMD [41]. The high omega-3 diet included 1.9 wt % of each EPA and DHA, 0.66 wt % α-linolenic acid (α-LNA) and 0.4 wt % docosapentaenoic acid (DPA). The omega-6/omega-3 ratio was 2.9, where the omega-6 source was from the linoleic acid (LA) only. Specifically, animals that ingested a high omega-3 diet for up to 8 months of age showed progression of retinal lesions compared with the low omega-3 diet group. This effect was suggested to be through a reduction in the AA metabolism, as demonstrated by the decreased pro-inflammatory derivatives (PGE_2 and LTB_4). High levels of dietary omega-3 PUFAs may result in the incorporation of EPA into cell membrane phospholipids at the expense of AA, leading to less substrate available for eicosanoid synthesis [42]. In contrast to PGE_2, higher serum levels of PGD_2 (an anti-inflammatory mediator [43]) were observed in the high omega-3 PUFAs group, indicating a protective effect against inflammation. In addition, there was lower ocular *TNF-α* and *IL-6* transcript levels in the high omega-3 group, suggesting that reactive mediators of omega-3 PUFAs may also regulate differential gene expression [41].

Following treatment with the AREDS2 formulation, Ramkumar et al. reported its effect on the *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$model [44]. This formulation included high doses of omega-3 PUFAs (54.9 mmol EPA/kg diet and 25.2 mmol DHA/kg diet), 17.6 mmol lutein/kg diet and 1.76 mmol zeaxanthin/kg diet. After 3 months of treatment, the animals fed with the high omega-3 PUFAs diet showed significant lesion regression, following fundoscopic examination and a great reduction in the ocular A2E concentration (a fluorophore found in lipofuscin and RPE phagolysosomes). Morphological changes were noted, where the outer nuclear layer thickness was greater in the high omega-3-treated group than that in the control one. The retinal expression of pro-inflammatory mediators, including *TNF-α*, *Cyclooxygenase-2* (*Cox-2*), *IL-1β*, *VEGF* and *inducible nitric oxide synthase* (*iNos*), was much lower in the high omega-3-treated group compared with the control. The AA concentration in the retina was found to be lower and the EPA higher in the high omega-3 group compared to the control, whereas the retina AA/EPA ratio was estimated to be 2.26. However, the serum concentration of PGE_2 which is a metabolite of AA did not significantly differ between the two groups [44]. This fact may indicate that the concentration of fatty acids (and their metabolites) in the serum and retina is not directly correlated.

Observational Studies in Age-Related Macular Degeneration

In addition to the preclinical studies, an open-label pilot study performed by Georgiou et al. investigated the therapeutic effect of controlled doses of high omega-3 PUFAs in patients with dry AMD. The supplement formulation included 3.4 g EPA and 1.6 g DHA, where patients followed this treatment on a daily basis for 6 months. Significant improvement in visual acuity was observed in all patients with dry AMD, and by 4.5 months of treatment, all patients had gained a minimum of 1 line of vision consisting of 5 letters on the Early Treatment Diabetic Retinopathy Study (ETDRS) chart and a third of patients gained 3 lines consisting of 15 letters [45].

Further observational studies took place with 24 eyes of twelve patients with dry AMD, both sexes and mean age of 75, where they were supplemented with 15 ml of omega-3 formulation divided into two daily doses. The omega-3 concentrates consisted of purified ethyl esters rich in EPA (400 mg) and DHA (200 mg) per gram for the liquid formulation. The dosage used in this pilot study provided approximately 5 g EPA and 2.5 g DHA per day. Patients were asked to continue with their current diet as normal and not to increase their consumption of food containing essential fatty acids.

Follow-up was performed at 1.5, 3, 4.5 and 6 months. Visual acuity was examined at each follow-up using the ETDRS chart, and the blood AA/EPA ratio was measured prior and during treatment using a gas chromatographic

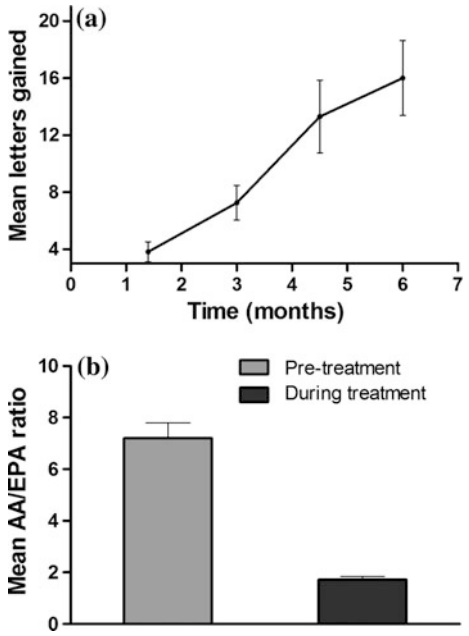

Fig. 20.1 The effect of omega-3 supplementation on **a** visual acuity in patients with dry AMD according to the letters gained and **b** the AA/EPA ratio pre-treatment and during treatment

technique. The optimum AA/EPA ratio is believed to be between 1 and 2 for maximum anti-inflammatory effects. The mean initial visual acuity of patients was 6/18 + 2 (36 %). The mean gain of letters at 1.5, 3, 4.5 and 6 months was 3.8 ± 0.7, 7.3 ± 1.2, 13.3 ± 2.3 and 16.0 ± 2.6, respectively (Fig. 20.1a). The mean AA/EPA ratio prior to treatment was estimated to be 7.2 ± 0.6, whereas during the period of treatment was reduced to 1.7 ± 0.1 (Fig. 20.1b). No side effects were reported by any of the patients treated in the study. The closer the AA/EPA to 1, the more pronounced the effect was.

Wet Age-Related Macular Degeneration

Additional animal studies have been performed using models of wet AMD and other ocular pathologies, including retinal angiogenesis or diabetic retinopathy. Yanai et al. demonstrated that metabolites derived from the omega-3 PUFAs, which are generated through the cytochrome P450 (CYP450), are potent inhibitors of intraocular neovascular disease, such as wet AMD [46]. It is known that the omega-6 PUFAs, including AA, generate CYP-metabolites, the epoxyeicosatrienoic acids (EETs), which are associated with the VEGF-activated signalling cascade, leading to angiogenesis [47]. On the other hand, the CYP-generated metabolites of EPA and DHA, namely 17,18-epoxyeicosatetraenoic acid (17,18-EEQ) and 19,20-epoxydocosapentaenoic acid (19,20-EDP), respectively, have shown anti-angiogenic properties [48]. Therefore, Yanai et al. investigated the dietary enrichment with omega-3 PUFAs in a mouse model of laser-induced CNV and demonstrated suppression of CNV (possibly through increased expression of peroxisome proliferator-activated receptor-γ, PPAR-γ), vascular leakage, immune cell recruitment and adhesion molecules (E-selectin and intracellular adhesion molecule-1, Icam-1) to the lesion site. In addition, VEGF expression was suppressed in the retina and choroid of the mice fed with the high omega-3 diet. A significant increase in the serum levels of anti-inflammatory eicosanoids was observed in the high omega-3 group, which was mediated through the CYP-metabolites, 17,18-EEQ and 19,20-EDP [46].

Similar studies by Connor et al. were previously performed, evaluating the therapeutic effects of omega-3 PUFAs on hypoxia-induced pathological neovascularisation in a mouse model of oxygen-induced retinopathy [49]. The results suggested that by increasing the level of omega-3 PUFAs either by dietary or genetic means (using *Fat-1* transgenic model which converts omega-6 to omega-3 PUFAs), there was a reduced hypoxic stimulus for neovascularisation. This effect was mediated through the bioactive metabolites neuroprotectin D1, resolvin D1 and resolvin E1, through reduction in the TNF-α expression which was found to be present in a subset of microglia within the retinal vessels [49].

Furthermore, in a clinical setting, Renzende et al. examined the effect of omega-3 PUFAs supplementation (1052 mg fish oil, 600 mg EPA and DHA) on the levels of vitreous VEGFA, in patients with wet AMD who were receiving intravitreal anti-VEGF therapy. Interestingly, the group of patients that was supplemented with omega-3 showed lower levels of VEGFA in the vitreous but similar levels in the plasma compared to the other groups [50]. This indicates that omega-3 PUFAs could also be useful in minimising progression of wet AMD.

Retinitis Pigmentosa

Retinitis pigmentosa (RP) refers to an inherited, genetically heterogeneous condition which can result from mutations in several different genes (>45 known genes), including the rhodopsin and cyclic guanosine monophosphate phosphodiesterase (cGMP) β-subunit genes [51–53]. RP is affecting approximately 1 in 3500 people pan-ethnically and is a major cause of blindness in adults [54]. What drives the disease progression is the dysregulation and degeneration of the photoreceptors through apoptotic signals, initiating from the rods followed by the cones at a later stage. The main characteristics of RP are night blindness, retinal pigmentary deposit [55] and gradual loss of peripheral vision. As the degeneration of the photoreceptors progress, the vision loss will be increased leading to eventual blindness.

Inflammation plays an important role in the pathogenesis of RP as demonstrated by Newsome et al. who found inflammatory cells infiltrate into the vitreous cavity of RP patients [56]. In addition, Yoshida et al. investigated the inflammatory response in the aqueous humour and vitreous fluid of RP patients by examining different pro-inflammatory cytokines and chemokines performed by ELISA analysis. There was a significant increase of MCP-1 and IL-8 in the aqueous humour, whereas in vitreous fluid, there was an increase in a variety of cytokines/chemokines, including IL-1α, IL-1β and MCP-1 [57].

Current Research in Retinitis Pigmentosa

At present, there is no available treatment which targets the regression of RP; thus, numerous studies are ongoing. The necessity of omega-3 fatty acids for proper retina functioning was demonstrated by Bush et al., where a reduced capacity for photoabsorption by rhodopsin could play a role in lowering retinal sensitivity to light in omega-3 PUFAs-deficient rats [58]. Omega-3 PUFAs and some anti-oxidants or vitamins have been used in several laboratory and clinical studies in order to examine their effect on RP progression, aiming to reduce inflammation.

The nature of the inflammatory response in the rd10 model of RP was evaluated by Yoshida et al., who found an increased expression of pro-inflammatory cytokines/chemokines in the retina, activated microglia and photoreceptor apoptosis. However, treatment of animals with an anti-oxidant, N-acetylcysteine (NAC), prevented the photoreceptor cell death and reduced the inflammatory response [59].

In addition, the effect of DHA was examined in a mouse model of inherited retinal degeneration ($Rs1h^{-/Y}$). Supplementation of animals with DHA demonstrated enhancement of the photoreceptors' survival, transformation of the activated microglia to a quiescent phenotype and reduction in the pro-inflammatory gene expression [60]. This indicated that the retinal DHA levels could control the activity of microglia and perhaps the extent of retinal degeneration.

A randomised, controlled, double-masked trial was performed in order to determine the effect of DHA in patients with RP receiving vitamin A treatment. Patients were given either 1200 mg/day of DHA or control capsules over a 4-year period, in addition to vitamin A. The end results showed that supplementation of DHA over a 4-year interval did not slow the course of disease in RP patients [61]. Similar disappointing results were obtained by a different clinical trial using DHA supplementation (400 mg/day) with X-linked RP patients [62]. In contrast, Berson et al. analysed questionnaires completed by patients with RP who were receiving vitamin A for 4–6 years. The difference in visual acuity was compared between those with high ($\geq$0.20 g/day) versus low (<0.2 g/day) omega-3 intake. The study concluded that patients receiving vitamin A and who consumed a diet rich in omega-3 fatty acids had slower decline in visual acuity than those who consumed a low omega-3 diet [63].

An overview of the clinical findings was presented by Hodge et al. which analysed 6 different studies involved investigation of the intake of omega-3 fatty acids. The review suggests that the data obtained from those studies did not provide a conclusive result as to whether or not the intake of omega-3 fatty acids could slow the progression of RP [64].

Stargardt Disease

The most common form of autosomal recessive macular dystrophy is Stargardt disease which affects the RPE and photoreceptor layer and is usually associated with mutations in the *ABCA4* gene (ATP-binding cassette 4) [65], which leads to accumulation of lipofuscin [66]. Stargardt disease is characterised by a juvenile onset (first two decades), a rapidly progressive course and a poor visual outcome. Loss in central vision is observed caused by a progressive central atrophy.

Another type of macular dystrophy is the autosomal dominant Stargardt-like macular dystrophy type 3 (STGD3), which is caused by 3 sets of mutations in the *ELOVL4* gene (elongation of very long chain fatty acids protein 4) [67, 68]. The ELOVL4 protein is involved in fatty acids' elongation which is necessary for the generation of long chain PUFAs [69]. Mouse models and human clinical studies indicated that a deficiency of very long chain PUFAs in the retina is likely to be a key factor in the macular pathology seen in STGD3 [70, 71].

Current Research in Stargardt Disease

The effect of omega-3 supplementation is believed to play a role in the disease progression. In particular, Dornstauder et al. used the ELOVL4 transgenic model (which displays extensive age-related retina dysfunction and A2E accumulation), to study the effect of dietary DHA supplementation. The data obtained indicated that following DHA supplementation for several months (starting at different stages), there was preserved retina function at mid-degenerative stages and reduced A2E levels in the mouse models [72]. Furthermore, Querques et al. reported a study where 840 mg/day DHA was given to 20 late-onset Stargardt disease patients for six months. A complete ophthalmological examination was performed pre- and post-treatment. The evidence from the study demonstrated

that DHA influenced some functional parameters in those patients; however, there was not significant short-term benefit [73].

Additionally, some emerging therapeutic options for Stargardt disease are being considered, including drugs which modify the functional activity of ABCA4 protein [74], which slow down the visual cycle [75] or gene-targeted therapy [76, 77].

Cone Dystrophies

Cone dystrophies are associated with genetic mutations (e.g. guanylate cyclase activating protein 1, *GCAP1*) which result in functional abnormalities in the cone photoreceptors and sometimes in their post-receptoral pathways [78]. This could be inherited as an autosomal recessive, autosomal dominant or X-linked recessive trait. Within these types of dystrophies, there are subtypes including the stationary cone dystrophy and the progressive (cone-rod dystrophy) which have a different time of the disease presentation. All forms of cone dystrophies show reduced visual acuity and colour vision deficiency, along with dysfunction of the cone electrophysiology [79, 80]. Although these are inherited disorders, chronic inflammation is an important component that contributes to the disease progression. Shiose et al. demonstrated the involvement of Toll-like receptor 3 (TLR3) in a cone-rod dystrophy animal model lacking the ABCA4 and RDH8 proteins (which are critical for all-trans-retinal clearance in the retina). These findings suggested that endogenous products from degenerating retina can stimulate TLR3, causing apoptosis and retinal inflammation. In addition, the loss of TLR3 showed a protective effect from cone-rod dystrophy [81].

Observational Studies in Macular Dystrophies

The strong evidence from the literature regarding the positive outcomes from the supplementation of omega-3 PUFAs in retinopathies and following the promising results from the preliminary study with dry AMD patients [45] led us to investigate the effect of omega-3 in patients with macular dystrophies. Observational studies included a total of 40 eyes of 21 patients with macular dystrophies, 9 patients with Stargardt disease with mean age of 33.6 ± 6.1, 6 patients with RP with mean age of 54.2 ± 6.4 and 6 patients with cone dystrophies and mean age of 41.3 ± 4.4. Patients were supplemented twice daily with an omega-3 formulation, and follow-up was performed at 1.2, 3, 4.5 and 6 months. RP patients were supplemented with 5 g/day of EPA/DHA (ratio 2:1), and the Stargardt's and cone dystrophy patients were supplemented with 7.5 g/day of EPA/DHA (ratio 2:1). Visual acuity was examined at each follow-up using the ETDRS chart, and the blood AA/EPA ratio was monitored. The mean initial visual acuity of all patients was 6/18 (33 %). The overall mean gain of letters for all types of macular dystrophies at 1.2, 3, 4.5 and 6 months was 5.2 ± 0.9, 9.7 ± 1.1, 10.5 ± 0.9, 11.2 ± 0.9, respectively (Fig. 20.2a). The mean AA/EPA ratio pre-treatment in patients with macular dystrophies was 9.9 ± 0.6, while during treatment, the ratio was reduced to 1.4 ± 0.09 (Fig. 20.2b).

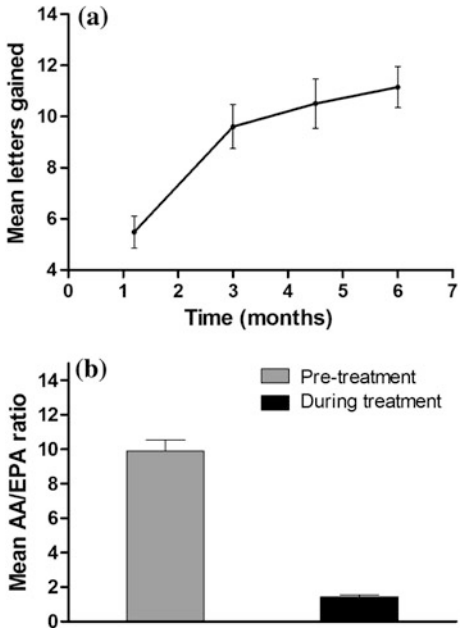

Fig. 20.2 The effect of omega-3 supplementation on **a** visual acuity in patients with macular dystrophies according to the letters gained and **b** the AA/EPA ratio pre-treatment and during treatment

Severe Dry Eyes

Dry eye disease is a multifactorial disorder of the tears and ocular surface that represents one of the most prevalent ocular conditions, and it is a frequent reason that people seek eye care [82]. Dry eye disease is caused by disequilibrium of tear film components and results in symptoms of chronic ocular discomfort, functional visual disturbance that interferes with quality of life [83–85]. Around 3.2 million women in the USA aged 50 years and older have dry eye disease [86], and depending on the population, age and definition, the estimates of the prevalence of dry eyes in the general population range from <1 % to 30 % [83, 87].

Although there has been some progress in understanding the natural history of dry eye disease, current treatment

options for severe dry eye disease have limited efficacy and there are no means of prevention. Artificial tears are the standard of care for the treatment of dry eyes [88]. However, they can provide only temporary symptom control and they do not affect the causative factors of the inflammation that occurs in dry eyes. As a result, artificial tears may only be suited for milder dry eye disease [89]. In more severe disease, multiple treatments are often required, in particular one survey of treating ophthalmologists found that patients with severe dry eye disease usually need to use around five different treatment approaches to manage this condition [90], with the most frequently used treatment being artificial tears, followed by anti-inflammatory drugs and secretagogues [89].

Restasis® (0.05 % cyclosporine ophthalmic emulsion; Allergan) is the only pharmacologic treatment approved by the FDA to increase tear production, but its licensed indication does not extend to the treatment of the symptoms associated with dry eye disease. The onset of action is 24 weeks and many patients are intolerant due to side effects, particularly ocular burning sensation, which occurs in up to 17 % of treated patients [91].

Inflammation, particularly of the ocular surface and meibomian glands, is thought to be an important contributor in the pathogenesis of dry eye disease [92, 93]. Patients with dry eye disease have an increased concentration of inflammatory molecules (such as TNF-α, VEGF, IFN-γ, IL-1, IL-6, epidermal growth factor and fractalkine) in the tear film [93]. Inflammation can be relieved by topical steroids and anti-inflammatory drugs, but most are unsuitable for long-term use and their use may be limited by side effects [94]. There is a clear need for additional options to provide continuous relief of severe dry eye disease symptoms and that are acceptable to patients.

Several studies have suggested that omega-3 PUFAs (including α-LA, EPA and DHA) systemic and topical administration is effective as adjunctive therapy in dry eye disease [95–100]. Possible mechanisms suggested effects on tear secretion, prevention of oxidative damage and reduction in inflammation.

In a cross-sectional observational study, Milanović et al. found that the prevalence of dry eyes was substantially lower among women who had a higher intake of omega-3 PUFAs, compared with those with a lower intake, where the prevalence was higher [95]. In this study, intake of omega-3 PUFAs was estimated by a questionnaire of dietary intake. To date, most studies to investigate omega-3 supplements in dry eye disease have been limited in design and size, and the doses of omega-3 PUFAs have varied, making meaningful comparisons difficult. There is a need for additional trials using omega-3 PUFAs to provide the evidential basis for their use as an adjunct to current therapies for dry eye disease.

Observational Studies in Severe Dry Eyes

We hypothesised that high-dose omega-3 PUFAs may provide a viable alternative treatment for dry eye disease. Therefore, an observational study was performed in order to investigate this hypothesis, where patients with severe dry eyes who had not responded to other standard treatment were supplemented with 10 ml of omega-3 formulation (providing approximately 3.4 grams of EPA and 1.6 grams of DHA per day). The dosage was divided into two daily doses of 5 ml each. Sixty eyes of 30 patients with dry eyes were included in the study, 26 females and 4 males, with mean age of 58 ± 9.9 years. Patients were asked to continue with their current diet as normal, and not to increase their consumption of food containing essential fatty acids. Visual acuity using the ETDRS chart, tear break-up time and fluorescein staining score was determined prior to the initiation of the study and then at 1.5 and 3 months post-treatment.

Eyes that had the greatest gain in visual acuities were those who started with the worst initial vision. The patients who had reduced vision (e.g. 6/15 or worse) had a significant ($p < 0.05$) improvement in vision (Fig. 20.3a). Prior to the omega-3 supplementation, the fluorescein staining was either moderate (17 %) or severe (83 %) in all patients, whereas at

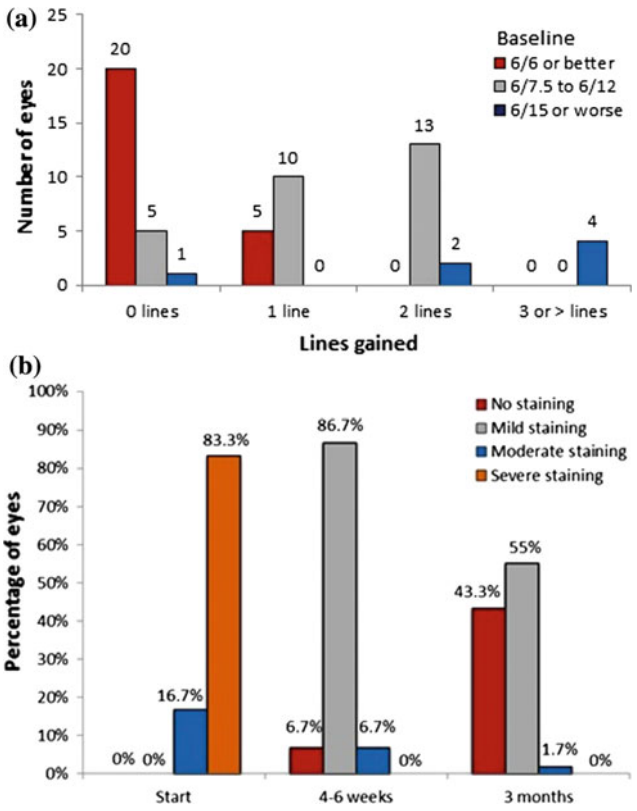

Fig. 20.3 **a** Number of ETDRS chart lines gained at 3 months in patients with severe dry eyes and **b** corneal fluorescein staining scores following supplementation with an omega-3 formulation

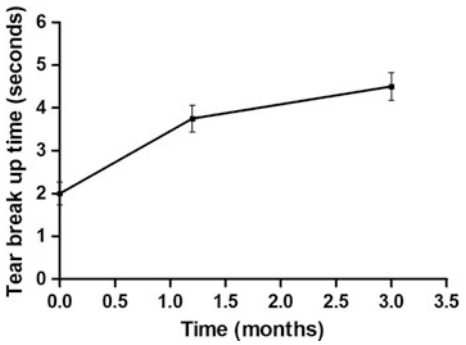

Fig. 20.4 Improvement in tear film break-up time in patients with severe dry eyes following supplementation with an omega-3 formulation for 3 months

the end of the study, no eye had severe staining, and only one eye had moderate staining, illustrating that staining scores improved significantly ($p < 0.001$, Fig. 20.3b). Tear break-up time increased significantly from a mean of 1.78–4.4 s at 3 months ($p < 0.001$, Fig. 20.4). No side effects were reported by any of the patients treated in the study.

In summary, evidence from the literature and from our observational studies suggest that supplementation with high doses of omega-3 PUFAs of patients with retinopathies and severe dry eyes results in disease regression by a substantial improvement in visual acuity. A point for consideration is the monitoring of the AA/EPA ratio, where this could not only provide the optimum therapeutic effect (between 1 and 2) but it could also prevent undesirable side effects by remaining in the safety range (≥ 1).

Conclusions

Chronic low-grade inflammation attacks the body's organs for years until enough damage occurs to produce a chronic disease. It is inflammation below the threshold of pain which is not initially noticeable until chronic organ damage occurs. As for the eyes, reduced vision is the first obvious symptom. Anti-inflammatory drugs, such as steroids and non-steroidal anti-inflammatory drugs (NSAIDS), could reduce chronic inflammation; although side effects such as gastric bleeding, immunosuppression, osteoporosis and heart failure do not make them ideal agents for long-term use. In addition, substantial long-term improvement in visual acuity has not been demonstrated with this class of drugs. Maintaining the AA/EPA ratio between 1 and 2 using omega-3 supplementation could provide an excellent therapeutic regimen for reducing inflammation in the retina and the optic nerve with no side effects. Further studies are ongoing in order to get a better optimisation of the anti-inflammatory effect of omega-3 PUFAs and to have an improved understanding on their mechanism of action and their long-term effects.

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DHA-Rich Algae Oil Is a Safe and Effective Vegetarian Source of Omega-3

21

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Introduction

Omega-3 rich algae oil is a safe and effective vegetarian food. *Schizochytrium* algae are derived from single strains cultured, grown, and extracted for their internal store of edible triacylglycerol oils. *Schizochytrium* oils are the most widely developed and characterized for their nutritional lipid components. These organisms produce both saturated and unsaturated fatty acids de novo, although principally produced in enclosed tanks for their long-chain polyunsaturated fatty acids [1, 2]. *Schizochytriums* are known colorless heterokonts, primitive members of the kingdom Chromista, which are natural to marine food chains or non-marine habitats found on decomposing plant material [3].

Algae oil effectiveness has been investigated in clinically relevant nutritional studies in reference to the very long-chain omega-3 fatty acid docosahexaenoic acid (DHA). Clinical levels of DHA in algae oils are reported to be 35–55 % DHA (wt/wt). However, along with the omega-3 DHA, the omega-6 docosapentaenoic acid, DPAn-6, is another significant fatty acid of importance in the oils, reported at 7–15 % DPAn-6 (wt/wt). In the human body, each bioactive lipid has distinct and relevant roles in the neuronal and cardiovascular systems for cell and tissue structure and function, also resident in lipoprotein phospholipids [4]. These lipids are both cell building blocks in neuronal plasma membranes, have twenty-two carbon chain lengths, and are terminal lipids in their respective synthetic fatty acid pathways [5]. Algae oil DHA and DPAn-6 together improve cardiovascular risk factors in healthy men and women [6, 7]. The effective clinical use as a vegetarian food oil has been repeatedly proven to substitute for clinical fish oils.

The value of vegetarian algae oil is also suggested because DHA and DPAn-6 of similar ratios are present in a variety of foods related to growth and development, abundant in eggs, and breast milk, for example. The ratio of DHA to DPAn-6 in human breast milk is reported to range normally from 1:1 to 1:6 [8]. The ratio of DHA to DPAn-6 in *schizochytrium* oil is about 1:3–1:6, within the range found in breast milk. Because algae oils and fish oils nearly always demonstrate equality in dose-dependent omega-3 studies, DHA alone is proven to be broadly effective compared to any other combination of long-chain omega-3s [9].

While more study is needed in persons, the value of the algal DHA/DPAn-6 ratio is not related to fish oils of different omega-3/omega-6 ratio compositions. The observation of native lipid composition in human tissues suggests algae oil lipid ratios, DHA with some DPAn-6, and with about 0.5–3 % eicosapentaenoic acid (EPA) is similar to the inherent order of abundance of these lipids in a person [4].

In contrast, fish oil EPA and DHA ratios are fundamentally different than how these exist in a person. EPA is generally two-thirds of the omega-3 in fish oil and DHA is only one-third of the long-chain omega-3s. What is increasingly being learned from omega-3 studies in people is that the lipid ratios of oils consumed will enter the blood and transiently exist for several hours. Because algae oil provides a preformed ratio of fatty acids more similar to steady-state tissue ratios and fasting ratios of these lipids, this is suggestive that algae oil may be less of a metabolic challenge for the systems to process.

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Supplementation

Natural oil supplementation with omega-3 fatty acids is increasingly considered for health. The omega-3 bond is essential to life and must be obtained from accepted foods or supplements. Normal fortification of the essential short-chain omega-3 called alpha-linolenic acid (ALA) may address nutritional needs. However, regular public health discussion emerges from the understanding that short-chain ALA is the most abundant omega-3 in the food supply, but one of the least abundant omega-3s in the human body. Ordinary public health practices generally now rely upon daily recommendations, but minimum omega-3 levels are currently not well supported by health policy. Minimum levels are also not intended to produce specific clinical effects.

Scientifically, the longest chain omega-3, DHA, is also the most abundant omega-3 fatty acid in human cells and tissues. Physiologically, DHA comprises approximately 80 % of all the omega-3s present in a person. Specifically, DHA is as high as 97 % of all the omega-3 fats found in the brain. Thus, clinical use of DHA-rich algae oils available in large countries like the USA, India, and China could benefit diverse populations [10].

Current unmet clinical needs for omega-3 supplementation include uses in health management for vascular health conditions such as with heart disease, dyslipidemia, prediabetes, or type 2 diabetes patients [11, 12]. However, medical intervention with omega-3 supplements may not affect, or may only indirectly affect, insulin signaling and glucose homeostasis [13].

The value of numerous algae oil studies has been to show equal benefits compared to fish omega-3s, without the need for fish oil. The pressure on animal sourcing of fish oils has led to public concerns over purity and sustainability and stimulates algae oil solutions in nutrition and medicine. Overall, the assessment of algae oil DHA supplementations provides clinical study substantiation.

The human benefit from algae oil defines greater natural value as an option that has proven safe and effective in clinical nutrition for vegetarians, type 2 diabetics, infants, children, pregnant and nursing mothers, the elderly, and those with fish allergies. Clinical DHA supplementation under the care of a doctor when used along with diet or medications could be considered an important co-therapy.

DHA in Pregnancy and Development

Especially in the final months of pregnancy, clinical reproductive health studies increasingly suggest DHA supplementation may be needed by pregnant mothers. The US population is one of the lowest natural consumers of DHA in the Western world. As part of a regular diet, Japan and Korea are among the highest natural consumers [14]. In the USA, adults usually consume about 1 g ALA daily from food and only about 60 mg DHA. Current information and Asian perspectives on long-chain polyunsaturated fatty acids in pregnancy, lactation, and infancy have been reviewed [15].

In late pregnancy, the woman's diet will not typically replace her own body's stores due to the fetal demands for long-chain polyunsaturated fatty acids, including DHA. Unique to the fetus, storage of DHA normally will take place in fetal adipose tissues in addition to the organ tissues. Storage of DHA and other fats naturally provides an important reserve for postnatal development during the critical first months of life. Heading to term, the fetus will generally add up to 90 % of its increasing mass in the form of fat.

A clinical DHA supplement program with algae oil could benefit the health of the pregnant mother, the pregnancy, and the infant at birth. Clinical research suggests a mother's DHA supplementation helps maintain her body's internal DHA stores. However, the pregnant mother cannot fully prevent significant depletion of her own DHA stores unless she supplements in the second half of the pregnancy with at least 600 mg DHA per day or more [16]. Vegetarian mothers with child are suggested to be at additional risk of DHA omega-3 deficiencies for her own needs at this critical time.

The value of preventative DHA supplementation during pregnancy could improve health outcomes for the mother and the pregnancy, although the consequence of declining maternal DHA stores during pregnancy is still understudied. At the time of birth, pregnant mothers who were supplementing with DHA are found to have slightly longer gestational durations. Scientifically, the data suggest these mothers tend to give birth to infants who have greater average birth weights, birth length, and a slightly larger head circumference [16].

At 30 weeks into term, DHA transfer to the fetus begins to double as the mother's own DHA stores drop about 10 % in pregnant women without additional supplementation. Calculations during the 3rd trimester show an average doubling of DHA demand by a growing fetal brain mass and DHA accumulations in the extra fat storage in the fetal body. Also, diversity in the function of the placenta may be eliminated by maternal DHA supplements that could otherwise affect neonatal fat stores and fetal levels of omega-3s in women with gestational diabetes [17]. Overall, the value of DHA levels measured in the umbilical cord blood is significantly higher in supplementing mothers compared to non-supplementing mothers.

Importantly, supplementation by the nursing mother after birth is valued because an infant's daily nutrition further supports the time in life when the brain grows fastest, rapidly

doubling in size. At this critical stage, the infant is utilizing DHA from its own reserves and from breast milk at rates greater than demanded during later periods of life.

In humans, one question is if only certain pregnancies are benefitted by DHA supplementation or is DHA supplementation important for all pregnancies in the same way? The results of this analysis are preliminary, but astounding in the context of public health. Although still an emergent finding, it is suggested that DHA algae oil supplementation by the pregnant mother may prevent preterm births, thus improving overall gestation time and developmental outcome across a population [16].

Possible mechanisms are only beginning to be understood, but these findings could suggest DHA supplementation can help protect the most at risk pregnancies. Progress in algae prenatal DHA research should be further considered. The original natural observation of pregnancy term was from women living in the Faroe Islands, where high fish oil intakes seemed to cause pregnancies to have longer gestational ages and infants to have higher birth weights [18]. Clinically, there is evidence that preterm births are associated with pregnancies complicated by maternal cardiovascular, immune, and metabolic disorders. Valuable research on the interactions between nutrition and risk of preterm birth may be needed, but variations in environments and demographics in studies complicate interpretation. Correlations to maternal fat stores are suggested. A mother's low body mass index has been associated with an increased risk of preterm birth, while maternal obesity has been shown to be protective [19]. Obesity, however, predisposes pregnant women to diabetes, preeclampsia, and cardiovascular issues.

One hopeful conclusion is that DHA supplementation findings indicate adequate preformed DHA intake by the mother could prevent a subset of premature births. Also, broader discussion on DHA available to infants under 2 years old has been investigated, suggesting minimum available dietary DHA sustains optimal health when in demand by the growing body, brain, and organs during this period. Thus, pregnancy and infancy are life-stages when higher DHA omega-3 demands may be best supported by the use of algae oil as a source of preformed DHA.

Is Algae DHA an Effective Co-therapy?

DHA could be standardized as an omega-3 co-therapy. A high fat diet is prone to elevated VLDL and LDL levels with low HDL concentrations. Lipoprotein diameter redistribution is observed with algae DHA intake. Clinical intake levels on average of about 1200 mg DHA increase the total omega-3 level in the body and positively affect VLDL, LDL, and HDL lipoproteins [4, 6, 20]. In addition, DHA facilitates chylomicron and lipoprotein particle clearance in the postprandial plasma [21].

For patients with mixed hyperlipidemia or dyslipidemia, statins have become widely used, but their reductions in lipoprotein particles are mainly on liver LDL. Statins may not control other atherogenic potentials in the postprandial state [22]. DHA impact is positive when added to a statin in young populations [23].

Of key significance is the entry of dietary fat into the VLDL pool. DHA is over-incorporated into this pool compared with EPA [24]. VLDLs contain much higher concentrations of DHA versus EPA. Selective partitioning of DHA in the early postprandial period could be principally determined by DHA levels in the liver, which increase with supplementation. DHA becomes the majority omega-3 in the VLDL fraction regardless of whether DHA only or EPA only is consumed in the diet [24]. DHA incorporated into the VLDL surface phospholipid pool informs how this lipid functions in forming lipoproteins, and this analytical physiology helps define part of the mechanism of how DHA is directly utilized in practice for the lowering of plasma triglyceride levels and other atherogenic potentials [4].

Unfortunately food components rarely will be considered together with drug treatments. Most studies of omega-3s are limited to healthy cohorts. Further confounding the issues are the number of forms of omega-3s found in foods and oils, reducing the likelihood of forming a single nutritional standard and limiting co-treatment studies that include omega-3 supplementation with a known medication.

Conclusion

DHA is uniquely utilized in the transport of fat in all people. In infants, the storage of DHA fat is an indicator of health. There is concern that weight gain in older children and adults is a cause of pathology. Effective use of the omega-3 bond can be a powerful co-therapy to broadly improve lipid transport and pregnancy outcomes. Medicines do not substitute for essential nutrients or healthy diets in the case of omega-3 deficiency, which is when omega-3 demand by the body is greater than the dietary supply and when there are low levels of these fats in red blood cells. A state of deficiency may arise during periods of greater metabolic demand, such as during pregnancy. Moreover, lipid transport is a common biological process inherent to a healthy metabolism and DHA is shown to be the most abundant structural and functional omega-3 in lipid transport mechanisms. The

liver and DHA have primary roles in the physical transport of fat to and from the body for maintaining tissue structure and function, with secondary clinical roles in health maintenance and prevention.

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Role of n-3 (*Omega-3*) Polyunsaturated Fatty Acids in Postpartum Depression: Mechanisms and Implications for Prevention and Treatment

22

Beth Levant

Introduction

Postpartum depression is a major depressive episode occurring in the first 3 months after giving birth and affects 10–20 % of childbearing women [1–4]. The symptoms are similar to depression occurring at other phases of the life span [5–7], and risk factors include a history of the previous depressive illness and psychosocial and other issues, such as lack of social support, marital stress, and difficulties with breastfeeding [5–10]. Many women with postpartum depression also experienced depressive symptoms during pregnancy [5].

Although there are many biological findings in non-puerperal depression, the pathophysiological basis of postpartum depression remains elusive. As with non-puerperal depression, the etiology is complex, involving the interaction of genetic and environmental factors, and is most likely heterogeneous [5, 6, 10–14]. However, due to the numerous physiological changes that occur during pregnancy, parturition, and the postpartum period, the underlying pathophysiology remains to be determined. The numerous hormonal changes associated with pregnancy and during the postpartum period appear to play a role, but, with the exception of the small subset of women with postpartum autoimmune thyroiditis, cannot explain the etiology of the disorder [7, 15–18]. Likewise, inflammation during pregnancy and after childbirth, and polymorphisms in genes encoding the serotonin transporter and serotonin metabolizing enzymes have been proposed as contributing factors, but current evidence is not sufficient to demonstrate a major causal role [19–21].

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Recent evidence suggests that n-3 polyunsaturated fatty acid (PUFA) status may be a contributing factor in the etiology of postpartum depression and other depressive disorders. This chapter reviews that literature and its implications for the treatment and prevention of depressive disorders with a focus on postpartum depression. The basic biology of n-3 PUFAs in the brain is discussed followed by a review of the literature on the regulation of maternal n-3 PUFA status during pregnancy and the postpartum period. The results of clinical trials with various n-3 PUFA preparations for the prevention or treatment of postpartum depression and depression during pregnancy are presented, as well as trials in non-puerperal depression. Finally, a review of studies examining neurobiological mechanisms, by which n-3 PUFAs may contribute to the pathophysiology of depressive illnesses and serve as a basis for further study of n-3 PUFAs in the prevention and treatment of postpartum depression, is presented.

Role and Regulation of n-3 PUFAs and the Brain

Long-chain PUFAs have important biological roles as part of membrane phospholipids and as signaling molecules. The most abundant, and thus presumably the most functionally important, long-chain PUFAs in the brain include the n-3 PUFA docosahexaenoic acid (DHA), which represents roughly 15 % of total fatty acids in that tissue, and arachidonic acid, the primary n-6 PUFA which comprises 10 % of brain fatty acids [22]. As constituents of the cell membrane, long-chain PUFAs are a determinant of the physicochemical properties of the membrane. Accordingly, variation in membrane fatty acid composition influences the function of membrane-bound proteins, such as transporters, ion channels, and receptors, as well as lipid rafts [23, 24]. In addition to this structural role, circulating PUFAs, derived from the diet or cleaved from cell membranes by phospholipases, serve in various signaling roles. These roles include acting as

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ligands at peroxisome proliferator-activated receptors (PPAR), retinoid X receptors (RXR), and liver X receptors (LXR), which regulate gene expression [25–27]. In addition, long-chain PUFAs are precursors for a variety of intra- and intercellular signaling molecules such the arachidonic acid-derived prostaglandins and thromboxanes [23]. Of particular note, n-3 PUFAs are metabolized into a variety of protectins, resolvins, and marisins that are involved in the resolution of acute inflammation [28].

DHA accumulates in brain phospholipids primarily during the late prenatal and early postnatal periods [29–31]. During this time, DHA is delivered to the fetus and infant by the mother through the maternal–fetal circulation or in the breast milk, respectively [32, 33]. Inadequate accumulation of DHA, which can occur as a consequence of low maternal stores prior to pregnancy and/or inadequate dietary consumption, results in the compensatory incorporation of the n-6 PUFA docosapentaenoic acid (n-6 DPA), which is normally present in brain in only trace amounts, thus qualitatively altering the membrane fatty acid composition [34]. The resulting change in membrane PUFA composition can lead to suboptimal visual, attentional, and cognitive development of the offspring [35–37]. Once DHA accumulates in the brain, the proportion of the fatty acid in membrane phospholipids appears to remain fairly stable. Notably, treatment with an n-3 PUFA-deficient diet for as long as 7 months failed to alter the percentage of DHA in brains of adult male rats [38]. However, subsequent studies in adult female rats or adult male mice found that prolonged consumption of diets with inadequate n-3 PUFAs caused a decrease in the percentage of DHA of the mature brain, suggesting possible sex and/or species differences in the regulation of brain fatty acid composition [39, 40].

Modulation of Maternal n-3 PUFA Status by Pregnancy and Lactation

As the primary source of nutrition for their offspring, pregnant and nursing females that do not have adequate diets can become depleted of key nutrients, including DHA [41–44]. Clinical studies in postpartum women show that the concentration of DHA in plasma decreased by as much as 50 % after a single pregnancy and did not return to prepregnancy levels at 26 weeks after giving birth [45–49]. Additional pregnancies resulted in further decreases in the DHA concentrations in maternal plasma and in breast milk [48, 50]. In agreement with these clinical studies, studies in animals found that the percentage of DHA in erythrocytes and liver of female rats decreases after pregnancy and lactation by an amount similar to that observed in humans even when the rats are fed a diet designed specifically for reproduction [51]. An even greater decrease in the DHA content of these tissues occurred when the animals consumed a diet low in n-3 PUFAs, demonstrating that diet and reproductive status interact to affect the DHA content of maternal peripheral tissues [51].

How pregnancy and lactation affect the fatty acid status of the brain has not been examined in humans; however, studies in animals show that nutrition and reproductive status also interact to affect the brain fatty acid composition. Notably, although the DHA content in the brain of female rats was not altered after producing multiple litters when the animals consumed a diet designed for reproduction, the levels of DHA in the brains of female rats that were fed a diet containing low levels of n-3 PUFAs were reduced by about 25 % after gestating and nursing a single litter [39]. This finding demonstrates, that although less affected than peripheral tissues, the DHA content in the brain is susceptible to depletion when the physiological demands of pregnancy and lactation are combined with inadequate dietary n-3 PUFAs. Furthermore, the magnitude of the decrease in DHA content of reproducing females was greatest in brain regions involved in cognition and mood such as the frontal cortex and temporal lobe [52], where many neurobiological alterations are observed in depressed patients. These reproduction- and diet-induced changes in the fatty acid composition of the brain were reversed by subsequent dietary supplementation with DHA [53]; however, it remains to be determined if restoration of brain fatty acid composition after a decrease in DHA content in brain reverses the neurobiological effect observed in these animals.

n-3 PUFA Status in Depression

Non-puerperal depression. Low dietary or tissue n-3 PUFA status, especially low DHA, is associated with major depressive disorder. A number of diet analysis studies support a relationship between higher incidence of depressive symptoms and lower intake of DHA and other n-3 PUFAs [54–61], though another study in adolescents did not [62]. Studies of the fatty acid compositions of serum, plasma, erythrocytes, or adipose tissue also indicate an association between lower tissue levels of n-3 PUFAs, particularly DHA, or increased ratio of n-6 to n-3 PUFAs, and depressive symptoms [63–77]. Another study found no relationship between erythrocyte DHA level and depression [78]; however, the relationship was supported by a meta-analysis [79] and improvement in depressive symptoms was associated with an increase in erythrocyte DHA levels in depressed patients treated with an n-3 PUFA supplement [80].

Decreased brain DHA levels have also been detected postmortem in studies of individuals that suffered from depression. Of note, tissue DHA levels were lower in several brain regions of individuals with diagnosed major depressive

disorder including the orbitofrontal cortex and cingulate cortex, but not in the entorhinal cortex or the amygdala [81–84]. DHA levels were also lower in the prefrontal cortex of individuals dying from suicide, at least some of whom likely suffered from depression, than in controls [85]. Expression of genes such as FADS1, which encode enzymes involved in the biosynthesis of long-chain PUFAs including DHA and arachidonic acid from their nutritionally essential precursors α-linolenic acid and linoleic acid, respectively, was also lower in suicide completers than in controls [86]. Other studies, however, failed to find alterations in the abundance of any n-3 PUFAs in the brains of suicide completers, even in those diagnosed with major depression [87, 88]. Interestingly, particularly in light of greater tendency of women to develop depression [89–91], in some studies, the association of low n-3 PUFA status with depression was greater in women than in men or was significant only in women [81, 92, 93].

Postpartum depression. As with major depression, the preponderance of epidemiologic studies of n-3 PUFA consumption supports an association of low intake and/or tissue levels with postpartum depression. A diet analysis in 23 countries worldwide found that greater intakes of fish, a major dietary source of long-chain n-3 PUFAs, resulted in higher DHA concentrations in the breast milk of postpartum women and was associated with lower prevalence of postpartum depression [94]. Similarly, an observational diet study found higher prevalence of postpartum depression in Brazilian women who consumed diets with at least 9 times the amount of n-6 PUFAs as n-3 PUFAs [95]. Studies of women in England and Canada also found associations between low consumption and/or tissue levels of n-3 PUFAs and the development of depression during pregnancy [96–100]. However, another study in Australian women failed to find a relationship between risk of postpartum depression and the quantities of n-3 PUFAs consumed [101].

Although brain fatty acid composition has not been determined in women with postpartum depression, a number of reports on the fatty acid composition of maternal peripheral tissues indicate the association between the disorder and low n-3 PUFA status. Notably, the levels of DHA, or the ratio of DHA to n-6 DPA, were lower in postpartum women experiencing depressive symptoms than in those who were not [99, 102]. Similarly, women who had lower serum or erythrocyte DHA levels during late pregnancy or at the time of delivery were more likely to become depressed in the postpartum period [103, 104]; however, this finding was not replicated in other studies [105–108], and another study, in which blood samples were collected at both pre- and postnatal time points, found only a weak association [109]. Risk of developing postpartum depression was also higher in women with who had undergone multiple pregnancies or waited less than 24 months between pregnancies [10, 110], which increases the likelihood of decreased tissue DHA content [45–49]. In addition, postpartum depression was associated with a single nucleotide polymorphism in the FADS1/FADS2 gene cluster that results in reduced long-chain PUFA biosynthesis [111]. Consistent with the association of low tissue n-3 PUFA status with this illness, women with this form of the FADS1/FADS2 gene had lower breast milk DHA concentrations even if they consumed fish or fish oil, a source of DHA and EPA [112].

Clinical Trials with n-3 PUFAs in Depression

Non-puerperal depression. A number of randomized controlled trials of various n-3 long-chain PUFA preparations have been performed in depressed patients (Table 22.1). Many of these studies found that eicosapentaenoic acid (EPA), the biologically active biosynthetic precursor of DHA, combinations of EPA and DHA, or fish oil reduced depressive symptoms when given in combination with antidepressant drugs [113–115]. Administration of various formulations of n-3 long-chain PUFAs without concurrent treatment with antidepressant drugs also decreased depressive symptoms at least at certain doses or in subsets of subjects [116–125], even in selective serotonin reuptake inhibitor (SSRI)-resistant patients [77]. Similarly, depressive symptoms in patients with Parkinson's disease improved after treatment with fish oil [126]. In addition, ethyl-EPA, a synthetic derivative of EPA, was as effective as the SSRI fluoxetine in improving depressive symptoms, and the combination of ethyl-EPA with fluoxetine resulted in greater reduction in depression score than fluoxetine alone [119]. Treatment with DHA alone also reduced depressive symptoms, at least at some doses, in a dose-ranging study [120]. However, other randomized controlled trials using fish oil, combinations of DHA and EPA, or DHA alone failed to find improvement in depressive symptoms [125, 127–131]. Likewise, a study treating patients with EPA failed to detect antidepressant effects for the fatty acid alone or when used in addition to antidepressant medication [132]. Despite the mixed results from these clinical trials, meta-analyses and other post hoc evaluation of these data generally support the beneficial effects of n-3 PUFAs in depression, particularly for EPA [133–139], although another meta-analysis suggests that this apparent positive finding may be the result of publication bias [140]. The basis for the apparent superior efficacy of EPA over DHA in depression remains to be determined.

Depression during pregnancy and the postpartum period. The effects of n-3 PUFAs on mood outcomes in pregnant and postpartum women have been assessed in several studies (Table 22.2). Although positive findings have not been reported in the randomized controlled trials, a systematic

Table 22.1 Randomized controlled trials of n-3 PUFAs in non-puerperal depression

Study	Disorder	Population[a]	Intervention	Treatment groups	n[b]	Dose	Duration	Major finding
Nemets et al. [114]	Major depressive disorder	Israel, 85 % female	Add-on to current antidepressant	Ethyl-EPA Placebo (not stated)	10 10	2 g/day	4 weeks	Improvement in HAM-D score over placebo
Peet and Horrobin [115]	Major depressive disorder	UK, 84 % female	Add-on to current antidepressant	Ethyl-EPA 1 g/kg 2 g/kg 4 g/kg Placebo (liquid paraffin)	17 18 17 14	1, 2, or 4 g/day	12 weeks	Improvement in HAM-D score over placebo at 1 mg/kg. No effect at 2 or 4 mg/kg
Su et al. [113]	Major depressive disorder	Taiwan, 82 % female	Add-on to current antidepressant	Fish oil Placebo (olive oil ethyl esters)	12 10	9.6 g/day (4.4 g EPA + 2.2 g DHA)	8 weeks	Improvement in HAM-D score over placebo
Marangell et al. [127]	Major depressive disorder	USA, 80 % female	Monotherapy	DHA Placebo (not stated)	18 7	2 g/day	6 weeks	No effect of treatment on HAM-D, CGI or MADRS scores over placebo
Silvers et al. [128]	Depression	New Zealand, 53 % female	Add-on to current antidepressant	Fish oil Placebo (olive oil)	40 37	8 g/day (0.6 g EPA + 2.4 g DHA)	12 weeks	Improvements in HAM-D and BDI scores were greater than, but not significantly different from placebo
Nemets et al. [116]	Childhood depression	Israel, gender ratio not stated, 6–12 years old	Monotherapy	EPA + DPA Placebo (olive or safflower oil)	10 10	1 g/day (400 mg EPA + 200 mg DHA)	16 weeks	Improvement in CDRS score over placebo
Grenyer et al. [129]	Major depressive disorder	Australia, 74 % female	Add-on to current antidepressant (74 % of subjects) or Monotherapy	Fish oil, n = 32 Placebo (olive oil)	32 28	8 g/day (0.6 g EPA + 2.2 g DHA	16 weeks	No effect of treatment on BDI score over placebo
da Silva et al. [126]	Depression in Parkinson's disease	Brazil, gender ratio not stated	Monotherapy or add-on to current antidepressant	Fish oil only Fish oil + antidepressant Placebo (mineral oil) Placebo + antidepressant	6 8 7 8	720 mg EPA + 480 mg DHA/day	12 weeks	Improvement in MADRS score compared to placebo or placebo + antidepressant.
Rogers et al. [130]	Mild-to-moderate depression	UK, gender ratio not stated	Monotherapy	EPA + DHA, Placebo (olive oil with 7.5 mg mixed tocopherols)	96 94	630 mg EPA + 850 mg DHA/day	12 weeks	No effect of treatment on DASS or BDI scores over placebo
Lucas et al. [117]	Psychological distress and depressive symptoms	Canada, 100 % female, 40–55 years old	Monotherapy	Ethyl-EPA Placebo (sunflower oil)	59 61	1.5 g/day (1.05 g EPA + 0.15 g DHA)	8 weeks	Improvement in HAM-D score was greater than placebo only for subjects not meeting criteria for major depression

(continued)

Table 22.1 (continued)

Study	Disorder	Population[a]	Intervention	Treatment groups	n[b]	Dose	Duration	Major finding
Mischoulon et al. [238]	Major depressive disorder	USA, 65 % female	Monotherapy	Ethyl-EPA Placebo (paraffin oil with 0.2 % α-tocopherol)	11	1 g/day	8 weeks	Improvement in HAM-D score was greater than, but not significantly different from placebo
Rondanelli et al. [121, 123]	Depression in elderly	Italy, 100 % female, 65–95 years old	Monotherapy	n-3 LC-PUFA Placebo (paraffin oil)	22 24	1.67 g EPA + 0.83 g DHA/day	8 weeks	Improvement in GDS score over placebo
Bot et al. [132]	Major depression in diabetes mellitus	The Netherlands, 52 % female	Add-on to current antidepressant	Ethyl-EPA Placebo (rapeseed oil with medium chain triglycerides)	12 12	1 g/day	12 weeks	No effect of treatment on MADRS score over placebo
Lespérance et al. [122]	Major depression	Canada, 69 % female	Monotherapy or add-on to current antidepressant	Omega-3 Placebo (sunflower oil)	218 214	1,050 mg EPA + 150 mg DHA/day	8 weeks	Improvement in MADRS score over placebo in patients without comorbid anxiety disorders
Sinn et al. [124]	Mild cognitive impairment	Australia, 18-53 % female depending on group, >65 years old	Monotherapy	EPA-rich fish oil DHA-rich fish oil Placebo (safflower oil	13 16 11	1.67 g EPA + 0.16 g DHA/day 1.55 g DHA + 0.40 g EPA/day	6 months	Improvement in GDS scores in EPA and DHA groups over placebo
Mozaffari-Khosravi et al. [125]	Mild-to-moderate depression	Iran, 61 % female	Add-on to current antidepressant	EPA DHA Placebo (coconut oil)	20 21 21	1 g/day 1 g/day	12 weeks	Improvement in HAM-D score in the EPA group over DHA or placebo
Michoulon et al. [131]	Major depression	USA, 53 % female	Monotherapy	EPA-rich oil DHA-rich oil Placebo (soybean oil)	51 50 53	1060 mg EPA + 274 mg DHA/day 900 mg DHA + 180 mg EPA/day	8 weeks	No effect of either treatment on QIDS-SR-16 or CGI scores over placebo

[a]Percentage of females in study cohort is averaged across groups unless groups were notably different
[b]Sample size at end of study
BDI Beck Depression Inventory, *CDRS* Childhood Depression Rating Scale, *CGI* Clinical Global Impression, *DASS* Depression, Anxiety, and Stress scales, *GDS* Geriatric Depression Scale, *HAM-D* Hamilton Depression Rating Scale, *MADRS* Montgomery–Åsberg Depression Rating Scale, *QIDS-SR-16* 16 Item Quick Inventory of Depressive Symptomatology Self-Report

Table 22.2 Randomized controlled clinical trials of n-3 PUFA for the prevention of treatment of depression in pregnant and postpartum women

Study	Population	Treatment period	Baseline mood status	Other	Treatment groups	n[a]	Dose	Duration	Major findings
Llorente et al. [146]	USA	Postpartum	Healthy	Breastfeeding	DHASCO Placebo (not stated)	44 45	200 DHA mg/day	4 months beginning within a week of delivery	No effect of treatment on BDI score 4-month postpartum over placebo
Doornbos et al. [147]	The Netherlands	Pregnancy or postpartum	Healthy	Vitamin and mineral supplement	DHA DHA + AA, Placebo (soy oil)	42 41 36	220 mg/day 220 mg/day	Week 16 of pregnancy through 3-month postpartum	No effect of either treatment on EPDS, HAM-D or MADRS scores over placebo
Krauss-Etschmann et al. [150]	Germany, Spain, Hungary	Pregnancy	Healthy	Vitamin and mineral supplement	Fish oil Folate Both Placebo (milk-based vehicle)	69 65 64 72	500 mg DHA + 150 mg EPA/day 400 μg/day	Week 22 of pregnancy through delivery	No effect of any treatment on EPDS score 1 at delivery over placebo
Mattes et al. [100]	Australia	Pregnancy	Healthy	–	Fish oil Placebo (olive oil)	36 37	4 g/day (56 % DHA, 27.7 % EPA)	Week 20 of pregnancy through delivery	No effect of treatment on BDI score during first-week postpartum over placebo
Makrides et al. [148]	Australia	Pregnancy or postpartum	Healthy	–	Fish oil Placebo (vegetable oil)	1197 1202	800 mg DHA +100 mg EPA/day	< 21 gestation through birth	No effect of treatment on the percentage of women with EPDS > 12 at 6-week and 6-month postpartum over placebo
Mozurkewich et al. [149]	USA	Pregnancy or postpartum	Past history of depression, EDPS score 9-19 (at risk of depression or mildly depressed)	–	EPA-rich fish oil DHA-rich fish oil Placebo (soy oil)	39 38 41	1060 mg EPA + 274 mg DHA/day 900 mg DHA + 180 mg EPA/day	< 20 gestation through 6-week postpartum	No effect of either treatment on BDI score 4-month postpartum over placebo
Freeman et al. [143]	USA	Pregnancy or postpartum	Major depression	Supportive psychotherapy	DHA + EPA Placebo (corn oil with 1 % fish oil)	31 28	0.8 g DHA + 1.1 g EPA/day	8 weeks	No effect of treatment on EPDS and HAM-D scores over placebo
Su et al. [118]	Taiwan	Pregnancy	Major depression	–	DHA + EPA Placebo (olive oil ethyl esters)	17 16	1.2 g DHA + 2.2 g EPA/day	8 weeks	Improvements in HAM-D, EPDS, and BDI scores, and higher response and remission rates
Rees et al. [144]	Australia	Pregnancy or postpartum	Major depression	–	Fish oil Placebo (Sunola oil)	13 13	6 g (27.3 % DHA, 6.9 % EPA, 80 mg vitamin E)/day	6 weeks during pregnancy or postpartum	No effect of treatment on EPDS, HAM-D, or MADRS scores over placebo

[a]Sample size at end of study

BDI Beck Depression Inventory, *DHASCO* DHA single cell oil, *HAM-D* Hamilton Depression Rating Scale, *MADRS* Montgomery–Åsberg Depression Rating Scale

review of these studies done in 2010 found them largely inconclusive due to issues such as small sample size, not being designed specifically to assess postpartum depression and/or not focusing on women with or at risk of postpartum depression [141]. One very large study and another in at-risk women have been done subsequently to that analysis; however, the therapeutic potential of n-3 PUFAs in this population still remains to be definitively determined (see below).

Studies of n-3 PUFA treatments specifically in postpartum depression have had mixed results. In a dose-ranging pilot study that was not placebo-controlled, treatment of women with postpartum depression for 8 weeks with DHA and EPA (1.5:1; 0.5, 1.4, or 2.8 g/day) improved depressive symptoms [142]. Randomized controlled trials in women with postpartum depression, however, failed to find an effect of n-3 PUFA treatments [143, 144].

Studies assessing the ability of n-3 PUFAs to prevent postpartum depression have yielded negative findings. An open-label study in which women were treated with fish oil (2960 mg/day; EPA:DPA, 1.4:1) from week 34 to 36 of pregnancy through 12 weeks after birth found no effect of treatment on the development of depressive symptoms [145]. Likewise, several randomized controlled trials of the effects of various n-3 PUFA preparations administered during, and sometimes also after, pregnancy found no difference in the incidence of postpartum depression from placebo [146–148], even in women considered at risk of postpartum depression and in whom the intervention produced an increase serum DHA level [149]. Furthermore, the negative finding in the study by Makrides et al. [148] cannot be attributed to inadequate statistical power as the sample sizes were over 1000 women per group. However, the authors report that the rate of depressive symptoms in the control group was lower than expected, and unlike numerous previous studies [35–37], the study also failed to find beneficial effects of DHA treatment on neurodevelopmental outcomes in the offspring.

Although not a distinct diagnostic category, pregnancy is increasingly recognized as a time of increased risk of mood disorders in addition to the postpartum period. In one randomized controlled trial, treatment of pregnant women with major depression with an n-3 PUFA supplement for 8 weeks increased erythrocyte DHA levels and resulted in lower depression scores, as well as higher response and remission rates [118]. In another study of pregnant women with depression, however, n-3 PUFAs failed to improve symptoms [144]. Similarly, in studies of otherwise healthy pregnant women, mood scores were not different between those treated with n-3 PUFAs or placebo [100, 150], although serum DHA and EPA concentration were higher in n-3 PUFA-treated groups [150].

Effects of n-3 PUFAs on Depression-Related Neurobiology and Behavior

Major findings in depression include alterations in the monoamine neurotransmitter systems, dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis, and decreased expression of brain-derived neurotrophic factor (BDNF) in the hippocampus. Neuroinflammation is also becoming increasingly recognized a contributor to the pathology of depression. The effects of variation in brain n-3 PUFA levels on these systems are discussed, as well the effects of n-3 PUFAs on rodent behaviors relevant to depression.

The preponderance of these mechanistic studies were done in animal models that vary widely with respect to the magnitude of the alterations in brain n-3 PUFA content, which is a function of the specific fatty acid composition the diet used and duration of treatment, as well as in the point in the life span when those alterations occurred. Accordingly, differing neurobiological outcomes may be anticipated. Only a few studies include female subjects and even fewer specifically model the postpartum period. Nevertheless, a number of consistent themes emerge that support a neurobiological basis for n-3 PUFAs in the pathophysiology of postpartum depression and other depressive disorders.

Serotonergic neurotransmission. Decreased concentrations of serotonin in brain regions such as the brainstem are notable findings in postmortem depressives and suicide completers [151–153]. Consistent with a role for n-3 PUFAs in depression, adult female rats with a diet-induced decrease in brain DHA of 25 % had lower concentrations of serotonin in the frontal cortex [154]. Likewise, male rats treated from birth with a diet that decreased brain DHA by 60 % exhibited increased serotonin turnover in the prefrontal cortex, as well as lower midbrain levels of tryptophan hydroxylase, the rate-limiting enzyme involved in the biosynthesis of serotonin [155, 156]. Moreover, in rats with low brain DHA, subsequently feeding a diet containing the n-3 PUFA α-linoleic acid reversed the effect on serotonin turnover [156]. Consistent with the effects of low dietary n-3 PUFAs, adult rats fed a diet supplemented with fish oil or treated with other preparations that increased brain DHA levels, exhibited increased serotonin concentrations in whole brain, the frontal cortex, and/or the hippocampus [157–160]. A diet supplemented with n-3 PUFAs also reversed the decrease in brain serotonin levels in mice produced by exposure to unpredictable chronic mild stress, a rodent model of depression [161]. However, serotonin concentrations were not altered in any brain region, including the frontal cortex, in postpartum female rats with a 25 % decrease in brain DHA resulting from consuming inadequate n-3 PUFAs during pregnancy and lactation [154].

Another major finding in postmortem depressives and suicide completers is increased densities of serotonin

receptors, notably 5-HT_{1A} and 5-HT_{2A}, in the prefrontal cortex, that down-regulate after treatment with antidepressant drugs [162–166]. Similar to these findings in depression, higher densities of 5-HT_{2A} receptors were observed in the frontal cortex of rats with a 70 % reduction in brain DHA resulting from being maintained for multiple generations on an n-3 PUFA-deficient diet [167, 168]. However, in adult virgin or postpartum rats, female rats with a 25 % decrease in brain DHA, the densities of cortical 5-HT_{1A} or 5-HT_{2A} receptors were not different from those of female rats with normal brain DHA levels [154]. Interestingly, the density of hippocampal 5-HT_{1A} receptors in postpartum female rats with decreased brain DHA was higher than that in postpartum females with normal brain DHA levels or in virgin females with normal or decreased brain DHA levels, indicating an interaction of brain DHA content with reproductive status [154]. Although studies of hippocampal 5-HT_{1A} receptors in non-puerperal depression indicate that densities of this receptor were either not altered or were decreased [169–172], several studies in male rats showed that the antidepressant-like effects of fish oil on hippocampal BDNF expression were blocked by a 5-HT_{1A} receptor antagonist, suggesting a role for this receptor in the BDNF-modulating effects of n-3 PUFAs [159, 160].

Noradrenergic neurotransmission. Notable alterations of the noradrenergic system in depression include increased density of α_1, α_2, and β-adrenergic receptors, which down-regulate after treatment with tricyclic antidepressants [162, 173, 174]. The few findings on the effects of n-3 PUFAs on the noradrenergic system include DHA-induced increases basal [^{3}H]-norepinephrine release and the density of β-receptors in cell culture systems [175, 176]. In animal studies, rats raised from conception on a diet containing inadequate n-3 PUFAs had lower concentrations of norepinephrine in the cortex, hippocampus, and striatum [177]. In contrast, regional norepinephrine concentrations were not altered in either adult virgin or postpartum female rats with a 25 % decrease in brain DHA, or in adult rats with a 70 % decrease in brain DHA resulting from multigenerational treatment with an n-3 PUFA-deficient diet [154, 168]. Effects of in vivo manipulations of n-3 PUFAs on noradrenergic receptors are yet to be determined.

Dopaminergic neurotransmission. Hypoactivity of the mesolimbic dopamine projection leads to decreased engagement in reward-oriented behaviors in animals and is hypothesized to contribute to the anhedonia and decreased motivation observed in depression [178]. Consistent with this hypothesis, lower levels of D_2 dopamine receptors or mRNA have been found in the ventral striatum of depressed women, as well as in several rat models of the disease [179–183]. Similar to these findings, postpartum female rats with a 25 % decrease in brain DHA content had lower densities of D_2 dopamine receptors in the nucleus accumbens, a target of the mesolimbic dopamine projection involved in reinforcement and reward [184]. Virgin females with a similar decrease in brain DHA content exhibited a smaller, near-significant decrease in the density of this receptor, suggesting that the interaction of decreased brain DHA and postpartum status modulates D_2 receptor density [184].

In addition to these effects, manipulation of brain DHA content can produce a number of other effects on the brain dopamine systems. Diet treatments in adult rats that increased brain DHA content increased brain dopamine concentration [158]. Likewise, in a clinical study of patients with major depressive disorder, serum concentrations of prolactin, which is inhibited by dopamine and thus serves as a marker of dopaminergic function, were negatively correlated with plasma DHA levels [185]. Large decreases in brain DHA content (70 %) in a multigenerational model of n-3 PUFA deficiency resulted in many dopaminergic effects including altered dopamine receptor expression in the nucleus accumbens and frontal cortex, as well as increased basal dopamine release, decreased vesicular monoamine amine transporter ($VMAT_2$) density, and decreased tyramine-stimulated dopamine release [168, 186–190]. However, in adult rats with smaller decreases in brain DHA content resulting from the treatment with reduced dietary n-3 PUFAs throughout the life span, no alterations in D_1 or D_2 receptor density or the concentrations of dopamine in the nucleus accumbens, frontal cortex, or striatum were observed [191]. The high variability of the dopaminergic alterations observed in various models in which brain n-3 PUFA status has been manipulated suggests that this system may be affected in different ways depending on the timing and magnitude of the change in brain DHA status, with only some changes being similar to those observed in depression.

HPA axis function. A number of abnormalities in the HPA system are noted in depression including increased basal cortisol levels and impaired feedback regulation [192]. Altered HPA axis function induced by a change in n-3 PUFA status has also been found in several animal studies, particularly during the postpartum period. In one study, postpartum female rats with a 25 % decrease in brain DHA content had higher stress-induced serum corticosterone concentrations, as well as greater relative increases in corticosterone secretion over basal levels, compared to postpartum females that had been fed a diet that maintained normal brain DHA levels [154]. In that study, a less pronounced increase in stress-induced corticosterone secretion was also observed in virgin female rats with decreased brain DHA relative to virgins with normal brain DHA levels, suggesting that postpartum status augments the effects of decreased brain DHA [154]. In agreement with these findings, treatment with fish oil in ovariectomized females that

were subjected to a hormone regimen that simulated the postpartum state resulted in lower corticosterone concentrations in both the plasma and the hippocampus [193].

Studies in humans and male animals also support a role for brain n-3 PUFA status in the regulation of the HPA axis. In a clinical study, abstinent alcoholics treated with fish oil supplements for 3 weeks had reduced basal cortisol levels and reported lower anxiety and stress levels than those treated with a placebo [194]. Likewise, in animal studies, an n-3 PUFA-enriched diet decreased the stress-like behavioral responses in rats treated with interleukin (IL)-1, an inflammatory cytokine that increases corticosterone levels [195]. Similarly, the effects of stressors such as tail suspension and restraint stress on stressed plasma corticosterone levels and behavior were reduced in rats fed diets supplemented with n-3 PUFAs for 2–3 months [196, 197]. Consistent with these effects of n-3 PUFA supplementation, a diet low in n-3 PUFAs resulted in higher serum corticosterone concentrations after restraint stress in male rats fed the diet from conception; however, this effect was not seem in male rats fed the deficient diet only between postnatal days 21 and 70, suggesting that the effects of n-3 PUFA deficiency on HPA axis function have a critical developmental window, at least in males [198].

In addition to affecting acute responses to stress, low dietary and/or tissue DHA levels also affect long-term behavioral responses. In a study in rats subjected to a diet-induced decrease in brain DHA content of 70 % and/or maternal separation during the preweaning period, the combined treatment resulted in greater reaction to novelty in the open field, increased anxiety in the classical fear conditioning and elevated plus maze tests, and increased sucrose consumption at adulthood compared to rats subjected to maternal separation without the low n-3 PUFA diet [199]. In another study, mice with a 50 % decrease in brain DHA content, resulting from multigenerational treatment with an n-3 PUFA-deficient diet, exhibited greater anxiety in the novelty suppressed feeding test after being subjected to the stress of individual housing than mice fed a control diet [200]. Similarly, rats fed a diet supplemented with n-3 PUFAs exhibited less weight loss, a smaller reduction in grooming, and lower peak plasma corticosterone levels after chronic restraint stress than those that consumed control or n-3 PUFA-deficient diets [201].

Hippocampal BDNF expression. The hippocampus is one of a few regions in the mature brain that exhibits neurogenesis, a process supported by BDNF [202, 203]. Decreased expression of BDNF in the hippocampus is strongly associated with depression, and antidepressant drug-induced increases in BDNF appear to contribute to their therapeutic mechanism [204].

In animal studies, manipulations that increase or decrease dietary and/or tissue n-3 PUFAs produce similar changes in BDNF in the hippocampus. Most relevant to postpartum depression, postpartum female rats with decreased brain DHA, had lower concentrations of both BDNF mRNA and peptide in the hippocampus of a magnitude similar to that reported in suicide completers [154, 205, 206]. Hippocampal BDNF mRNA levels were also decreased in virgin female rats with a diet-induced decrease in brain DHA, but the concomitant decrease in BNDF peptide was not quite significant [154]. Likewise, reduced serum and hippocampal concentrations of BDNF peptide were observed in male rats maintained on an n-3 PUFA-deficient diet of 15 weeks beginning on postnatal day 35 [207].

Higher levels on n-3 PUFAs likewise resulted in greater expression of hippocampal BDNF. In clinical studies, higher consumption of n-3 PUFAs correlated with serum levels of BDNF in adolescents [208]. Likewise, treatment with n-3 PUFA-enriched diets or α-linoleic acid injections increased BDNF expression in the hippocampus of mice or rats [157, 159, 209–212]. Consistent with those observations, a diet enriched with DHA increased concentrations of calmodulin kinase II and activated Akt, which are involved in BDNF signaling [213]. Dietary supplementation with n-3 PUFAs also resulted in increased levels of mediators of the increases in BDNF induced by antidepressant drugs, such as cAMP response element-binding protein (CREB) [214]. Increased hippocampal volume and neurogenesis, which are supported by BDNF, have also been observed in mice or rats treated with n-3-PUFAs, as well as in *fat-1* transgenic mice that are able to convert n-6 PUFAs into n-3 PUFAs, thus eliminating the requirement for n-3 PUFAs in the diet [211, 215, 216].

Neuroinflammation. Notable neuroinflammatory findings in depression include higher levels NFκB-regulated inflammatory mediators such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-1β, and IL-6 [217]. N-3 PUFAs mitigate inflammation in a variety of ways that could contribute to the prevention or reversal of depression [218]. For example, N-3 PUFAs inhibited the NFκB-mediated inflammation cascade through actions at PPARs and the toll-like 4 receptor [219, 220]. In BV-2 microglia, DHA reduced IFN-γ-induced expression of nitric oxide synthase, cyclo-oxygenase-2, IL-6, and TNF-α, [221]. In addition, DHA and EPA can be metabolized into the D- and E-series resolvins, respectively, which modulate the duration and magnitude of the inflammatory response [222]. Other DHA derivatives include neuroprotectin D1, which reduced the production of TNF-α and IFN-γ in activated T cells, and maresin R1, which blocked capsaicin-induced inward current in neurons [28, 223, 224].

To date, only a few studies have examined the effects of n-3 PUFAs on neuroinflammatory responses in vivo. In the most relevant study to postpartum depression, ovariectomized females subjected to a hormone regimen to simulate the postpartum state and also treated with fish oil exhibited

decreased plasma concentrations of IL-1β, IFN-γ, and TNF-α compared to similarly prepared females treated with vehicle [193]. Likewise, male rats that were fed an n-3 PUFA-deficient diet from birth had higher plasma concentrations of IL-6, TNF-α, and C-reactive protein, which were reversed by feeding a diet containing α-linoleic acid [156]. However, in a study in aged mice, expression of IL-6 and IL-10 was not affected by dietary n-3 PUFA content [225].

Depression-related behaviors. The forced swim test, a behavioral despair model that assesses the behavior of rats or mice when placed in a cylindrical tank of cool water that is sufficiently deep that the animal cannot balance on the bottom or escape, is a highly validated drug screen for antidepressant activity. Drug treatments that decrease the time spent in floating immobile, or increase the latency to immobility, are likely to have antidepressant activity in humans [226, 227].

A variety of diet treatments that manipulate n-3 PUFA levels alter performance in the forced swim test. Of greatest relevance to postpartum depression, postpartum female rats with a 25 % decrease in brain DHA content had shorter latencies to immobility compared to postpartum females with normal brain DHA levels [154]. Consistent with that finding, ovariectomized female rats treated with a hormone regimen to model the postpartum state also exhibited less immobility in the forced swim test after being treated with fish oil [193]. Studies in a number of other animal models have also found that treatments that decrease n-3 PUFA levels produced effects in the forced swim test that are opposite to those associated with antidepressant efficacy [198, 225, 228]. Similarly, treatments that result in higher levels of n-3 PUFA levels produced less immobility in the test consistent with the prediction of antidepressant activity [157, 160, 196, 197, 210, 211, 214, 229–234]. Treatment with fish oil also potentiated the effects of the antidepressant drugs fluoxetine and mirtazepine in the test [233]. In addition, the increase in immobility resulting from olfactory bulbectomy, a rodent model of depression, was reversed by the treatment with fish oil [235]. Some studies, however, failed to find an effect of reduced n-3 PUFA status, notably in adult male rats that had been maintained on an n-3 PUFA-deficient diet for multiple generations and in adult virgin female rats with a 25 % decrease in brain DHA [154, 199].

Effects of n-3 PUFA treatments consistent with potential antidepressant activity have also been found in several other behavioral paradigms that are predictive of antidepressant efficacy. In the tail suspension test, another behavioral despair model, animals with higher n-3 PUFA levels exhibited less immobility, consistent with findings in the forced swim test [197, 210]. Likewise, treatments involving decreased n-3 PUFAs resulted in anhedonia, a symptom of depression, in some studies [236, 237], though another did not find this effect [199].

Conclusion

Multiple lines of evidence suggest that reduced abundance of DHA, and perhaps other n-3 PUFAs, may represent a factor that, when combined with genetic and environmental factors, creates a susceptibility to the development of depression. Although relatively little is known about the brain DHA status of women with postpartum depression, epidemiologic data and findings in animals support the association of lower levels of brain DHA content, and perhaps also circulating n-3 PUFAs levels, and physiological and behavioral changes associated with depression. Despite the widely varying animal models, a number of consistent findings emerge that provide mechanisms through which a change in n-3 PUFA status could play a part in the pathobiology of depressive illness, with higher levels leading to phenotypes that are less consistent with depression in humans, and lower levels leading to phenotypes that are more similar to depression. Moreover, animal studies show that the effects of decreased brain DHA content interact with reproductive status to produce a greater number of depression-associated alterations, and effects of greater magnitude, in postpartum females than in virgin females. The low n-3 PUFA content of the typical North American diet and genetic polymorphisms that result in reduced abilities to biosynthesize and/or utilize long-chain PUFA may put some individuals at risk. When combined with the physiological demands of pregnancy and lactation, reduced levels of DHA and perhaps other n-3 PUFAs, and/or the compensatory increase in incorporation of n-6 PUFAs, may lead to particular vulnerability in postpartum women. However, although the majority of clinical trials of n-3 PUFAs in non-puerperal depression have shown promising effects, this has not been the case in postpartum depression suggesting the possibility of differing pathophysiologies and perhaps also the necessity of differing treatment strategies for these disorders. Future studies must assess whether the neurobiological effects of reproduction-associated changes in maternal n-3 PUFA status are reversible and thus determine whether treatments should target maintenance of maternal PUFA status or reversing deficits. Optimal formulations, doses, and duration of treatment must also be determined.

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Influence of Omega-3 Fatty Acids on Bone Turnover

23

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Introduction

Bone Turnover

Bone is a metabolically active tissue that undergoes continuous remodeling to cope with the body's Ca and P requirements and to repair microscopic damage in a dynamic process where osteoblasts are responsible for bone formation and osteoclasts for its resorption. Bone resorption and formation are tightly coupled with each other, so that the amount of bone removed is always equal to the amount of newly formed bone, and this balance is achieved and regulated through the action of various systemic hormones (e.g., PTH, vitamin D, other steroid hormones) and local mediators (e.g., cytokines, growth factors). In contrast, somatic growth, aging, metabolic bone diseases, states of increased or decreased mobility, therapeutic interventions, and many other conditions are characterized by more or less pronounced imbalances in bone turnover. This dynamic process of bone turnover is regulated by controlling osteoblast and osteoclast cell number and activity.

Osteoclasts are the cells that degrade (resorb) bone during normal bone remodeling and in pathologic states in which bone resorption is increased. Osteoclasts form microscopic trenches on the surfaces of bone trabeculae in the spongy bone by secreting hydrochloric acid and proteases, such as cathepsin K, into an extracellular lysosomal compartment beneath a ruffled part of their basal cell membrane to dissolve the mineral and matrix components of bone simultaneously. Precursors of osteoblasts, the cells that form bone, are recruited to these trenches from the adjacent bone marrow stromal cell population and differentiate into osteoblasts, which lay down new matrix and mineralize it [1]. Bone remodeling can be increased in response to many influences, including mechanical strain, cytokines, hormones, and growth and dietary factors. Osteoclastogenesis is in large part regulated by a triad of proteins consisting of a ligand, receptor-activated nuclear kappa-β ligand (RANKL), its receptor, RANK, and a decoy receptor, osteoprotegerin (OPG). RANK is a membrane-bound protein expressed on osteoclast precursors and mature osteoclasts [2]. RANKL exists in both a membrane-bound and soluble form and is produced by a range of cells including osteoblasts and activated T cells. Binding of RANKL to RANK leads to osteoclastogenesis and inhibits osteoclast apoptosis [3]. Osteoblasts are specialized mesenchymal cells that have also a key role in the regulation of bone resorption through RANKL, that links to its receptor, RANK, on the surface of preosteoclast cells, inducing their differentiation and fusion.

On the other hand, osteoblasts are specialized mesenchymal cells that undergo a process of maturation where genes such as core binding factor α-1 (Cbfa-1) and osterix (Osx) play

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a very important role. Moreover, it was found recently that the Wnt/b-catenin pathway plays a part on osteoblast differentiation and proliferation. Osteoblasts secrete OPG that blocks RANK/RANKL interaction by binding to RANKL and, thus, prevents osteoclast differentiation and activation. Therefore, the balance between RANKL and OPG determines the formation and activity of osteoclasts [4, 5].

Oxidative Stress, Inflammation, and Bone Remodeling

Recent evidences in the scientific literature suggest a link between bone metabolism and redox balance regulation, indicating that reactive oxygen species (ROS) play a key role in osteoporosis by inhibiting osteoblast generation. ROS are also involved in cartilage homeostasis and degradation, leading to the development of osteoarthritis. Rheumatoid arthritis is an autoimmune disease characterized by chronic inflammation leading to joint destruction, and it has been suggested that the level of ROS in patients with rheumatoid arthritis is higher than in healthy subjects. Recent studies have also reported that ROS could be a key modulator of bone cell function and play a role mediating intracellular signaling [6].

At the molecular level, cellular stress response pathways are controlled by four categories of molecules and transcriptional regulators: insulin/IGF-1 signaling, sirtuins, target of rapamycin, and AMP-activated protein kinase-dependent pathways. All these pathways have one molecular target in common, named FoxO. FoxOs play an important role in bone biology by enabling the maintenance of a physiologically appropriate lifespan of mature osteoblasts through their defense activities or via modulating the activity of other transcription factors such as β-catenin [7]. The antioxidant defense provided by FOxOs is overwhelmed by high levels of oxidative stress or oxidative stress-activated pathways that interfere with the activity of FoxOs, or both.

RANKL activates mature osteoclasts and mediates osteoclastogenesis. It acts by binding to its receptor, RANK, stimulating their differentiation into mature osteoclasts. OPG acts as a decoy receptor for RANKL, preventing it from binding to and activating RANK. Abnormalities of the RANK–RANKL–OPG system with an unbalanced increase in RANKL activity have been implicated in the pathogenesis of various skeletal diseases, including osteoporosis and bone disease secondary inflammation. The increased osteoclastic activity may increase the superoxide anion generation and/or inhibit superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities with concomitant bone destruction [8].

Osteoblasts can produce antioxidants, such as glutathione peroxidase (GPx), to protect against ROS, as well as transforming growth factor-ß (TGF-ß), which is implicated in reduction of bone resorption. Bai et al. [9] have reported that intracellular ROS suppressed the differentiation process of osteoblasts, manifested by a reduction of differentiation markers such as type 1 collagen and colony-forming unit osteoprogenitor (CFU-O) formation. Induced oxidative stress is also able to inhibit bone cell differentiation of pre-osteoblastic cell line (MC3T3-E1) and of a marrow stromal cell line (M210B4) that undergoes osteoblastic differentiation. Estrogens decrease oxidative stress and antagonize ROS-induced osteoblast apoptosis and the pro-survival effects of receptor activator of RANKL on osteoclasts. It has been proposed that elucidation of these mechanisms provides a paradigm shift from the "estrogen-centric" account of the pathogenesis of involutional osteoporosis to one in which age-related mechanisms intrinsic to bone are central to the disease process [10, 11].

In response to oxidative stress, FoxOs up-regulate enzymatic scavengers and DNA damage repair genes avoiding the adverse effects of ROS, thereby representing an indispensable homeostatic mechanism for bone health [12]. In addition, FoxOs may control the generation of new osteoblasts from their mesenchymal stem cell progenitors by modulating their proliferation and/or differentiation through their antioxidant properties. Experimental studies have shown a diminution in antioxidant activity in patients with osteoporosis. GPx1 is highly expressed in osteoclasts, and its expression in bone marrow macrophages by RANKL and in osteoclasts by estrogens. Overexpression of GPx in osteoclast progenitors abolishes osteoclast formation and suppresses RANKL-induced NF-kB activation and increased resistance to oxidation [13]. Oxidative stress appeared as an independent risk factor for osteoporosis linked to the increase of superoxide dismutase (SOD)/GPx ratio. In this sense, the imbalance propitiated by greater SOD activity with respect to GPx favors the increase in H_2O_2 levels and consequently greater oxidative stress [11].

Several clinical studies have provided wide evidence to implicate the crucial role of osteoclasts in the pathogenesis of bone erosions in patients with rheumatoid arthritis. The important role of osteoclasts in bone resorption has been found to be also crucial in other inflammatory arthritis. In this sense, Ritchlin et al. [14] reported that patients with psoriatic arthritis, who have elevated serum TNF-α levels, have a significant increase in the osteoclast precursor cell pool within the peripheral blood mononuclear cells populations that in turn correlated with the extent of bone destruction. The demonstration that osteoclasts are largely responsible for focal bone erosions has increased efforts in the researchers to elucidate the exact role played by a number of cytokines and inflammatory mediators that have the capacity to induce the recruitment, differentiation, and activation of these osteoclasts [15].

RANKL plays a role in controlling osteoclastogenesis. Inflamed synovial tissue produces a variety of other cytokines and hormones that can also influence osteoclastogenic activity. These factors include interleukin-1α (IL-1α), IL-1β, TNF-α, IL-6, macrophage colony- stimulating factor (M-CSF), IL-17, and parathyroid hormone-related peptide (PTHrP) [15]. The role of these cytokines in inflammation and bone erosions provides further evidence to a link between immune system activation and bone resorption. Once the function of OPG/RANK/RANKL in bone remodeling was perceived, it was hypothesized that RANKL may be of major pathophysiological importance in the bone and joint destruction observed in inflammatory rheumatoid arthritis. Some studies have provided compelling evidence that activated T cells from the rheumatoid arthritis synovium and synovial fibroblasts produce RANKL. Indeed, it is currently assumed that activated T cells, which play a central role in the pathogenesis of rheumatoid arthritis, may contribute to the osteoclast-mediated bone resorption via RANKL expression [16].

In bone destruction associated with rheumatoid arthritis, IL-17-producing helper T cells (TH17) play a major role by inducing receptor activator of nuclear factor-κB ligand (RANKL). RANKL stimulates osteoclastogenesis through nuclear factor of activated T cells cytoplasmic 1 (NFATc1), which is a crucial regulator of immune response [17]. In addition to cellular interactions via cytokines, the immune and skeletal systems share various molecular pathways, including transcription factors, signaling molecules, and membrane receptors.

Influence of Omega-3 Fatty Acids on Bone Turnover

Beneficial Effects of Omega-3 Fatty Acids on Bone Turnover

Dietary fat has a clear influence on bone health. Long-chain polyunsaturated fatty acids (LC-PUFAs), especially the omega-3 (ω-3) fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are beneficial for bone metabolism. Several studies in humans have reported that LC-PUFAs can increase bone formation, affecting peak bone mass in adolescents and reducing bone loss, because LC-PUFAs reduces inflammatory cytokines, increases calcium absorption, and enhances skeletal calcium levels. EPA and/or DHA treatment has showed inhibition of osteoclastogenesis [18–20] and protection of osteoblastogenesis [21–23]. The cellular mechanisms of action of the LC-PUFAs are complex and involve modulation of fatty acid metabolites such as prostaglandins, resolvins and protectins, cytokines, growth factors, and some other molecular signaling pathways [24].

Many lipid mediators have a critical role in the signaling bone turnover pathway. Immediately after mechanical loading of bone tissue, prostaglandin E_2 (PGE_2) is released by osteocytes and mature osteoblasts [25]. Phospholipase-mediated membrane releases fatty acids (FA), including arachidonic acid (AA, 20:4n-6), the substrate for PGE_2 synthesis; in addition, an increased expression of cyclooxygenase 2 (COX-2), which oxidizes AA to PGE_2, is featured as an early response [26]. PGE_2 promotes osteoclastogenesis by stimulating expression of both RANKL and RANK and inhibiting expression of OPG. PGE_2 also activates the Wnt signaling pathway and promotes Cbfa-1 and insulin-like growth factor 1 (IGF-1) expression, stimulating osteoblastogenesis [27]. PGE_2 therefore is a potent modulator of bone turnover, affecting bone resorption, by stimulating osteoclastogenesis through RANKL. Both processes, formation and resorption, are linked to PGE_2. High levels of PGE_2 suppress osteoblast, and promote differentiation and bone resorption by osteoclasts via RANKL, whereas low levels stimulate bone formation by osteoblasts [24].

There are a good number of studies about PUFA and its impact on bone health, and many of them suggest that DHA may have more potent bioactivity in bone than EPA [19, 28–30]. DHA features more potent anti-inflammatory effects relative to EPA, with marked attenuation of NF-κB activation and TNF-α secretion in macrophages [31–33]. DHA specifically enhances anti-inflammatory IL-10 secretion and reduces the expression of pro-inflammatory M1 macrophages [32]. In addition, DHA is more potent inhibitor of bone resorbing osteoclast formation than EPA [19, 34]. DHA supplementation in rat showed that DHA accumulated in the osteoblast-rich and nerve-abundant periosteum of femur and appears to be a vital constituent of marrow and periosteum of healthy modeling bone [35, 36]. A few studies have also suggested preventive effect of DHA against ovariectomy-induced bone loss in rat [22, 28, 37].

The healthy recommended ratio of omega-6/omega-3 fatty acids is approximately 1:1; however, actual estimations indicate that it is in fact much higher: between 15:1 and 16.7:1. According to evidence, a reduction in the omega-6/omega-3 proportion decreases the risk of suffering cardiovascular pathologies, tumors, and other chronic diseases, including osteoporosis [38]. It is hypothesized that these polyunsaturated fatty acids also act on bone formation, because the products of the metabolism of omega-6 and omega-3 fatty acids can act on precursor cells of osteoblast and adipocytes [39]. A diet rich in omega-6 fatty acids, which rises omega-6/omega-3 ratio, seems to cause not only cardiovascular problems, but also an increase in the adiposity of the bone marrow, by enhancing the adipogenic

differentiation of mesenchymal cells, inhibiting their differentiation into osteoblasts. Consequently, this could have a negative impact on bone metabolism. Furthermore, a diet with a suitable proportion of omega-6/omega-3 fatty acids appears to avoid pathologies in the bone health associated with aging, such as osteoporosis [38]. This is because omega-3 fatty acids do not exert a strong adipogenesis induction capacity as the one of the omega-6 fatty acids, thus allowing the osteoblastogenesis. That fact, together with their inhibitory effect on osteoclastogenesis, may improve the maintenance of the bone mineral mass.

Omega-3 Fatty Acids and Bone Density

Growing evidence in the scientific literature suggests a lower incidence of bone fracture in neonates maintained on intravenous fish oil compared to soybean oil. In this sense, previous studies have demonstrated beneficial effects of omega-3 PUFAs on bone strength [24, 40, 41]. The available results indicate that DHA provides the best protection against trabecular bone loss, a fact that is reflected by increased bone volume fraction, increased bone surface density, increased trabecular number, thickness, and connectivity density together with decreased trabecular spaces. In this sense, Li et al. [42] reported that DHA accumulates in the osteoblast-rich and nerve-abundant periosteum of femur in growing rats and appears to be a vital constituent of healthy modeling bone. In addition, other study report improved long bone microarchitecture in female rats administered high-fat diets containing m omega-3 polyunsaturated fatty acids. Fallon et al. [43] have reported an increase in the trabecular number and connectivity density values, suggesting an increase in the number of trabecular elements and subsequent strengthening of the trabecular network in the animals fed a DHA diet. Sun et al. [44] found that growing female mice fed fish oil had a lower bone mineral density loss after ovariectomy, and Watkins et al. [37] similarly found a positive response between omega-3 PUFAs and bone mineral content in femur bones of ovariectomized rats. The protective effects of the PUFAs appear to affect bone microstructure alone, as no change in bone mineral density values was found in trabecular bone. Interestingly, bone mineral density of the DHA group was higher compared with the group fed soy and hydrogenated coconut oil; however, it is unlikely that increased bone mineral density alone could play a significant protective role for cortical bones. The lower fracture risk observed in fish oil fed neonates in combination with the protective effects of DHA observed in the femurs of young mice suggest an important role for omega-3 PUFAs on bone development [43].

In a cohort study 78 healthy young men were enrolled. Bone mineral density of the total body, hip, and spine was measured at baseline and at 22 and 24 years of age. Fatty acid concentration was measured at 22 years of age. The results showed that ω-3 fatty acids, especially DHA, are positively associated with bone mineral accumulation and therefore with peak bone mineral density in young men [40]. In the first controlled feeding study in humans, the effect of dietary plant-derived ω-3 PUFAs on bone turnover was assessed. In this study, 23 subjects consumed each diet in a randomized, three-period crossover design. Each diet consisted of different amounts of saturated and unsaturated fatty acids. Bone turnover was assessed by the differences in serum concentrations of NTx and BSAP at baseline and six weeks later. There was no change in the levels of BSAP across the three groups. The N-telopeptide levels (a key biomarker of bone resorption) showed a positive correlation with the pro-inflammatory cytokine TNF-α in all the groups. The authors concluded that plant sources of dietary ω-3 PUFAs may have a protective effect on bone metabolism via a decrease in bone resorption in the presence of consistent levels of bone formation [24, 45].

Omega-3 Fatty Acids, Breast Cancer and Bone Turnover

Rahman et al. [46] have demonstrated that the DHA and not the EPA attenuates the breast cancer metastasis to bone by inhibiting proliferation and invasion of breast cancer cells, as well as osteolysis by inhibiting breast cancer-stimulated bone resorbing osteoclast formation. The results of this study showed that bones treated with DHA had a reduced expression of CD44 protein compared to those treated with EPA when injected with MDA-MB-231 breast cancer cells, providing a mechanism for the attenuation of breast cancer cell migration/invasion by DHA. Furthermore, they demonstrated that DHA prevents the formation of osteolytic lesions in the bone more potently than EPA when breast cancer cells are injected. Moreover, CD44 has been shown to bind and retain the protein MMP-9, which is present on the surface of breast cancer cells and cleaves collagen I in the bone matrix and may contribute to induce osteolysis found in bone, resulting from breast cancer metastasis. DHA inhibits MMP-9 expression in breast cancer, and therefore, DHA-mediated prevention of osteolysis in breast cancer metastasized bone is likely to be associated with the modulation of MMP-9 expression in bone microenvironment, thereby attenuating bone resorbing osteoclast formation in bone.

Hutchins-Wiese et al. [47] conducted a study in 38 postmenopausal breast cancer survivors on aromatase inhibitors therapy, supplying 4 g of EPA and DHA daily for 3 months. This study was different compared to other investigations of the effects of omega-3 PUFA on bone turnover, because in this report the subjects were 100 %

postmenopausal women at increased risk for fracture and with a high dose of EPA + DHA (4 g). In a study of Appleton et al. [48], no benefits of 1.48 g EPA + DHA were found compared to an olive oil placebo in depressed individuals. However, Hutchins-Wiese et al. [47] reported that bone resorption was inhibited in the fish oil responders compared to placebo. In this study, several inflammatory markers were analyzed as a potential mechanism of action for the effects of fish oil on bone resorption. However, there was no effect of high dose fish oil on inflammatory markers in breast cancer survivors on aromatase inhibitors therapy. Breast cancer survivors have high hs-CRP levels [49]. In light of the lack of changes of the other inflammatory markers tested, Hutchins-Wiese et al. [47] postulated that the excessive rise in hs-CRP demonstrated an acute-phase response outside of what was tested or examined in the study. An alternate mechanism of action for the reduced bone resorption recorded in the study could be via resolvins formed from EPA and DHA [50]. EPA may affect bone resorption via resolvin E1 [51] and/or DHA via the inhibition of osteoclastogenesis by resolving D1 [34]. Therefore, this study reports alternative mechanisms, in which n-6 PUFA acts as pro-inflammatory precursors and n-3 PUFA as anti-inflammatory, explaining some inflammatory-associated bone turnover impairments [18, 19, 30, 34].

Negative Effect of Omega-3 Fatty Acids on Bone Turnover

As mentioned above, several PUFAs have shown to have an effect on bone cells, modulating bone turnover process including formation, re-absorption, and density [19, 52, 53]. On the other hand, fatty acids are essential in the process of induction of mesenchymal stromal cells (MSC) to adipocyte differentiation, as well as in adipose tissue hypertrophy, because they activate the peroxisome proliferator-activated receptor gamma (PPARc), a transcription factor which induces adipogenesis [54]. It is accepted that the amount and nature of fatty acid residues have a key role for human health. In fact, the nutritional and metabolic factors, which modulate the PPARc and the MSC differentiation, such as the amount and nature of PUFA, may influence the bone health [55]. In this sense, Casado-Diaz et al. [39] have demonstrated that there is an increase in the number of adipocytes when the MSC cultures are supplemented with DHA or EPA. On the other hand, other authors report that the consumption of low concentrations of PUFA is positive for bone formation, although a high consumption may not have beneficial effects [56]. Additionally, Casado-Diaz et al. [57] have also found that the addition of PUFA to the culture medium of the MSC induced to differentiate into osteoblasts increases the number of adipocytes and PPARc gene expression. This is in agreement with the effect of lipid oxidation on bone metabolism, in which lipoxygenase activity is partially responsible for the loss of bone mass with aging. Thus, ß-catenin does not bind T cell-specific transcription factors to induce the MSC differentiation into osteoblasts, due to the increase in oxidative stress caused by such enzyme, but instead, it binds to the forkhead box O (FoxO) transcription factors. This induces PPARc gene and therefore triggers the MSC differentiation into adipocytes [58]. Therefore according to Casado-Diaz et al. [57], a high intake of PUFA (omega-6 or other activators of the lipoxygenase activity) may have a negative impact on the bone formation. These findings suggest that nutritional alternatives are needed for improving nonoptimal omega-6/omega-3 ratios and that an increase in the omega-3 of fish (EPA and DHA) or plant (linolenic acid) origin in the diet may be regarded as a valuable strategy for the prevention of and treatment for bone metabolism pathologies, such as osteoporosis.

The NHANES III data have established the correlation between dietary fat and hip bone mineral density in cohort of men and women. There was no significant association between total fat intake and bone mineral density. On the other hand, saturated fat intake was negatively associated with bone mineral density in the femoral neck across all subjects and the greatest effect was observed in men younger than 50 years of age. This indicates the probability of more vulnerability of men to the undesirable effects of saturated fat on bone density [59]. This study is in agreement with another study which was conducted with 20 men and 3 women using a controlled feeding protocol. Significant reduction was reported in N-telopeptide when the subjects consumed diet containing 8 % saturated fatty acid (SFAs) and 17 % PUFAs compared with a diet containing 13 % SFAs and 9 % PUFAs [38].

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Interactions Between Omega-3 Fatty Acids and Iron

24

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Introduction

Iron Metabolism

Fe is an essential micronutrient, required for many biological processes. Apart from its role in hemoglobin, it is central to many redox processes throughout the body. However, in addition to being essential, Fe has the capacity to generate free radicals, which, in turn, can cause severe cellular damage to the main biomolecules including protein, lipids, and DNA [1]. Furthermore, since there is no natural pathway for excreting excess Fe from the organism, systemic Fe homeostasis must be very tightly controlled in order to ensure coordinated Fe absorption by enterocytes, reutilization in macrophages of the reticuloendothelial system, and correct Fe redistribution to its site of utilization (mainly for erythropoiesis) or storage (in hepatocytes) [2]. Various molecules are involved in Fe uptake and storage by hepatocytes and its export from hepatocytes, and systems describing the Fe cycle have evolved.

Several regulatory proteins, such as transferrin, ferritin, hemosiderin, hepcidin, and ferroportin, exert crucial functions in the maintenance of systemic Fe homeostasis. Reticuloendothelial system or macrophage system also plays an important role in executing the regulatory events that lead to changes in systemic Fe levels [3]. The main site of Fe absorption is the duodenum, but most Fe is recycled by the monocyte–macrophage system via phagocytosis of senescent erythrocytes. In the bloodstream, Fe is usually bound to transferrin (Tf), and most of the Tf-bound Fe is utilized for erythropoiesis in the bone marrow [4]. The vectorial transport of Fe from the lumen of the gut into systemic circulation involves an orchestrated interplay of an apical ferrireductase, luminal and abluminal ferro symporters, and a ferroxidase. The components of this transport system-comprising duodenal cytochrome b (Dcytb) and divalent metal transporter 1 (DMT1) operate in a responsive and regulatory manner for the Fe absorption in mammalian [5].

Within cells, Fe is stored in ferritin or hemosiderin. Fe absorption can be precisely adjusted to the needs of the individual, i.e., enhanced when erythropoiesis is increased or in pregnancy, or suppressed in conditions of Fe overload. The key molecule in this regulation is hepcidin. Hepcidin is predominantly synthesized in hepatocytes, secreted from hepatocytes, and excreted through the kidney. In Fe deficiency or in hemorrhagic or hemolytic anemia, the production of hepcidin in hepatocytes decreases markedly. When this happens, both Fe absorption in the duodenum and the release of Fe from stores are greatly increased. In patients with anemia or chronic disease, hepcidin is over-expressed [6]. Intracellular Fe is released into the circulation via ferroportin (FPN). The Fe is donated to Tf and reutilized for bone marrow erythropoiesis. Hepcidin binds to the Fe exporter FPN and leads to its degradation, thereby inhibiting

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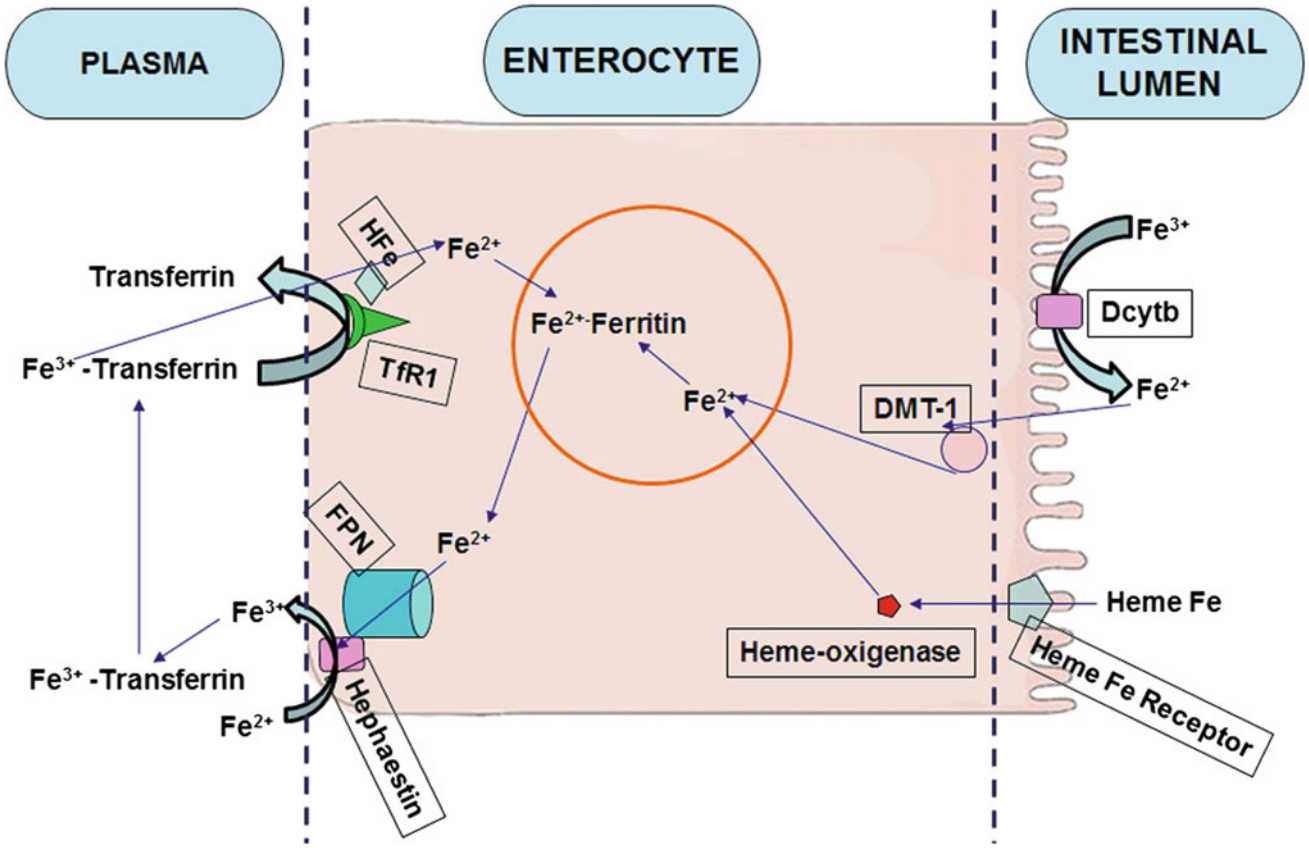

Fig. 24.1 Fe absorption pathways in the enterocyte. Nonheme Fe: All nonheme Fe is ultimately taken up from the lumen by divalent metal transporter (DMT-1) situated on the microvillus membrane, before joining the labile Fe pool in the cytoplasm. Ferric Fe must first be reduced to the ferrous form by Dcytb before uptake. Ferrous Fe in the labile Fe pool is then transferred to the circulation by ferroportin (FPN), which requires hephaestin for oxidation to the ferric form in order to bind to circulating transferrin. Heme Fe: Heme Fe is taken up by receptor-mediated endocytosis. Internalized heme Fe is degraded by hemeoxygenase, releasing nonheme Fe. The nonheme Fe is then transported to the cytoplasm, joined to the labile Fe pool, and is transferred to the bloodstream by FPN in the same manner as nonheme Fe

intestinal Fe absorption, cellular export, and reticulo-endothelial Fe release [7] (Fig. 24.1).

Omega-3 Fatty Acid Metabolism

Dietary fat is an essential macronutrient in all mammals since it participates in a number of biological functions in the maintenance of homeostasis. These compounds are also classified depending on the number of double bonds present in their hydrocarbon chains as saturated or unsaturated. Saturated fatty acids do not have any double bonds in their chemical structure and thereby are constituted by a straight chain of methylene groups [8].

On the other hand, unsaturated fatty acids contain one or more double bonds in their structure and are further classified as 'monounsaturated' (containing one double bond) or 'polyunsaturated' (PUFA, containing more than one double bond). The omega-3 (n−3) and omega-6 (n−6) fatty acids are long-chain PUFAs (LC-PUFAs) with the first of the double bonds in the cis configuration starting from the third and sixth carbon atom, respectively, from the methyl terminus group [9]. The n−3 LC-PUFAs and n−6 LC-PUFAs are essential fatty acids since they cannot be synthesized de novo and are essential for homeostasis and humans need a continuous dietary supply [10], being one of the most important n−3 LC-PUFA DHA.

LC-PUFA DHA is found in considerable amounts in fish and fish oil, and they are commonly considered to be marine sourced fatty acids. Thus, regular fish intake in our diet guarantees an optimal supply of n−3 LC-PUFAs in human metabolism since in vivo conversion of ALA to EPA and DHA might be limited. Rich sources of ALA are flaxseed meal and oil, while rich sources of EPA and DHA are salmon, tuna, mackerel, anchovy, and sardines [11].

DHA is abundant in excitable membranes in retina and brain. In particular, 22:6 n−3 is high in phospholipids in the retina and in synaptic vesicles and plasma membrane of neurons [12]. DHA is essential for the function of retina, and a deficiency of n−3 PUFAs causes impairment in visual

function [13]. In addition, inclusion of 22:6 n−3 PUFAs in milk formulas accelerated the development of visual functions in preterm infants [14]. Also, improvement of neuronal cell survival by n−3 fatty acids suggests that 22:6 n−3 has a key role in differentiated functions of neurons [15]. In this sense, it has been also reported that 22:6 n−3 plays a similar role in both retina and brain [12] and there is a clear involvement of 20:4 n−6 in neurotransmitter recycling [16], a finding that implies 22:6 n−3 may also have the same function in neurons containing high 22:6 n−3.

Moreover, EPA and DHA are precursors of important pro-resolving autacoids, resolvins (designated E and D, from EPA and DHA), protectins, and maresins, which are powerful bioactive agents, involved in the inflammatory signaling and also have anti-inflammatory and immune regulatory activities, as they inhibit the production of inflammatory cytokines and decrease the leukocyte recruitment and diapedesis [17]. Long-chain PUFA DHA also plays important roles in neuronal growth and differentiation, mainly by modulating physical properties of biological membranes and gene expression of various proteins, as well as in myelination and neurotransmission [18, 19].

Omega-3 Fatty Acid and Iron Interactions

Schonfeld et al. [20] demonstrated in nerve growth factor-responsive PC12 cells that PUFA supplements enhanced Fe uptake by modulating Fe transporters, and accelerated apoptotic death. In many cell types, cellular Fe metabolism is controlled by the coordinated action of proteins that are crucial to Fe transport (i.e., TfR; DMT-1, FPN1) and/or Fe storage (i.e., ferritin). In order to determine whether the DHA supplement-induced changes in the Fe storage capacity of the cells, Brand et al. [21] performed a series of experiments quantifying possible changes in ferritin in OLN-93 cells under different culture conditions.

Brand et al. [21] investigated the effects of acute and long-term treatments of DHA-enriched OLN-93 cells with Fe^{2+}, demonstrated a DHA-dependent, robust over-expression of DMT-1 Fe transporter gene that is accompanied by elevated intracellular Fe levels, and also provided evidence for a cellular rescue mechanism activated by preconditioning with low Fe concentrations leading to an increase in cellular antioxidant machinery.

Sub-acute levels of Fe appear to facilitate activation of essential components of the cellular antioxidative machinery in DHA-enriched cells as showed by the increase in GPx and GR enzymatic activities. Accumulation of intracellular Fe is modulated by DHA supplements. Genes associated with Fe transport are over-expressed upon enrichment with DHA, accompanied by a marked increase in intracellular Fe. Taking into account the role of DMT-1 modulating Fe uptake, the explanation of this effect seems easy. Similarly, TfR was significantly elevated following DHA supplement; however, its responsiveness to changes in Fe concentration was down-regulated after Fe addition. Ferritin is also involved in Fe metabolism; however, no changes in ferritin levels were revealed in DHA-supplemented OLN-93 cells by qRT-PCR analysis [21] (Fig. 24.2).

The results reported by Baumgartner et al. [22] revealed that the combined deficiencies of Fe and (n−3) fatty acids disrupt brain monoamine metabolism and produce greater deficits in reference memory than Fe deficiency or (n−3)

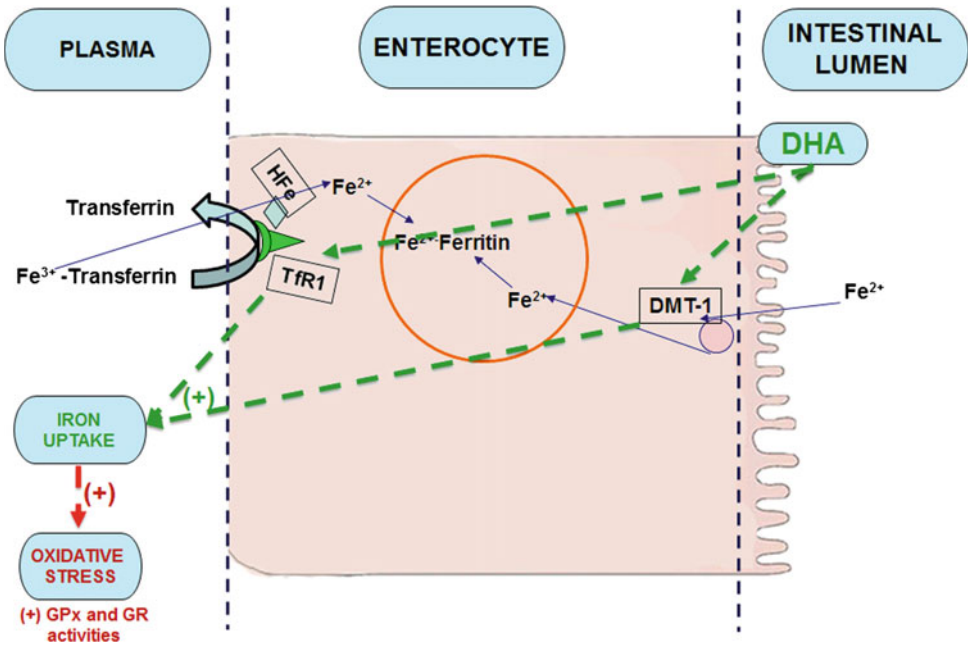

Fig. 24.2 DHA induces an over-expression of DMT-1 Fe transporter gene that is accompanied by elevated intracellular Fe levels and also increases GPx and GR enzymatic activities. Similarly, TfR is elevated following DHA supplement

fatty acids deficiency alone. Fe is a cofactor for desaturase enzymes required for the conversion of EFA into EPA, DHA, and arachidonic acid, fact that explains why DHA concentrations in the Hippocampus and olfactory bulb were lower in Fe-deficient rats, and in the striatum, the arachidonic acid increasing effect of (n−3) fatty acids deficiency was attenuated in combination with Fe deficiency (antagonistic effect). Lately, Baumgartner et al. [23] found in the hippocampus a synergistic interaction between Fe and alpha-linolenic acid with higher DHA levels, fact that was explained because Fe is also a cofactor of hepatic elongases [24], which are responsible for the conversion of alpha-linolenic acid to EPA and DHA. Depletion of DHA in cell membranes may adversely affect membrane function and impair receptors involved in Fe uptake [19]. This may explain why in the hippocampus, Fe concentrations were significantly reduced by (n−3) fatty acids deficiency [22].

The mechanisms that underlie the adverse effects of providing either DHA/EPA or Fe alone to rats with combined deficiency are uncertain but may involve the disruption of neurotransmitter balance in a brain that has adapted to chronic deficiency. In addition, the exposure of cells to moderate amounts of Fe may be necessary to adapt cells to oxidative stress. Because oxidative stress can trigger inflammation that may cause cognitive impairment [25], it is possible that the provision of either Fe or DHA/EPA alone produces greater inflammation than does the combined provision of Fe and DHA/EPA. These mechanisms may also potentially explain the negative effects of DHA/EPA supplementation when given alone to children with Fe deficiency anemia [23].

Interactions Between DHA and Iron During Gestation

Maternal nutrition during gestation will influence the subsequent development of the newborn child. Therefore, it is of great importance to know of the effect that nutrients might have during this stage of our life, as well as that of possible interactions between these nutrients. Two of these key nutrients, with a major role in the neuronal development of the newborn child, are iron and DHA [26, 27].

Iron is involved in early development through its role in oxygenation and energy metabolism. Numerous iron-containing enzymes are involved in brain/energy metabolism, neurotransmitter synthesis, and myelination. Iron uptake by the brain is maximal during its rapid growth, which coincides with the peak of myelinogenesis. Hence, the developing brain requires a continuous and regulated supply of iron across the blood–brain barrier. On the other hand, DHA is found in particularly high concentration in neural tissue, e.g., the photoreceptor cells of the retina and the gray matter of the brain. During the last trimester of pregnancy, fetal requirements of DHA are especially high owing to rapid synthesis of the brain and retinal tissue [26]. Nevertheless, though in a separated way, the role of these nutrients has been extensively documented, the interactions existing between both of them are less known.

Several studies have suggested that Fe deficiency is associated with an alteration in tissue fatty acid content [28, 29] and hypomyelination [30]. Fe deficiency in children is associated with lower levels of DHA in the red blood cells [31]. In animal models, LeBlanc et al. [32] reported that moderate Fe deficiency in dams during gestation and lactation resulted in changes in the fatty acid composition of the brain, liver, and red blood cells of the offspring. However, the most striking changes were in brain PUFA composition, where total (n−3) PUFA was significantly enriched by ∼20 % in phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol in offspring born to Fe-deficient dams, being the DHA content the main responsible for this increase. The altered fatty acid profile observed in the study of LeBlanc et al. [32] may, therefore, be part of a larger spectrum of neuronal Fe-dependent biochemical pathways that are altered by perinatal Fe deficiency, which may have a lasting impact on neurodevelopment. Previously, it was proposed that the decreased tissue long-chain PUFA content associated with Fe deficiency in adults is due to a reduction in D6 desaturase activity [29]; however, the overall brain PUFA composition in the study of LeBlanc et al. [32] and others [28] does not support a reduction in brain D6 desaturase activity with Fe deficiency. Nevertheless, in the developing brain, the capacity to desaturate and elongate PUFA is elevated, suggesting a greater capacity to locally produce highly unsaturated fatty acids during early brain development [33].

Iron and Eicosanoid Metabolism

As previously commented, n−3 and n−6 LC-PUFAs are precursors of different types of eicosanoids and their proportions in the diet will determine the synthesis of a type of eicosanoid and therefore different effects on the organism.

Prostanoids, consisting of prostaglandins (PGs) and thromboxanes (TXs), are the metabolites of arachidonic acid, with prostacyclin (PGI2) and thromboxane A_2 (TXA_2) being the prostanoids that have a key role in the cardiovascular system [34]. The opposite actions of PGI_2 and TXA_2 on the blood vessels and platelets are well known, and their balance is critical in the development of various thrombotic diseases, including acute myocardial infarction [35]. Both PGI_2 and TXA_2 are expressed in heart tissue; PGI_2 and its analog have been reported to exert beneficial effects on cardiac ischemic injury [36], whereas TXA_2 is involved in vascular atherosclerosis and has pro-inflammatory characteristics.

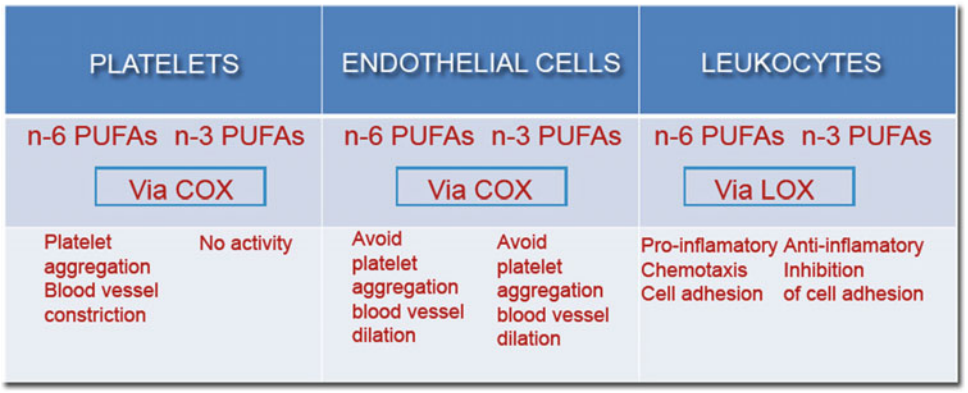

Fig. 24.3 Eicosanoids and biological effects of their metabolites

In a study conducted by Mattera et al. [37], it was reported that Fe overload increases arachidonic acid release, as well as cyclooxygenase (COX) activity, COX-2 induction, and eicosanoid production, in neonatal rat ventricular myocytes (NRVMs). Release of arachidonic acid is catalyzed by phospholipase A2 enzymes and/or diacylglycerol lipase. In addition, Fe overload sensitized NRVMs to COX-2 induction. Fe-overloaded NRVMs stimulated with interleukin (IL)-1α displayed increased production of several prostaglandins (PGs) including PGE_2, $PGF_{2\alpha}$, and 6-keto-PGF1α. Interestingly, Fe overload not only potentiated IL-1α-induced eicosanoid release but also modified the relative ratio of eicosanoid production in cytokine-stimulated NRVMs. Although PGI_2 was the most abundant product in resting NRVMs (controls or Fe-overloaded), a relatively higher increase in PGE_2 was observed after IL-1α treatment of Fe-overloaded cells compared with smaller changes in either PGI_2 or the less abundant $PGF_{2\alpha}$. Consequently, PGE_2 was the predominant eicosanoid in cytokine-stimulated Fe-overloaded NRVMs. This is significant because the specific pattern of eicosanoid production in Fe-overloaded cardiomyocytes may be ultimately responsible for the final balance of pro-arrhythmogenic or anti-arrhythmogenic effects, suggesting a causal connection between these signals and electromechanical alterations in Fe-overload-induced cardiomyopathy.

Lin et al. [38] reported that TXA_2 was the major eicosanoid that mediates iron-overload cardiomyopathy. TXA_2, a metabolite of arachidonic acid, is produced in a catalytic reaction by cyclooxygenase and TXA_2 synthase (TXAS). It acts through the thromboxane prostanoid (TP) receptor, a G-protein-coupled receptor, to regulate Ca^{2+} entry on the cells, with subsequent increase in cytosolic Ca^{2+}. TXA_2 is highly expressed in platelets and exerts potent effects on platelet aggregation and smooth muscle contraction in vasculature [39]. Although the role of TXA_2 in vascular disorders, such as atherosclerosis and thrombus formation, is clear, its direct action on the heart was less certain [40]. However, Lin et al. [38] indicated that TXAS-TP signaling-induced NFAT (a transcription factor that controls lymphocyte development and function) translocation plays an important role in cardiac inflammation after Fe loading. This study was the first to elucidate that TXA_2 mediates Fe-overload cardiomyopathy through TNF activation.

The high sensitivity of PUFAs to oxygen is apparently used to induce stepwise appropriate cell responses. Lipoxygenases (LOX) transform PUFAs bound to membrane phospholipids to lipid hydroperoxides [41]. If an outside impact exceeds a certain limit, the catalyzing bivalent iron ions in LOXs are liberated. They cleave the enzymatically generated lipid hydroperoxides and induce a switch to nonenzymatic lipid peroxidation reactions that produce peroxyl radicals. Peroxyl radicals are much more reactive than lipid hydroperoxides molecules and attack all types of biological molecules. PUFA-phospholipids are apparently the precursor molecules of a signaling that respond in a dose-related inducing an appropriate gene response. The deleterious reactions include epoxidation of cholesterol–PUFA esters, plasmalogens, and sphingolipids, as well as the release of hydrogen peroxide by the reaction of peroxyl radicals with alcohols and amines [42] (Fig. 24.3).

In conclusion, Fe is an essential micronutrient, required for many biological processes. Apart from its role in hemoglobin, it is central to many redox processes throughout the body. The long-chain polyunsaturated fatty acid (PUFA) docosahexaenoic acid (DHA; 22:6 n−3) is ubiquitously present in the plasma membrane of biological cells and has a key role as an essential macronutrient in all mammals since it participates in a number of biological functions in the maintenance of homeostasis. There is a need of more research to elucidate the interactions of these two nutrients, especially due to the great abundance of studies reporting supplementations with both nutrients separately in important stages of the life such as gestation and/or lactation.

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Role of Omega-3 Fatty Acid in Major Cardiovascular Events—A Current View

25

Yanting Wen and Qian Gao

Introduction

Nutritional intake of long-chain omega-3 polyunsaturated fatty acids (omega-3 PUFAs) derived from marine sources, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [1], is widely consumed as supplements within the community and has long been recognized to be effective in protecting cardiovascular health. It was reported as early as 1971 that Greenlandic West-coast Eskimos who consume a diet rich in whale, seal, and fish have a very low incidence of coronary artery disease (CAD) [2], indicating the cardiovascular-risk-reducing potential of omega-3 PUFAs, a compound always found in fish and fish oils. From then on, on the basis of numerous epidemiologic and interventional studies, health authorities have long recommended intake of omega-3 PUFAs to provide cardiovascular protection. World Health Organization recommended an adequate intake of PUFAs in the range of 6–10 % of daily energy intake to promote cardiovascular health [3]. American Heart Association recommended that all adults eat fish (particularly fatty fish) at least twice a week; patients with documented coronary heart diseases (CHD) intake 1 g of EPA and DHA (combined) per day, and in patients with hypertriglyceridemia, an EPA + DHA supplement may be useful [4].

However, the controversies surrounding the dietary intake of omega-3 PUFAs always exist. Numerous reported meta-analyses [5–17] on the basis of randomized controlled trials (RCTs) have investigated the effects of dietary supplementation with omega-3 PUFAs on the reduction in major cardiovascular events and mortality, demonstrating conflicting clinical statements, which may be associated with diverse patient populations included. This chapter summarizes the clinical evidence addressing the potential benefit of marine omega-3 PUFAs in major cardiovascular events in the diverse patient populations.

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Mechanistic Insights on omega-3 PUFAs in Cardiovascular Protection

Although the mechanisms underlying the effects of omega-3 PUFAs in cardiovascular protection are not completely understood, omega-3 PUFAs is known to function as natural HMG-CoA reductase and ACE enzyme inhibitors, antiarrhythmic, antihypertensive, anti-atherosclerotic, anti-inflammatory, cyto-protective, and cardio-protective molecules [18]. A growing body of evidence suggests that one of its major mechanisms of omega-3 PUFAs in preventing the cardiovascular diseases (CVD) could be its endothelial defensive action, including improving endothelial function, preventing the pathogenesis of vascular endothelial dysfunction, and inhibiting platelet aggregation [19, 20]. In patients with various cardiovascular and metabolic disorders [21–23], omega-3 PUFAs have been demonstrated to have counter-effects on the processes involved in atherosclerosis. It stabilizes plaques through attenuating platelet aggregation caused by various stimuli, decreasing triacylglycerol, hepatic LDL-C, VLDL-C as well as cholesterol, increasing HDL-C, improving triglyceride clearance, and reducing IL-1β and TNF levels. Omega-3 PUFAs are believed to have anti-platelet and antithrombotic properties as well; for instance, omega-3 PUFAs supplementation markedly increases the eicosapentaenoate content of phospholipids from red blood cells as well as platelets and alters their pattern of thromboxane and prostacyclin synthesis [24, 25]. On the other hand, omega-3 PUFAs can exert protective effects comprised the cells of the innate as well as the adaptive immune systems, which play important roles in the immune and inflammatory processes that take part in CVD [26–28]. Omega-3 PUFAs may suppress T-cell proliferation and cytokine production when the dendritic cells (DC) were

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added to naive T cells and hence block the activation and function of DC [29–31]. Omega-3 PUFAs induce a shift in the Th1/Th2 balance toward a Th2 prone response [32]. Furthermore, omega-3 PUFAs have been shown to reduce mast cell degranulation [33, 34], which may interfere thrombus formation [27].

Different Roles of Omega-3 PUFAs in Major Cardiovascular Events According to the Diverse Patient Populations

The association between omega-3 PUFAs supplementation and major cardiovascular events is controversial. In subjects previously free of known coronary heart disease (CHD) at baseline (though risk factors for CHD, such as obesity, hypertension, dyslipidemia, type 2 diabetes mellitus, and metabolic syndrome, may have been present), data support a significant 35.1 % reduction in the risk of sudden cardiac death and a near significant 16.6 % reduction in the risk of nonfatal coronary events with the consumption of ≥250 mg/d of the omega-3 PUFAs relative to the consumption of <250 mg/d [11]. A meta-analysis of RCTs evaluating omega-3 PUFAs supplementation lasting over one year in adult participants failed to associate omega-3 PUFAs administration with a lower risk of all-cause mortality, cardiac death, sudden death, myocardial infarction (MI), or stroke based on relative and absolute measures of association [5]. In this circumstance, the use of omega-3 PUFAs as a structured intervention in daily clinical practice or guidelines supporting dietary omega-3 PUFAs administration [3, 4] is not justified. However, combined types of participants or conditions in this meta-analysis may result in potential statistical heterogeneity. In fact, when including a definite group of patients' populations, some beneficial effects of omega-3 PUFAs supplementation may be demonstrated. Some benefit found is largely due to those which enrolled high-risk patients; for example, reduction in platelet aggregation is found after omega-3 PUFAs supplementation when the participants were at poor health status, but not in healthy persons [12]. The next subsection addresses this important issue.

According to the report from American Heart Association [35], an estimated 83.6 million American adults (>1 in 3) have ≥1 types of CVD. Although omega-3 PUFAs have been recommended to serve as a complementary and alternative medical remedy in patients with CVD, supplementation with omega-3 PUFAs did not reduce the risk of overall cardiovascular events, all-cause mortality, sudden cardiac death, MI, congestive heart failure, or transient ischemic attack and stroke when involving 20,485 patients with a history of CVD (including CHD, MI, lower limb atherosclerosis, implanted cardioverter defibrillator (ICD), and stroke survivors) [10]. When the patients population is limited to those with CAD, AMI, peripheral vascular disease (PVD), ICD, hypercholesterolemia, and congestive heart failure (CHF), dietary supplementation with omega-3 fatty acids significantly reduced the risk of cardiovascular deaths, sudden cardiac death, all-cause mortality, and nonfatal cardiovascular events [14]. In this meta-analysis, the mortality benefit found is largely due to the studies which enrolled high-risk patients, while the reduction in nonfatal cardiovascular events was noted in the moderate-risk patients (secondary prevention only). While the patients are limited to those with CHD, supplement of omega-3 PUFAs is not associated with a protective effect on major cardiovascular events, while it does exert beneficial effects in reducing death from cardiac causes, sudden cardiac death, and death from all causes [9]. A subgroup meta-analysis on the effect of omega-3 PUFAs in patients with CAD or after MI [7] showed a 26 % reduction in sudden cardiac death (SCD) and a significant 20 % reduction in deaths from cardiac causes with fish oil when compared with placebo.

Cardiac arrhythmias are widely spread. Estimated by the American Heart Association, bradyarrhythmias affect approximately 4 % of the population, and SCD occurs in >400,000 Americans annually [35]. The management of cardiac arrhythmias has advanced enormously, including anti-arrhythmic drugs, implantable devices, and other invasive or noninvasive therapies [36]. Omega-3 PUFAs, which serve as one of the complementary and alternative medical remedies, may possess clinically significant anti-arrhythmic properties, especially in serious ventricular arrhythmias [37, 38], tested by a number of clinical studies [39–43]. However, according to a recent meta-analysis including a total of 32,919 patients from nine trials, dietary supplementation with omega-3 PUFAs does not affect the risk of SCD or ventricular arrhythmias [6]. The sub-analysis of studies that enrolled patients with recent myocardial ischemia showed a trend of greater reduction in sudden death or ventricular arrhythmias than the main analysis, and some sub-analysis [6, 7] of the these trials that included patients with only a prior history of ventricular arrhythmias and ICD showed no difference in the risk of sudden death and ventricular arrhythmias between omega-3 PUFAs supplementation and placebo, although with a significant heterogeneity. Based on their study, there is no additional benefit to treat those patients with a prior history of ventricular arrhythmias and ICD with omega-3 PUFAs.

Peripheral arterial disease (PAD) is most commonly caused by atherosclerosis obliterans and thromboangiitis obliterans and is associated with a higher ischemic cardiovascular risk than the general population [44, 45]. With significant morbidity and mortality, PAD can lead to claudication and critical limb ischemia (CLI), resulting in major amputation and subsequent death. Current guidelines for the

management of patients with PAD recommend lifestyle changes, medicines, and surgery or procedures [46]. Medicines recommended include lipid-lowering therapy with a statin to achieve a goal LDL <100 mg/dL (or <70 mg/dL in high-risk patients), antihypertensive therapy to achieve a systolic blood pressure below 140 mm Hg, and antiplatelet therapy [47]. Given the high incidence of cardiovascular events in individuals with peripheral arterial disease [48] and the potential cardiovascular benefits of omega-3 PUFAs, it has been used in the PAD population in several clinical trials, showing some improvements in vascular function [49, 50] or inflammatory status [51]. However, after meta-analysis including adults with PAD, insufficient evidence exists to suggest a beneficial effect of omega-3 PUFAs supplementation with regard to cardiovascular events and other serious clinical outcomes, such as need for revascularization or amputation, pain-free walking disease, or quality of life [16]. Hence, there is very little indication for recommending omega-3 PUFAs as a therapeutic approach in individuals with PAD.

Individuals with impaired glucose metabolism (IGM) are at high risk, not only to develop diabetes mellitus, but also to experience an adverse cardiovascular event later in life [52–54]. As a result, interventions targeted at those in an early stage of IGM are recommended. When concerning to adult IGM patients, which include impaired fasting glucose patients and impaired glucose tolerance patients and type 2 diabetic patients, omega-3 PUFAs supplementation can reduce triglyceride level, but have no protective effect on cardiovascular mortality, major cardiovascular events, all-cause mortality and composite end point of all-cause mortality or hospitalization for cardiovascular cause [17]. The recommendation of omega-3 PUFAs supplementation in patients with PAD is worth debating.

In sum, omega-3 PUFAs supplementation appears to be a low-risk and cost-effective strategy to improve cardiac health. It may be recommended in primary prevention of major cardiovascular events in those free of known CHD, but with high risk. Furthermore, patients with CAD and previous MI may potentially benefit on causes of mortality from omega-3 PUFAs. However, omega-3 PUFAs may not be effective in preventing major cardiovascular events in patients with PAD, cardiac arrhythmias, and IGM. Larger RCTs with optimal dosage and supplementary duration of omega-3 PUFAs need to be carried out to confirm the present findings. In addition, the findings from laboratory studies on omega-3 PUFAs, which often dealt with acute cellular and animal settings, need to be carefully translated to the clinical situations. Moreover, the effect of omega-3 PUFAs sourced other than fish and fish oils and therefore may have very different compositions of unsaturated long-chain fatty acids, which may be worth to explore in various classified cardiovascular conditions.

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Modulation of Immune Response by Omega-3 in Health and Disease

26

Sudha Gangal

Introduction

The Immune System

Survival is the foremost instinct of all living organisms. The pragmatic danger for survival is the life-threatening diseases caused by millions of disease-causing pathogens present in the environment. As evolution progressed, animals adopted an uncanny way of combating with invading microorganisms with the help of rapidly evolved built-in defence system called immune system. In higher vertebrates, especially mammals, it represents an extremely finely tuned exquisite, well-orchestrated system with memory.

Immune system functions in two ways: innate or pre-existing immune system which is composed of phagocytic cells such as granulocytes and monocytes/macrophages; and natural killer (NK) cells, which act in coordination with soluble mediators which are the components of complement system, chemokines and monokines (produced by monocytes and macrophages), acute phase proteins produced by hepatocytes and mediators of inflammation to get rid of offending pathogens [1]. This is the first line of defence which acts immediately after the microbe enters in the body and has no memory. On the other hand, adaptive or acquired immunity needs to 'educate' the system to defend the microbes, which takes a few days, but is very specific, long lasting and has memory, which means it remembers the pathogen against which it is developed and when the same pathogens infects the host again, acts quickly and more efficiently. Adaptive immunity consists of antigen-presenting cells, responding T and B lymphocytes and a host of cytokines produced by antigen-activated cells. Cytokines are small molecular weight mediators of immune response which can be helpful in initiating, amplifying and channelizing the immune response or can at times be harmful and can induce pathological conditions such as autoimmunity, allergy and several inflammatory conditions.

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Long-Chain Fatty Acids and Immune Response

Ingested foods are known to influence immune response. Some nutritional components called food allergens can cause severe fatal hypersensitivity reactions [2] sometimes, while micronutrients are the essential features for development and functioning of immune response [3]. Recently, a great deal of emphasis is being given on the ability of long-chain polyunsaturated fatty acids such as omega-6 and omega-3 to modify immune functions [4]. These fatty acids form the framework of cell membranes and help in maintaining membrane fluidity. They also act as precursors of molecules involved in the regulation of inflammation and immunity [5]. Omega-6 is known to induce pro-inflammatory response, while omega-3 fatty acids EPA and DHA produce substances that can favourably regulate inflammation [6–10].

The anti-inflammatory effect of fish oil, a well-accepted source of omega-3, has been shown in many diseases such as cardiovascular disease, ulcerative colitis, Crohn's disease, inflammatory bowel disease and arthritis [11–15]. Immunomodulatory effect of omega-3 is recorded in healthy individuals, pregnant women and nursing mothers [16–18]. Even in basic immunology, modulatory effects of omega-3 on functions of T and B cells, potent antigen-presenting dendritic cells, natural killer cells, macrophages, etc. and cytokine production by these cells are well documented [19, 20]. The literature on effect of omega-3 on immune system in health and in diseases, especially those caused by excessive inflammatory response, is very large. In this chapter, an overview is provided for the effect of omega-3 on different components of immune system, on health and on reducing pathological manifestations of diseases, in particular,

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inflammatory diseases. Although extensive literature is available on effect of omega-3 in animal models in inflammatory diseases, this chapter relates more to human studies.

Effect of Omega-3 PUFAs on Components of Immune System

The two arms of immune system, the innate and adaptive immunity, function to destroy microbes by non-specific and specific means, respectively. The main feature of innate immunity is inflammation, while that of adaptive immunity is to produce specific antibodies and cytotoxic cells to get rid of microorganisms and foreign substances, although inflammation plays a major role in adaptive immunity as well. Inflammation is a common mechanism in both arms of immune response, guided by cytokines and other soluble mediators. The main steps in inflammation are release of cells from circulation to the site of inflammation, which involve chemotaxis, cell adhesion and release of cells from vascular endothelium, the process called 'extravasation', essential in both innate and adaptive immunity. Steps involved in adaptive immune response are antigen presentation, activation, proliferation and differentiation of responding cells into antibody-producing (B cells) and helper/cytotoxic cells (T cells). Both innate and adaptive responses are guided by a host of cytokines [1]. Therefore, to understand the modulatory effect of fatty acids on immune system, the effect needs to be considered on all these aspects of immune response.

Several factors may be involved in modulation of immune functions by PUFAs, but the main event could be changes in the cell membrane due to dietary fatty acid manipulations. They can be incorporated in the cell membrane after dietary intake, changing the composition of lipids in the membrane leading to changes in membrane fluidity and expression of surface molecules, affecting the receptor–ligand interactions so essential for initiating the immune response. The lipids may also be released from the membrane or may suffer from biochemical degradation resulting in modulation of immune functions [21].

Several studies have reported that n-3 PUFAs are able to manipulate many immune functions in favour of controlling diseases caused by excessive inflammatory response and have opened up possibilities of modifying diets to restrict many inflammatory disorders. Classical epidemiological study conducted on Eskimos, whose main diet is fish (a premier source of PUFAs), shows low incidence of inflammatory and autoimmune disease [22].

A number of studies reported on the effect of polyunsaturated fatty acids on modulation of immune response are either in vitro studies or those conducted using animal models. Therefore, translating them in human system has to be done with caution, as the dose of PUFA in the diet and its metabolic activation differs in these model systems and man [23]. Modulation of immune system by fatty acids depends heavily on quantity and nature of lipids ingested [24]. It is suggested that omega-3 PUFAs modify membrane lipid composition by decreasing arachidonic acid and increasing EPA. EPA can suppress eicosanoid (prostaglandins, thromboxanes and leukotreines)-associated systemic inflammatory response. Eicosanoids are known to influence TH1/TH2 balance by decreasing the production of TH1 cytokines IFN-ɣ and IL-2. They also enhance TH2 cytokine production (IL-4, IL-5) and stimulate IgE synthesis [24]. The mechanisms involved could be membrane fluidity as mentioned earlier [25], production of lipid peroxides [26] and eicosanoid synthesis [27]. Omega-3 PUFAs are known to regulate gene expression in several ways. They affect signalling pathways by directly interacting with nuclear receptors [28]. They can bind to peroxisomal proliferator-activated receptor gamma and regulate immune and inflammatory responses [29].

Over last three decades, extensive literature has enriched this aspect of PUFA research, and only the important contributions are discussed below.

Inflammation

The main clinical manifestations of inflammation are swelling, pain, fever and erythema. Arachidonic acid lowers pain, and omega-3 fatty acids are known to prevent fever caused by bacterial lipopolysaccharides [19]. At the cellular level, inflammation involves the release of neutrophils, lymphocytes and monocytes from circulation at the site of inflammation by extravasation [1]. The phagocytic cells released and killed the microbes. The inflammation gets aggravated by the release of cytokines and soluble mediators. Effect of PUFAs on these aspects of inflammatory response is considered below.

Extravasation

As mentioned earlier, the cellular and molecular events in inflammation are chemotaxis, adhesion and diapedesis (release of cells from capillary endothelium). Expression of adhesion molecules on neutrophils and capillary endothelial cells sets the neutrophils rolling and are released from between the endothelial cells at the site of inflammation [1]. Omega-3 fatty acids and oleate reduce expression of adhesion molecules such as VCAM-1, E-selectin and ICAM-I on endothelial cells, thus preventing the rolling of cells, and can suppress the chemotactic responses of neutrophils to chemoattractants such as LTB4 and FMLP [30].

Effect on Phagocytosis

In order to kill phagocytosed bacteria, neutrophils and macrophages need to be activated to generate reactive

oxygen and nitrogen species. Excessively produced free radicals, however, can cause tissue injury, a side effect of chronic inflammatory response [23]. Patients treated with dietary omega-3 show decreased production of reactive oxygen species by activated neutrophils, which can, at times, hamper the destruction of infective organisms [31]. Similarly, production of NO by macrophages is also inhibited by omega-3 PUFA, particularly DHA [32]. It is shown that omega-3 fatty acids enhanced anti-parasitic activity against P. *falceperum*, when added in cell cultures [33].

Production of Cytokines and Other Soluble Mediators

Activated macrophages, lymphocytes and some other cell types produce small molecular weight mediators called cytokines which are essential for activation inflammation and maturation of immune response [1]. Pro-inflammatory cytokines such as IL-1, IL-6, IL-8, and IL-10 are produced by activated macrophages, while anti-inflammatory cytokines (IL-2, IL-12, IFN-γ etc.) are produced by activated T-helper cells. A potent inflammatory cytokine TNF-α is produced by activated T cells as well as by activated macrophages. Production of pro-inflammatory cytokines such as IL-1 and TNF-α is reduced by dietary supplementation by fatty acids [34]. Reports on effect of dietary PUFA on TNF-α production are contradictory. De Luca et al. have shown that omega-3 lowers the production of TNF-α both in vivo and in vitro [35], while Chevali et al. have shown increased levels of TNF-α in vitro in mouse splenocytes supplemented with omega-3-rich medium [24]. These discrepancies could be due to different PUFA actions on cell populations of various species and different methods used [36].

Omega-3, especially EPA, can decrease IL-2 production and reduce expression of IL-2 receptors, both together affecting lymphocyte proliferation [37]. These authors have also shown that lymphocytes treated with omega-3 fatty acids show decrease in IFN-γ production.

Omega-6 increases expression of chemokine IL-8 on endothelial cells, a step essential for extravasation of cells from capillaries [38], while omega-3 reduces the expression of IL-8, IL-6 and IL-10 on endothelial cells and monocytes [39]. Mice fed with diet containing coconut oil show low TNF production, whereas Il-10 production is significantly increased [40].

Omega-3 fatty acid-rich diet lowers the level of PGE2 and also lowers neutrophil chemotaxis [24]. Production of platelet-activating factor by granulocytes, important for limiting inflammation, is also reduced in rats fed with omega-3 [41]. Omega-3 PUFAs are also known to reduce histamine release in mast cell cultures, the mechanism by which the fatty acids can control hypersensitivity reactions [42]. Attempts have been made of unravel the molecular mechanisms underlying cytokine response modulation by PUFAs [43, 44].

Antigen-Presenting Cells (APCs)

Diet rich in omega-3 lowers the ability of APCs to present antigens required for activation of T cells in adaptive immunity, perhaps due to the reduced expression of MHC class II molecules [45]. Dendritic cells (DCs) are the most potent APCs as they constitutively express MHC class II molecules and second signal molecules, and can activate naïve T cells [1]. A class of DCs can be differentiated from monocytes. DHA can act at multiple stages on differentiating/differentiated DCs. It can influence expression of co-stimulatory molecules, inhibit IL-6 expression and secretion of IL-10 and IL-12 by lymphocytes and can also influence the lymphoproliferative capacity [46].

Expression of Adhesion Molecules

For interaction of APCs with T cells, T and B cell interaction, T and target cell interaction and cell migration, several adhesion molecules are responsible to establish the adhesion and make it firm [1]. Animals fed with diet supplemented with fish oil and olive oil show decreased expression of LFA-1, ICAM-1 and CD2 on immune cells [30]. Reduced expression of adhesion molecules L-selectin and LFA-1 is seen if lymphocytes are pre-treated with DHA or EPA [47]. These authors have also shown that interaction of endothelial cells with lymphocytes is hampered due to reduced expression of VCAM-1 in the presence of DHA or EPA. Fish oil containing diets increase TGF-β1 expression on T cells modulating the immune response [28].

Lymphocytes

Lymphocyte Proliferation

When the antigen is presented to T cells, the latter undergo extensive proliferation and differentiate into a variety of helper T cells to activate specific humoral (antibody production) and cell-mediated (inflammation and cytotoxic T cells) immune response as well as regulatory T cell response, all of which are essential to induce, expand, modulate and regulate adaptive immune response [1]. Lymphocyte activation is inhibited by PUFA, as demonstrated by in vitro proliferation of T cells [48, 49]. EPA and DHA incorporated in lymphocyte membrane can alter membrane fluidity, suppress signal transduction and affect T cell proliferation. Protein composition of T cell membrane, changed by omega-3 PUFAs may result in activation-induced apoptosis of T cells [50]. However, in vitro proliferation data need to be readdressed as in clinical trials, and no correlation was found in in vivo effect of dietary administration of PUFAs and in vitro lymphocyte proliferation [23].

Antibody Production

Antigen-activated B cells undergo proliferation and differentiate into antibody-producing plasma cells with the help of T-helper cells [1]. Omega-3 fatty acids are shown to inhibit antibody production by rat lymphocytes [30]. Diets rich in omega-3 PUFAs causes alterations in membranes of APCs, down regulating their function and recognition by T cells [51].

NK and LAK cell Activity

Natural killer (NK) cells comprise a major cytotoxic cell type in innate immunity [1], while lymphokine (IL-2)-activated killer cells (LAK cells) can efficiently kill transformed tumour cells. Dietary lipids have been shown to negatively affect NK cell activity [52]. It has been demonstrated that human volunteers injected with triacylglycerol containing eicosapentaenoic acid show suppressed peripheral blood NK cell activity [53]. It is also reported that diet containing docosahexaenoic acid reduces NK cell activity in humans [54]. Diets rich in omega-3 PUFAs can reduce NK and LAK cell activities and can also reduce the number of circulating NK and LAK cells [55]. Human peripheral blood mononuclear cells treated in vitro with omega-3 PUFA suppress both NK and LAK cell activities [37].

Regulatory T Cells

Favourable immune response depends upon the balance between T-helper and T regulatory cells [1]. Patients with solid tumours, fed with PUFA-rich diet show decrease in regulatory cells, a response favourable to patents [56]. Th17 has now been identified as a key T regulatory subset, which produces cytokine IL-17, implicated in inflammatory diseases such as autoimmune diseases and allergy [1]. Recently, using genetic mouse models, it has been shown that TH17 cell-mediated inflammation promoted by PGE2 can be antagonized by n-3 PUFA [57]. It is suggested that obesity associated with inflammatory diseases such as colitis can be attributed in part to TH17 cells. Using colitis-induced mouse model, the same group has shown that with the use of fish oil-rich diet the percentage of TH17 and TH1 cells was reduced in spleens, along with colitis-associated disease severity [58].

Thus, the foregoing account shows that polyunsaturated fatty acids do influence the immune response by complex mechanisms. The effect appears to influence almost all features of immune response including inflammation, chemotaxis, phagocytosis lymphocyte activation, production of cytokines and expression of surface molecules, and controls the ultimate result of immune response, namely end products such as antibody production and generation of cytotoxic cells and regulatory cells. Generally, the fatty acid-modified responses seem to be favourable for translation into clinic for diseases involving excessive inflammatory response; it should be kept in mind that interference in almost all essential steps in activation of immune response may hamper the immune response required to take care of infections, which is one of the major functions of immune system. Also, most of the anti-inflammatory effects of PUFAs have been shown using animal models and in vitro systems. Care needs to be exercised in deciding the dose and length of treatment as in many animal systems the effect seems to be reverted when dietary administration is terminated. Also, while reducing inflammation, the immune system should not be injured to the extent that it is unable to cope up with infections.

Effect of Omega-3 PUFAs in Health

Although it is feasible to study the effect of dietary omega-3 on disease condition in terms of failure to express the disease, or delay in the onset of the disease and alterations in the severity of the disease, using animal models of inflammatory diseases, such experiments cannot be conducted in healthy volunteers. Even in the disease-prone individuals, intervention studies are difficult as all prone individuals may not express the disease, and the period required to express pathological changes may vary. Healthy volunteers have been used to establish the basis for interventions by dietary omega-3 in inflammatory diseases, and the end points are measured either by in vivo challenge or in vitro reactivity of immune cells. However, information obtained from animal experiments and in vitro system cannot be directly applied to human situation for various reasons, aptly reviewed by Fritsche [23]. Firstly, genetic heterogeneity is much higher in human population than inbred animals. Secondly, background diet control is in the hands of experimentalists in animal studies than in man. It is likely that impact of dietary PUFAs on individuals before or during development of the disease could be more important than when it is supplied after the disease is established. Also, the experiments conducted to establish the effectiveness of treatment in terms of immune restoration are mostly in vitro assays, which may have problems. In vitro assays are often optimized to obtain measurable responses. Many stimulants such as mitogens, anti-CD3 antibodies and bacterial lipopolysaccharides are used as stimulants amplifying the responses, which perhaps give exaggerated responses than those occurring in vivo. In vivo tests generally represent DTH responses, which is just one component of immune response.

Therefore, effect of omega-3 supplementation on health will be covered in this chapter in terms of: 1. trials conducted to assess effectiveness of omega-3 diet supplementation in healthy volunteers using in vivo assays, 2. to study the effect of omega-3 diet supplementation in pregnancy and lactation

in relation to infant allergies and 3. to study the adverse effects, if any, on aged population.

Effect of Omega-3 Fatty Acid Supplementation on Healthy Donors: In Vivo Data

Fritsche has given an extensive account of effect of omega-3 diet supplementation on immune parameters in healthy volunteers [23]. He has taken into consideration several clinical trials on healthy volunteers receiving omega-3 fatty acids and their effects in immune parameters tested in vivo and in vitro. Considering the in vivo responses, few studies have shown increase in EPA/DHA content in peripheral blood mononuclear cells (PBMC [54, 59, 60]), change in serum lipid content [61], no change in circulating pro-inflammatory cytokines [62], adequate DTH response to recall antigens, adequate antibody response after vaccination with w/3 strains [63] and no change in circulating T, B and NK cell numbers and DTH response [64]. These volunteers were given PUFA supplementation from 1 to 52 weeks. Several parallel in vitro studies conducted simultaneously did not corroborate with in vivo responses.

Effect of Omega-3 Diet Supplementation on Pregnancy and Lactation in Relation to Infant Allergies

Omega-3 containing diet of pregnant women influences newborn's susceptibility common respiratory illnesses and allergy [65–67]. Kremmyda et al. have systematically reviewed the effect of omega-3 in maternal diet during pregnancy and the risk of atopic and allergic diseases in infants and children [68]. According to this review and other studies [69, 70], maternal intake of fish during pregnancy showed negative correlation with eczema, asthma and sensitization to food, dust and mites in infants. The effect appears to extend till early childhood [71].

Increasing amounts of cytokines and chemokines adversely affecting the allergic responses are shown to be present in the cord blood of women consuming omega-3 PUFAs during pregnancy [72, 73]. Fish oil supplementation of lactating mothers also showed favourable cytokine production in toddlers inhibiting inflammatory response [74]. Romieu et al. showed that fish intake during pregnancy was protective against the risk of eczema at the age of 1, a positive skin pick test for house dust mite at the age of 6 and atopic wheeze at the age of 6 [75]. Furuhjelm et al. showed that maternal n-3 fatty acid supplementation may decrease the risk of food allergy and IgE-associated eczema during the first year of life in infants with a family history of allergic disease [76]. In a 16-year follow-up study, it was found that the hazard rate of allergic asthma was reduced by 87 % in children born of mothers who received n-3 PUFAs in late pregnancy [77]. However, in another study conducted in Australia on a large cohort of children showed that supplementation with high-dose omega-3 PUFAs in pregnant women did not reduce IgE-associated food allergy in the first year, while incidence of eczema and egg allergy was reduced [78].

Diet Supplementation with Fatty Acids in Older Population: Adverse Effects?

Although dietary supplementation with fatty acids is proving to be important as complementary medicine in various diseases as will be discussed further, because of its anti-thrombotic properties it could adversely cause bleeding problems, especially in older population and post-partum bleeding in pregnant women. A comprehensive review of fish oil administration in aged population in terms of severe and mild adverse effects has been published by Villani et al. recently [79]. Severe adverse effects (SAE) are death, stroke, bleeding or major bruising, while non-SAEs are GI tract disturbances, headache, skin irritation, vertigo, malaise, weight gain, polyurination, restlessness, blurred vision, sore throat and skeletal muscle pain.

None of the studies reported SAE attributable to consumption of n-3 by older adults. GI tract disturbances were reported by many [80, 81], but Holguin reported significantly higher number of subjects consuming fish oil diet with GI tract problems [80]. Rondanelli et al. reported headaches in old subjects treated with oral administration of n-3 PUFAs compared to controls [81], while skin irritation was described as minor adverse effect by Gruenwald et al. [82]. Symptoms such as weight gain, polyurination, restlessness, blurred vision, sore throat and skeletal muscle pain were also reported to be due to dietary intake of n-3 PUFAs [83]. Fakhrzadeh et al. have reported vertigo and malaise in old people after dietary intake of fatty acids [84]. This meta-analysis agreed with earlier expert opinion by Harris [85] that n-3 fatty acid supplementation does not show serious adverse effects and risk of bleeding in older population even when they are on anti-coagulation medication.

Thus, several reports have favoured the use of dietary fatty acids as a supplementary modality of treatment of various inflammatory disorders which are fatal, disabling or producing chronic lifelong illnesses with miserable quality of life for the suffers. On the basis of this extensive information, dietary PUFAs are now being used to control the diseases caused by excessive inflammation such as autoimmune diseases and allergies which are considered below.

Omega-3 in Inflammatory Diseases

Several animal studies and reports published by US Food and Drug administration have concluded that PUFAs as edible fats are safe for human consumption [86]. Calder has provided a list of inflammatory diseases [87] which may have beneficial effect by dietary PUFAs, some of which are considered here.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disorder that primarily affects joints. The abnormal immune response causes inflammation that can damage joints and organs. It may result in deformed and painful joints, which can lead to loss of function. The disease may also show signs and symptoms in organs other than joints. RA is one of the target diseases which have shown beneficial results by introduction of treatment with dietary PUFAs. Earlier animal dose-response studies showed that DHA was more effective than EPA, and both together showed synergistic effect [88]. In a study carried out on patients by Kremer et al. [89], suppression of inflammatory mediator LTB4 was seen even after termination of PUFA treatment. When pro-inflammatory cytokines were studied in patients treated with fatty acids, it was seen that IL-1α, β and TNF remained suppressed even after discontinuation of treatment, suggesting that reduction in IL-1 may be responsible for reduction in disease symptoms [90]. Recently, Calder et al. studied the effect of PUFAs on inflammatory process in rheumatoid arthritis [91]. A meta-analysis conducted earlier on several clinical trials showed positive clinical benefits in joint pain and tenderness [92].

Asthma

In asthma, pulmonary inflammation is caused by the production of several inflammatory mediators such as prostaglandins and leukotreinesin blood and bronchial lavage by mast cells, causing bronchoconstriction. This condition is more common in childhood. Broughton et al. studied the effect of omega-3 added in different proportions with omega-6 in asthmatic subjects and showed that with increased proportion of omega-3 ingestion the respiratory symptoms could be ameliorated [93]. Okamoto et al. have also shown that n-3 fatty acid supplementation in asthma patients suppressed generation of inflammatory mediators and improved pulmonary function [94]. On the other hand, Schachter et al. have demonstrated that no conclusion can be drawn regarding the efficacy of n-3 fatty acid (FA) treatment in children/adults with asthma [95].

Inflammatory Bowel Disease (IBD)

Crohn's disease and ulcerative colitis are both inflammatory bowel diseases. They are complex disorders in which immunologic, genetic and environmental components are involved. Ulcerative colitis is a disease where characteristic ulcers are seen only in colon; while Crohn's disease can affect the whole gastrointestinal tract. Both ulcerative colitis and Crohn's disease are characterized by T cells infiltrating the colon and GI tract. Crohn's disease is now implicated to be associated with impaired cytokine secretion by macrophages, thus being classified as a disease caused by impaired innate immunity, leading to a sustained inflammatory response in the colon, where the bacterial load is high. Another theory is that the inflammation in Crohn's disease may also be caused by an overactive TH1 and TH17 cytokine response [96]. Inflammatory cytokines and other mediators of inflammation are actively produced at the site the site of inflammation in IBD [97]. It was suggested by Shoda et al. that in Japan, increased consumption of n-6 compared to n-3 PUFAs may be implicated in increased incidence of Crohn's disease in the country [98]. Clinical usefulness of dietary PUFAs has been demonstrated by many, as reviewed by Calder [99]. A meta-analysis on 13 studies showed that delay of relapse appears to be a consistent finding in ulcerative colitis patients after dietary fish oil treatment [100]. Feagan et al. have also shown longer maintenance of remission in Crohn's disease patients after the treatment with PUFAs [101].

Psoriasis

Psoriasis is an immune-mediated disease characterized by dermal inflammatory response and epidermal hyperplasia. The disease has a risk of developing severe metabolic disorders such as diabetes, hypertension, obesity or even cardiovascular disorders [102]. Increase in C-reactive protein due to elevated levels of IL-6 in the sera is often noted in patients with psoriasis. TH17 and TH1 cells responsible for producing inflammatory cytokines are detected in psoriatic lesions, which are implicated in the pathogenesis of psoriasis [103].

Several studies have shown that omega-3 fatty acids EPA and DHA can control symptoms in psoriasis and limit spreading of inflammatory process [104–106]. Leukotriene A4 produced by polymorphonuclear leukocytes can be converted into leukotriene B4 in psoriatic skin, which is a primary inflammatory mediator. Omega-3 LC-PUFA supplements help the topical treatment of psoriasis by significantly reducing the psoriasis area and severity index (PASI). Diet supplementation with PUFAs also improves the dermatological life quality index, lesions on the scalp, itching, erythema and desquamation in patients with psoriasis, compared to topical treatment without supplementation with PUFA [104–106].

Cardiovascular Diseases

Beneficial and protective effects of omega-3 on cardiovascular system have been shown by many clinicians and scientists [106–109]. PUFAs are shown to be useful in reducing

cholesterol levels, thus reducing the risk of atherosclerosis. Involvement of immunological impairment and clinical evaluation of intervention with omega-3 in cardiovascular diseases has been aptly reviewed by Simopoulus [88] and Gogus and Smith [106].

Inflammation and IL-6 appear to be important in pathogenesis of cardiovascular diseases. Inflammatory markers such as C-reactive protein and fibrinogen are raised in patients suffering from chronic coronary artery disease [110, 111]. Cytokine IL-6 is known to stimulate synthesis of all acute phase proteins including C-reactive protein [110]. Using in vitro system, Khalfoun et al. showed that EPA and DHA significantly reduce the production of IL-6 by endothelial cells [112]. Mutations in IL-6 gene supplies the link between IL-6 and cardiovascular diseases [113].

Acknowledging the protective efficacy of PUFAs, they have been incorporated in the diet of astronauts to protect their cardiovascular system from oxidative stress of space [114]. Intake of EPA and DHA is recommended in daily diet as a treatment of post-myocardial infarction and to prevent sudden cardiac death [109].

After a systematic search of randomized clinical trials associating dietary intake of fish, marine n-3 PUFAs and coronary heart disease, a moderate association was shown by Mente et al. [115]. This observation needs serious consideration as daily supplementation of fish oil caused significant increase in heart beats of patients with VF (ventricular fibrillation) and VT (ventricular tachycardia), which could have fatal consequences. Another meta-analysis conducted by Preiss and Sattar suggests that although omega-3 fatty acid supplementation has cardiovascular benefits, more data are needed to use it in routine clinical practice [116].

Diabetes

Patients with diabetes have three- to fourfold increased risk of CHD [117], due to hyperglycaemia and insulin resistance and other risk factors such as excessive weight, hypertension and dyslipidemia. Epidemiological studies have shown that populations such as Eskimos, Japanese and Alaskans, whose main diet is fish, show reduced mortality from CHD [118]. They also show low prevalence of diabetes [119]. Immigrants to India show high incidence of diabetes and CVD, as their n-6 intake is more than n-3 fatty acids [120]. Dietary n-3 fatty acids lower the plasma triglyceride levels by 20–50 %in diabetic patients [121]. Diet supplementation of type 1 diabetic patients with n-3 fatty acids is shown to improve neutrophil chemotaxis [122].

Effect of Polyunsaturated Fatty Acids on Complications Associated with Diabetes

In order to establish risk factors for CVD in diabetic patients, a meta-analysis was conducted by systematically searching databases from 1966 to February 2006. Changes in C-reactive protein, IL-6, TNF-alpha, platelet function, fibrinogen, factor VII, von Willebrand factor, endothelial function, heart rate and blood pressure were recorded [123]. The analysis concluded that in addition to dyslipidaemia, n-3 PUFA decreases diastolic blood pressure and appears to increase factor VII as risk factors for CVD. Another meta-analysis conducted to identify risk factors for CVD in diabetic patients treated with omega-3 fatty acids confirms the triglyceride-lowering effects of omega-3 PUFAs, along with improvement in thrombogenesis. Omega-3 PUFAs raise LDL levels. However, changes seen in conventional risk factors seem to be insufficient to explain the cardiovascular disease risk reduction, implicated to occur with omega-3 PUFAs [124].

Diabetic neuropathy is a degenerative complication of diabetes related to abnormal nerve conduction due to decreased activity of Na, K ATPase. It has been demonstrated that fish oil supplementation can modify the fatty acid composition of sciatic nerve membranes of diabetic rats treated with fish oil [125].

Effect of n-3 fatty acids on controlling nephropathy in type 2 diabetic mouse model seems to be at the level of down regulation of protein MCP-1, a chemoattractant for macrophages, restricting migration of macrophages to kidneys [126].

Diabetic retinopathy is the most common complication in diabetes resulting in macular oedema and blindness. Reactive neovascularisation and neural degeneration can be modulated by n-3 fatty acids [127].

Deficiency of Polyunsaturated Fatty Acids as a Causative Factor for Diabetes

Type 2 diabetes is a highly prevalent disease with a lifetime risk. Modifiable lifestyle factors, including diet, are thought to play an important role in the development of diabetes and its cardiovascular consequences [128] Among dietary components, long-chain n-3 fatty acids (FAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are found to be beneficial for reducing the incidence of cardiovascular diseases in older adults [129]. Dietary fatty acids also play an important role in reduction of cardiovascular events in diabetic patients [130]. In contrast, controversial data have been reported on the effects of n-3 FAs on risk of diabetes. Several prospective cohort studies have reported significant, positive associations between dietary n-3 FA consumption and incidence of diabetes [131], whereas others have shown no significant connection between the two [132]. No significant association has also been reported on plasma concentrations of marine n-3 FAs and incidence of diabetes [133]. Another study, with the use of objective biomarkers, has shown that consumption of long-chain n-3 FAs and

ALA was not associated with a either high or low incidence of diabetes. In fact, individuals with the highest concentrations of both types of FAs showed lower risk of diabetes [134].

Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune inflammatory disease affecting central nervous system and spinal cord. The damage results in a wide range of signs and symptoms, including physical, mental and psychiatric problems. It is thought to be caused by either destruction by the immune system or failure of the myelin-producing cells to produce myelin sheath. Proposed causes for this condition include genetics and environmental factors. The disease usually begins between the ages of 20 and 50 and is twice as common in women as in men. The disease is manifested as multiple plaques or lesions in the white matter of the brain and spinal cord. There is no known cure for multiple sclerosis. Treatments can improve function after an attack and prevent/delay new attacks. The approved immunomodulatory therapies for MS have shown modest efficacy, with wide range of side effects [135]. Therefore, an unconventional approach undertaken recently is the dietary supplementation with polyunsaturated fatty acids [136].

Several clinical trials on the use of polyunsaturated fatty acids in the diet of MS patients have been reported [137]. A study conducted in Norway showed inverse relationship between consumption of fish and risk of developing MS [138]. In many studies, it is shown that intake of high levels of vitamin D, present in cods, reduces the risk of developing MS [139]. However, studies on large cohort of female nurses, high risk with saturated fatty acids and protective effect of unsaturated fatty acids could not be demonstrated [137].

At the laboratory level, several studies showed reduction in PUFA content in sera, brain white matter, erythrocytes and lymphocytes of omega-3 untreated MS patients [140]; however, whether in such condition supplementation with fish oil will correct the deficit is not clear. Derivatives of PUFA such as D-series resolvins are known to control inflammation in neural tissues and inhibit neutrophil activity [141]. Protectin C1 exerts anti-inflammatory response by reducing TNF-α and IFN-ɣ production and also induces apoptosis of T cells [142]. Expression of nuclear factor NF-κB, involved in production of pro-inflammatory cytokines and chemokines is shown to be inhibited by omega-3 PUFAs [143]. They can also promote in vivo, the synthesis of myelin basic protein [144]. PUFAs are natural ligands for peroxisome proliferator-activated receptors (PPARs), which regulate genes involved in lipid metabolism and also play a role in anti-inflammatory responses. PUFAs can act as PPAR antagonists and reduce inflammation in animal models of MS and can well be doing the same in humans [145].

Clinical studies conducted so far have generated inconclusive results [146] in spite of excellent laboratory data. This could perhaps be due to main limitations such as inappropriate placebo, insufficient information of adequate dosage and absence of proper outcome measures.

Systemic Lupus Erythematosus (SLE)

Systemic lupus erythematosus (SLE) is an autoimmune disease in which the body's immune system makes antibodies against many self tissues. It can affect the skin, joints, kidneys, brain, heart and other organs. The underlying cause of this autoimmune condition is not fully known. The common symptoms of SLE are joint pain and swelling, and sometimes arthritis. The patients typically show a 'butterfly' rash on cheeks and swollen lymph nodes. Other symptoms such as headaches, abdominal pain, arrhythmias, coughing up blood, swelling in the legs and weight gain are noted, depending on affected part of the body. The immunological abnormalities are circulating antinuclear antibodies (ANA), anti-thyroglobulin antibodies, anti-thyroid microsomal antibodies, complement components (C3 and C4), anti-Ig antibodies. There is no cure for SLE. The goal of treatment is to control symptoms. Severe symptoms that involve the heart, lungs, kidneys and other organs often need specialist's attention.

Since it has been often demonstrated that omega-3 affects production of C-reactive proteins, pro-inflammatory cytokine, chemokines and other pro-inflammatory mediators, which are the features of SLE [147], it was felt that supplementation with these lipids may offer additional modality of treatment for SLE as well [148]. Several studies report beneficial effects of dietary supplementation with PUFAs on pathogenesis and relapse events in SLE, the recent ones are quoted here [149, 151].

At the laboratory level, Aghdasi et al. showed the presence of low levels of omega-3 EPA and DHA in red cell membranes of omega-3 untreated women suffering from SLE compared to controls. Whether this is due to low intake of omega-3 PUFAs or whether it is the consequence of the disease was not clear [152]. In another study, higher levels of EPA/DHA found in the fat cells of SLE patients negatively correlated with disease activity assessed by SLE Disease Activity Index (SLEDAI) and presence of atherosclerotic plaques [150].

However, these studies were conducted on small number of patients and were of short duration. These limitations warrant further studies to evaluate the effectiveness of omega-3 PUFAs on amelioration in SLE, a difficult-to-treat disease.

Summary and Research Leads

Over the last two decades, dietary consumption of omega-3 PUFAs has been advocated for many diseases characterized by excessive chronic inflammation, based on the laboratory findings that polyunsaturated fatty acids can, in a major way, modulate the immune system. The immune parameters assessed for modification by PUFAs are many, which include changes in fatty acid composition in cell membranes and membrane fluidity; cell signalling affecting expression of surface receptors such as adhesion molecules and MHC molecules; cytokine production, which control the key events in immune response; phagocytosis; and antigen presentation, T and B cell proliferation and differentiation, and activity of regulatory cells. Most of these events have been studied on in vitro culture system, where all conditions can be optimized by the researcher, including dose and quality of PUFAs and their derivatives to be added, favourable culture conditions, amplified end points (e.g. proliferation induced by polyclonal mitogens as a measure of proliferation ability of lymphocytes stimulated with antigen) and non-interference of inherent influences present in an intact host such as hormonal, psychological, environmental, seasonal and lifestyle influences. In vitro culture system is complex, and variations are seen with cell types, donors of cells, media especially sera used, oxygen tension and so forth. It is therefore necessary to keep in mind that direct extrapolation of these results to humans should be made with caution.

Same holds true with animal studies, where inbreeding has removed integral genetic variations that exist in human situation. Also, perfect diet control is possible in animal studies, while in humans controlling the ratio between omega-6 and omega-3 and other fatty acids to the desire level is difficult to achieve. Also, the animal studies do not provide us the exact dosage and duration of treatment, as many human studies have shown that withdrawal of added omega-3 PUFAs in the diet reverted the beneficial effects as assessed clinically, as well as in the laboratory.

Many mechanisms appear to be involved in understanding the effect of PUFAs on immune system, as the system itself is very complex. Although a great deal of attention is being paid recently to understand the underlying mechanisms, a lot is yet to be resolved. Membrane fluidity is one such aspect, while few others are activation of specific genes involved in expression of receptors and mediators required for cell migration, cell activation receptors expression and cytokine production, although we know the effects are not very well aware of the molecular mechanisms.

The effect of omega-3 PUFAs on inflammatory conditions is now unequivocal, proved in the laboratory as well as in the clinic. However, immune system is a double-edged sword. When changes in phagocytic cells are in favour of control of inflammation, their phagocytic activity is jeopardized and the host might get immunocompromised and might show susceptibility to infections. Also, although the effectiveness of dietary PUFAs on existing inflammatory diseases has been proved, further research is needed in controlling dose and duration of treatment to have sustained effect. Also, effectiveness of PUFA diet on onset of the disease in susceptible hosts has been shown in experimental models, in human situation, it is a difficult proposal.

Inclusion of PUFAs in the diet of expecting mothers, especially those who are prone to develop atopy and other autoimmune and inflammatory diseases, appears attractive but is still in its infancy. Further research is needed in respect of time point to start the treatment, doses to be given, continuation of treatment in lactation, etc. This is important for pregnant women as there is a risk of bleeding involved. It is most important to investigate whether the infants of treated mothers, although free from asthma, are susceptible to other infectious respiratory diseases. It also needs to be seen whether infants and children of mothers susceptible for inflammatory diseases should be given oral PUFAs, although some literature is already available on this aspect, confirmation is needed. Also, whether formula/breast feed supplemented with PUFA can help preventing infections in preterm and IUGR babies needs to be seen.

It is shown recently that fatty acids may have a role in inducing cell death in cultures. This observation may provide a lead to test them in inducing cancer cell death.

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The Linoleic-to-Linolenic Dietary Intake Ratio: The Fundamental Implications of Imbalance and Excess Looked at from Both a Functional and an Evolutionary Perspective: An Overview

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Robert Andrew Brown

Terms	
AA	Arachidonic acid (Omega 6 20 carbon derivative of LA)
ACOX	Acyl-CoA oxidase (first step in peroxisomal beta-oxidation)
ALA	Alpha-linolenic acid (Omega 3 18 carbon plant polyunsaturated fat)
ATP	Adenosine triphosphate (enzyme used as an energy carrier)
COX	Cyclooxygenase (enzymes catalysing oxidation of fatty acids)
CO_2	Carbon dioxide (common atmospheric gas)
DHA	Docosahexaenoic acid (Omega-3 22 carbon derivative of ALA)
GLA	Gamma Linoleic acid (Omega 6 18 carbon plant polyunsaturated fat)
LA	Linoleic acid (Omega 6 18 carbon plant polyunsaturated fat)
LOX	Lipoxygenase (enzymes catalysing oxidation)
MDA	Malonaldehyde (non-exclusive LA and ALA oxidation product)
O_2	Oxygen (common atmospheric gas)
PPAR	Peroxisome proliferator-activated receptor (Protein regulating gene expression)
Oxo-HODE	Oxooctadecadienoic acid (oxidation product of 13HODE)
SO_2	Sulphur dioxide (atmospheric gas)
TRPV1	Capsaicin receptor (pain and temperature-related receptor)
UV	Ultraviolet light (UVC, B and A being subgroups)
VEGF	Vascular endothelial growth factor (A protein signalling angiogenesis)
4HNE	4-hydroxynonenal (exclusive lipid Omega 6 peroxidation product)
13HODE	13-hydroxyoctadecadienoic acid (oxidation product of linoleic acid)

Evolutionary Importance of LA and ALA

Introduction

This overview seeks to gather, within an evolutionary context, diverse research highlighting the fundamental importance of Omega 3 and 6 plant-based fats, LA (linoleic acid), ALA (alpha-linolenic acid) and their oxidised derivatives (oxylipins) in early vesicles that facilitated the emergence of life and more widely; membrane structures, cellular signalling, UVB protection, energy and oxidative pathways including; photosynthesis, mitochondrial membrane function, peroxisomal activity; as well as immune function, defence, fat deposition, reproduction and wider function. LA and ALA are the predominant fats in plant material; without assistance from longer 20 and 22 carbon chain polyunsaturated fats, they have the capacity to support flourishing plant life. Some photosynthetic cyanobacteria and microalgae have developed the ability to make EPA and DHA, but many have not. This chapter has been written to be read in conjunction with a subsequent chapter on the relevance of the oxidised products of LA and ALA to mammalian and particularly human physiology.

The particular structural, polar and electrochemical properties of fats, and their inherent chemical stability, as variably moderated by the introduction of double bonds, make them ideal structural components of membranes, a compact medium for energy storage and a substrate for messengering systems. Discussion of their structural roles is limited to the evolutionary importance of simple lipid vesicles to the development of life.

GLA has been the subject of much research in humans, but is not central to metabolism in the same way as LA and ALA, is not a primary plant fat, and is less influential than

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ALA because of the additional distance of the first double bond from the methyl group, hence the inner membrane. Importantly, GLA likely has a reduced potential ability to transfer protons and/or electrons to an inner membrane. Ultimately, GLA is simply beyond the scope of the space and time available for this overview.

Basic organic reactions and energy production, hence the development of life, require the sufficient 'unassisted' concentration of the necessary substrates including simple organocompounds, minerals, sulphur and halides, within lipid membrane-enclosed vesicles. Lipids have bipolar 'amphipathic' characteristics that are conducive to the formation of membranes. Research shows even a mix of 'simple' lipids are capable of forming simple membrane-based vesicles. Clearly for the development of life, vesicle membranes must have the capacity to allow;

- Ingress and egress of the building blocks of life.
- Protection from excessive external oxidative pressures including ultraviolet light.
- Feedback of information about external environmental conditions.
- Sufficient integrity to contain reactions, including changes of pH and pressure.

The environment must contain sufficient concentrations of substrate, to allow lipid vesicles to accumulate reaction-viable concentrations of substrate by simple mechanisms such as concentration gradient diffusion, evaporation and accumulation in crystalline mineral structures.

Conditions of Existence

The creation of life demands conditions that will support it, including the potential for reactions that create organic molecules; catalysts; mechanisms for the organisation of structure including polymerisation; a fluid medium; fluid evaporation and so concentration; a protective enclosed yet accessible vesicle environment for those processes to happen; controlled temperature ranges consistent with life; time; a suitable pH range; and a harnessable not overly destructive energy source to power the necessary processes but at the same time sufficiently destructive to facilitate evolutionary adaptation.

Conditions of existence arguably selected LA and ALA as the predominant 18 carbon polyunsaturated fats best capable of supporting functional lipid vesicle membrane structures with the characteristics necessary for the development of surface sunlit terrestrial life forms.

Womb and Cradle of Life

The womb of life, those niche area(s) of earth likely to best meet the conditions of existence needed for the emergence of terrestrial forms, are suggested to be sunlit surface terrestrial locations exhibiting volcanic activity; likely thermal spring outfalls combined with gaseous vents; possibly adjacent to, or subject to periodic ocean inundation, faulting, subduction and eruption [1], providing a mix of magma mineral profiles so mineral solutes; together with varied shallow interlinked mineral and halide rich water pools originating from volcanic sources; and more specifically providing;

- **Raw materials**—Development of life probably depends on vent emissions, pools or pockets of fluid in crystalline structures, containing simple organic chemicals [2], minerals, halides, sulphurs and other raw materials present or accumulated in concentrations, consistent with life and adequate to support concentration gradient diffusion into and out of simple vesicles, without membrane pumps or pores, all energised primarily by UVC. Ion concentrations in modern cells may and logically would reflect early evolutionary solute concentration availability [3].
- **Light**—light exposure, particularly UV, is needed to shift electrons into higher orbitals and so power chemical reactions, but must not be so powerful as to destroy new material faster than it can be created. Basic building blocks, including halides, minerals and ions, catalyse organic elimination and substitution reactions, magnifying the effect of the longer, less destructive wavelengths of UV. The action of UVC particularly, but also UVB, on mineral-rich water and dissolved organic matter in oceans produces a mix of free radicals, including peroxide, singlet oxygen, hydrated electrons, superoxide, hydroxyl radicals, and a range of bromide- and iodide-based products, simple organic compounds [4] and oxidised lipids, and by biodegradation products such as acetate, acetone, citrate and formate. Circular polarised light of celestial origin, and (or) following passage through chiral mineral crystals [5] and/or mineralised organified water, as well as reaction conditions, may have helped create the necessary product chirality for the formation of complex sugars and proteins (see below).
- **Reactive substrates**—Volcanic pools, and/or trapped ocean water, rich in electron acceptors and donors, including transition metal ions, halides, particularly the more 'reactive' iodides and bromides and likely sulphur derivatives with their greater inherent 'reactivity' than hydroxyl groups, were key elements in reducing the energy input required for, hence catalysing, basic organic

elimination and substitution reactions. The large size, and number of electrons possessed by iodine; its tendency to bind covalently with double bonds in organic compounds or by simple non-bonding association without the abstraction of hydrogen [6]; by creating large diffuse electron clouds, may assist interaction with photons, so accounting for iodine's evolutionary importance in water-based organic reactions; and the existence of the 'iodine cycle' by which iodine is cycled between the ocean and land, via the atmosphere and rain. Significantly, attachment of iodide, but not bromide, appears to confer UVB photon absorption properties, giving iodine/iodide particular relevance post-oxygenation of the atmosphere and potential for increased atmospheric UVC absorption [7, 8]. Hydrogen sulphide will reduce iodate to iodide, which would facilitate iodo-organic reactions [9]. There are also other pointers to iodine being a key element in the emergence of life [10].

Larger size, so more loosely bound electrons, also confers sulphur derivatives in solution with willingness to react more readily than hydroxyl ions. Hydrogen sulphide and likely other related 'small' molecules cross membranes by simple diffusion and could provide a reductive or oxidative source within vesicles. The potential reductive power of sulphur products is seen in the action of glutathione and might have provided mechanisms to support energy production and cycles of non-assisted membrane oxidative reductive LA/ALA flip-flop [11, 12], as a possible basis of charge, ion, proton/electron and substrate, import and export, across early lipid vesicle membranes. Glutathione is particularly important in human physiology, because it can reverse initial phospholipid oxidation in situ in the membrane; could this unusual characteristic hint at an early evolutionary membrane's redox role for hydrogen sulphide of volcanic origin. Interestingly, in humans, hydrogen sulphide reduces hydroperoxide fats [13], potentially helping regulate flip-flop and hence the transport of substrate and/or of electrons/protons.

- **Evaporation**—Desiccation and other related drying/concentrating processes encourage nutrient aggregation, polymerisation, vesicle formation, chiral product enhancement and transfer of substrate into vesicles.
- **Protective environment**—A crucial evolutionary requirement for the emergence of 'life' is a reaction environment, that is temperature and light protective, and facilitates the necessary delicate balance between change and stability, for example, mineral-rich muds or more likely mineral structures crystallised from volcanic waters, such as zeolites, which are potentially mini chemical-processing plants:
  - hydrated, sometimes highly absorbent,
  - potentially crystalline chiral UV light filters, of varied thicknesses and opacity,
  - reaction vessels, containing catalytic mineral atoms and interstitial pores of varied size that are sufficiently large to allow ingress and egress of water, hydrogen sulphide, sulphur dioxide, mineral ions and small-diameter organic molecules,
  - organised crystalline structural filters, with pore diameters ranging between 3 and 10 angstroms, which can differentiate, for example, between branched and linear organic chains [14]; matter with diameters close to the pore size can potentially squeeze through because of vibrational dynamics [15], thus conceptually providing very specific product sifting,
  - capable of alternate desiccation and hydration, resulting in breakdown and reformation of the hydrated zeolite crystal structures, thus potentially allowing egress of newly formed but matrix-trapped molecules; or incorporation of new material for processing prior to the recrystallisation that follows rehydration,
  - contain large amounts of hydrogen-bonded water, also possibly hydrogen sulphide or carbon dioxide, in their structures, which, on drying and consequent matrix shrinkage, would likely give rise to polar charges. The effects would be magnified by physical constrainment and, in addition by the catalytic effects of transition metals in the crystalline structure, and by semiconductor inclusions such as manganese sulphate [16], thus multiplying the potential for organic reactions in the restricted limited diameter tubular pores, including the possible formation of organic molecular chains and chiral products,
  - and at modest temperatures, e.g. 20 °C, dry zeolites are very good absorbers of sulphur dioxide, [17] absorption increasing with gaseous SO_2 content, potentially increasing gaseous concentration gradients within the crystal matrix, so potentially changing zeolite catalytic properties; the paper did not look at the effects of moisture on absorption,
  - further, physical constriction of reaction spaces, such as in the longitudinal tubular pores in zeolite crystalline structures, could determine the spatial structure and nature of products, including selecting for long-chain rather than bifurcated complexes. By encouraging and facilitating repeatable geometrically spaced catalytic function, as determined by the mineral crystal structure of pores; zeolites could potentially give rise to protein production and polymerisation, or long-chain lipid production.
- **Organic chemical plant**—The volcanic environment as a whole forms a matrix of environments, including mineralised shallow pools, muds, mineral matrix pores and interstitial spaces between crystals, whose area is accessible to light and raw material substrates and subject

to temperature pressure and diurnal variation, the whole allowing a series of interconnected processes, together comprising a natural organic chemical plant.

- **The oceans**—Early endothermic prototype chemical-processing vesicles could, subject to favourable substrate concentration gradients, have arisen in energy providing oceanic thermal vents. However, an exothermic capacity, based on the ability to make and modulate energy production through stored substrate, is a key requirement of sophisticated cellular function and diurnal terrestrial surface 'life', as further discussed below.

  Some suggest that exothermic life first developed in thermal ocean vents however on the balance of probabilities, the temperature conditions in the ocean vents would be fairly stable and provide sufficent constant energy to power life forms, but in the absence of regular cyclical temperature changes, there would be no environmental pressure to develop an exothermic capacity, which suggests that ocean vents were unlikely to have been the womb of exothermic life.

  In contrast, it seems a reasonable proposition that following the intial emergence of life, likely in a terrestial surface volcanic rather than oceanic volcanic environment, as life further developed in the longer time frame, life moved into and was nurtured by the deep oceans, which provided relatively stable protective and varied environments, and where penetrated by UV also a possibility for change and evolution. Sea water may contain 3 % lipids [18], including a high proportion of oxylipins and other simple organic molecules, which when added to the free radical-generating properties of UV, the catalytic action of minerals and halogens, particularly iodine, sulphur and water radicals; points to oceans [19] being fruitful fertile chemical soup cradles, that provided the necessary long-term support for the onward development of life [20].

Terrestial life holistically depends on earth; being a planet of a particular size and composition, having a molten magnetic core, being at a particular location in the solar system, providing the necessary elements, containing liquid water and providing a protective atmosphere. The particular characteristics of the magnetic metallic core and atmosphere, created a precise neither to 'hot' nor too 'cold' light energy window on early earth, that allowed longer UVC to reach the earth's surface and interact with organic matter including proteins and polyunsaturated fats, in addition conditions permitted the passage of the longer wavelengths that power photosynthesis; so together allowing the evolution of the complex structures that underlie life.

Light and Energy

Sunlight [21] through photons powers most terrestrial life. Photons energise electrons into higher orbits, fuelling chemical including redox reactions. The wavelength of light determines its energy levels; a UVC photon with a wavelength of 200 nm has 3 times more energy than a UVA photon of 400 nm.

Light wavelengths most relevant to the development of 'life' on earth are those produced in greatest amount by the sun. Sunlight, and especially the UVC waveband between 200 and 290 nm, was key. By design or good fortune, the early atmosphere of earth created a filter window that selectively allowed this critical solar UVC waveband, along with UVB, UVA, visible light and infrared, to reach the surface of the earth and a subset thereof to penetrate the oceans, which have very complex optical properties [22]. UVC, UVB and UVA have different biological effects. UVC is capable of directly attacking the bonds of the individual substrate; UVB results in lipid and DNA damage by direct and indirect pathways; UVA increases oxidative stress assisted by photolysis, enzymes and free radicals, largely by indirect pathways. Overall, UVC is the most damaging. At the end of the Archean era 3.8–2.5 billion years ago, following the evolution of photosynthetic cyanobacteria, the introduction of oxygen [23] into the atmosphere blocked UVC from reaching the surface of the earth. An oxygen-rich atmosphere altered the balance of solar UV penetration at the earth's surface to 0 % UVC, 5–10 % UVB and 90–95 % UVA. Subsequent to oxygenation, to varying extents, organisms developed antioxidant and protective pathways against UVB and UVA [24], which allowed the development of sophisticated, if not always sage, surface terrestrial life forms including humans.

Shorter UVC under 200 nm or so will lift sigma electrons into higher orbit, hence breaking key structural bonds. UVC (200–290 nm), and UVB (290–320 nm) through to visible light (390–700 nm), will lift pi or unpaired electrons into higher orbitals, hence catalysing reactions. The energy required to lift a pi electron is dependent on the configuration of the organic molecule, in particular, the number and interrelationship of double bonds and structural ionic charge, hence their conjugation and hyperconjugation. This is discussed further below, including the less recognised, more subtle 'hyperconjugation' effects seen in the polyunsaturated fats LA, ALA and DHA. In general terms, the more symmetrically placed double bonds in the same plane of a ring or chain structure, subject to bond spacing and ion presence, and the greater the conjugation, the lower the energy

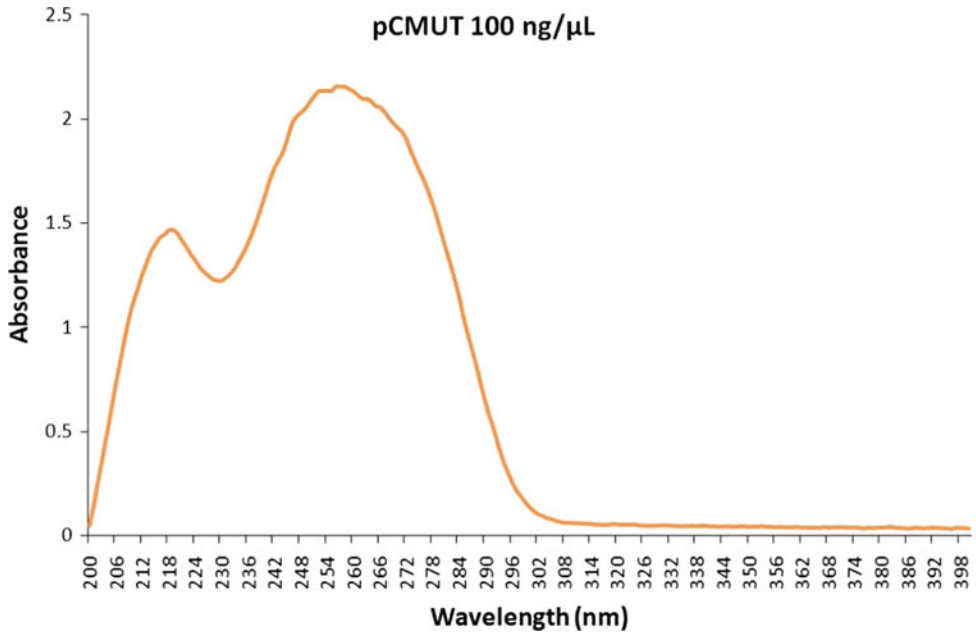

Fig. 27.1 'The absorption spectrum for the DNA molecule'. The graph powerfully makes the generalised point that DNA is potentially very susceptible to UVC in the range 200–290 nm and to a much lesser extent to UVB 290–320 nm. This arguably had significant evolutionary implications imposing faster evolutionary change prior to oxygenation and more stable conditions post-oxygenation, hence scope for more sophisticated life forms. This graph is from 'Biological Sensors for Solar Ultraviolet Radiation' by Yagura et al. [28]. I am very grateful and thank them for their kind permission

required to lift a pi electron into a higher orbit, hence the very useful and felicitous ability, of less powerful and so less biologically 'damaging' visible light, to energise pi orbitals in conjugated photosensitive material and power terrestrial and marine plant photosynthesis.

Shorter UVC under 200 nm is destructive, and can by displacement of sigma electrons break bonds; carbon to carbon, carbon to hydrogen, carbon and hydrogen to oxygen; so directly destroying essential organic compounds. Arguably, the very destructive potential of shorter UVC exceeds its creative potential. Opportunistically, the early terrestrial surface was over time likely shielded from UVC under 200 nm by atmospheric CO_2 accumulation, probably of volcanic origin.

Longer UVC (200–290 nm) was able to penetrate CO_2, thus reaching the surface of early Archean [25] earth. Crucially, for the creation of life, longer UVC will interact strongly with a range of relatively simple biological molecules, including importantly proteins [26], peptide chains including DNA at 200–296 nm (Yagura) (Fig. 27.1) and double- and triple-bonded polyunsaturated lipids. For example, UVC stimulates the creation of proteins from simpler molecules (at 254 nm) [27] and their disassociation, and so potential of proteins to form peptide chains; it also oxides polyunsaturated fats to biologically important derivatives. The presence of; transition metal ions, halides, hydroxyl ions, sulphur radicals and other electron acceptors and donors would magnify the effects of UV by influencing bond configurations and polarity, so making pi bonds more reactive to radiation of longer wavelength, so lower power.

UVB (290–320 nm) [29] can penetrate both CO_2 and O_2, so both the pre- and post-oxygenated atmosphere, but has limited ability to pass through cloud or dust haze. UVB has significantly lower energy than UVC, but importantly does lead to the photolysis of mineralised and iodine-containing water, creating the potential for elimination and substitution reactions, and so UVB remains a very relevant and important source of photo-oxidation in ocean water and aqueous cellular milieu. It will, for example, oxidise lipids including LA and ALA in plant membranes, thus affecting photosynthesis [30] and in humans cause skin 'burning', as well as oxidising oils in aqueous emulsion and indeed, oils alone, although results vary [31]. The optimal absorption spectrum for LA is around 217 nm [32], and for oxidised and conjugated ALA derivative products around 270 nm; the impact of UVB (290–320 nm) on oil may well also be moderated via other longer products in oils, such as polyphenols, or via catalytic pathways. UVB will also directly and indirectly damage DNA, as does UVA; both mechanisms provide potential for evolutionary change.

UVA (320–390 nm) can penetrate CO_2, O_2, cloud and dust haze, but has lower energy than UVB; it does not lead to the photolysis of pure water, or direct oxidisation of lipids, including LA and ALA and their derivatives in skin tissue; but it does lead to indirect oxidation, presumably via UVA photosensitive chromophore material in tissue such as

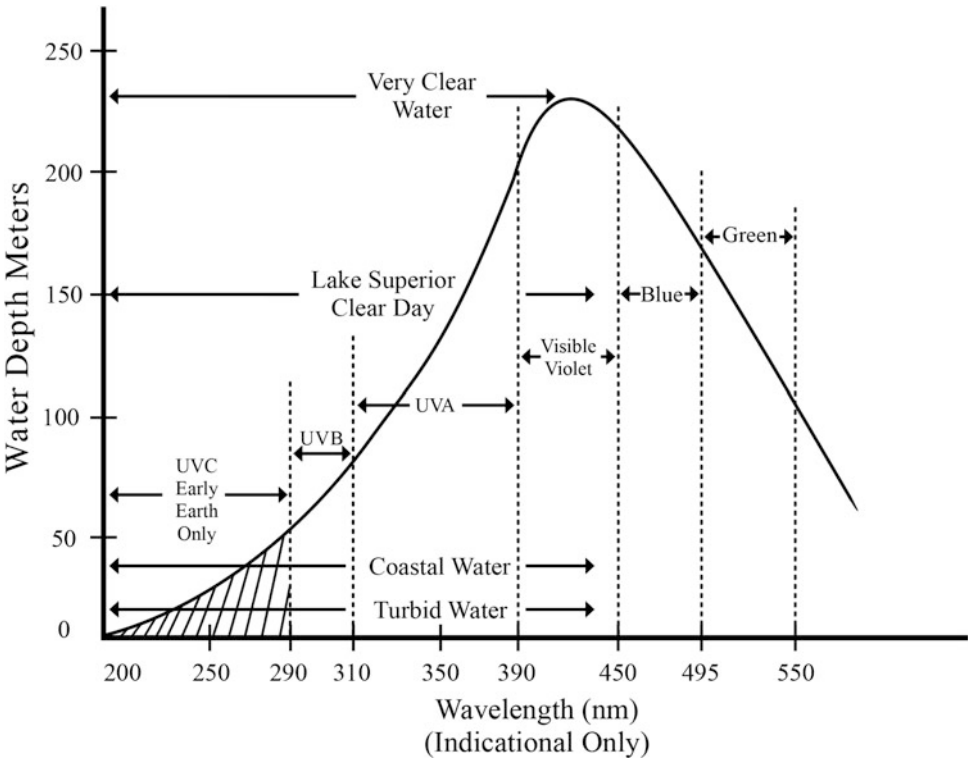

Fig. 27.2 Water is partially transparent to UV and visible light; the absorbance varies tremendously depending on the particular content of the water, thus providing a wide selection of possible UV exposures. (The diagram is indicative and drawn from a variety of sources, including 'UV penetration in the water column' by Vantrepotte and Mélin [36].) I am very grateful and thank them for their kind permission.)

porphyrins, proteins, flavins, vitamin B6 and vitamin K2 [33], ultimately triggering oxidative cascades, hence uncontrolled damaging transfer of electrons. In evolutionary terms, if early earth was obscured by clouds for long periods, UVA photo-oxidative products may have had an as-yet-under-recognised relevance in shaping membrane properties, solar protection and signalling pathways. There is a growing recognition of the role of UVA-based DNA damage in skin cancer incidence [34]. Different wavelengths, including UVA, effect differently composed DNA differently, and importantly produce different types of damage, with different consequences in different circumstances [28]; in other words, biological systems and interactions generally are vastly more complex and nuanced than widely portrayed.

Visible light (390–700 nm) will excite electrons in heavily conjugated lipids and other organic substances sufficiently to emit photons, and so power visual systems; it is self-evident but important that visible light is not as destructive as UVB and UVC. Infrared, whilst of lower energy, still affects cellular function, for example having physiological effects on cytochrome-C, and hence energy production and repair [35].

Light-Protective Evolutionary Niches

In the Archean era, penetration of the atmosphere by longer UVC (200–290 nm) likely provided the necessary energy to power the formation of lipids, complex proteins and wider organic matter. However, UVC is also destructive. Arguably, the evolutionary requirement for conservation of positive structural change; such as of peptides, hence enzymes and the DNA development necessary for the existence of more sophisticated life forms, makes it likely that early life forms could 'regulate' their UVC exposure; potential mechanisms include:

- Ascent or descent in the volcanic or oceanic water column allows short-term, random and/or controlled exposure to UVC, UVB, UVA and visible light (Fig. 27.2). The UV transparency of water is altered by a wider range of factors, including concentration of mineral and organic content as varied by terrestrial run-off and upwelling, temperature changes, and mixing as seen in surf [36]. Polyunsaturated fats are used by marine organisms to control buoyancy. Interestingly polyunsaturation of lipids changes density, as does UV oxidisation of them, thus creating an early buoyancy feedback mechanism in response to UV exposure. Absorption of UV energy through oxidation would also provide vesicles with protection from UV.
- Sheltering in an aqueous media containing UV co-absorbers, such as mud and slime, would provide further protection from UV. Some mineral products such as zinc sulphide may also increase UV protection, as well as enhancing chemical reaction potential, thus helping create fertile territory for the evolution of life forms.

- Sheltering, in clear or opaque crystalline structures, in vertical longitudinal voids or shadows between crystals, creates panoply of options for varying wavelength of light, intensity and exposure time.

Anoxic Environment

The Archean early earth atmosphere is believed to have been low in, or absent oxygen. In an anoxic, volcanic water, and vapour, related environment, rich in; minerals, sulphur compounds and halides; all sorts of 'unusual' reactions and stable products may have been routinely possible, that would be unstable in an oxygen based atmosphere, including:

- Ether-bonded lipids as possible components of lipid membranes [37].
- Organozinc complexes, some of which are relatively non-polar, so may have crossed early lipid membranes into the vesicle, as well as having a capability of interesting linkages with aryl cyclical ring groups [38].
- Organomagnesium complexes are used in the preparation of a variety of organic compounds [39] and have the ability to introduce carbon-to-carbon bonds, thus to make chained products; if such reactions took place in a mineral structure such as a zeolite matrix, physical pore constraints and zeolite catalytic function conceivably might have resulted in the production of non-bifurcated polyunsaturated fats. Magnesium will also form soaps with lipids; magnesium association with the carboxyl group might allow transfer of it by flip-flop across the membrane into a vesicle.
- In a volcanic anoxic, high-UVC incidence world, sulphur dioxide and hydrogen sulphide based, rather than phosphate-based reactions, may have been key to energy production in simple vesicles, and hence 'life'. To make energy, 'modern' diurnal sulphur-based organisms utilise daylight to metabolise hydrogen sulphide or sulphur to sulphate and at night metabolise sulphate and/or hydrogen sulphide to sulphur. Analysis of sulphur isotope fractions in sandstone deposits believed to contain early sulphur-based life forms supports the idea that '*there existed multistep bacterial sulphate reduction. That is, bacterial sulphate reduction to sulphide, re-oxidisation of the sulphide to sulphate, reduction of the sulphate to sulphide*' [40]. Importantly, hydrogen sulphide is relatively non-polar as is sulphur dioxide, so both easily cross membranes according to relative concentration gradients; providing they are permitted to do so by the temperature-related permeability of the vesicle membrane. Sulphurs likely played pivotal roles in early metabolism;
  - **Sulphur**—Why was sulphur a key element in the development of early anoxic life? Sulphur exists in a wide range of oxidation states from reduced −2, to oxidised +6, and so presents a wide scope for reactions. It is larger than oxygen, so the electrons are less well bound and probably structurally less physically able to form stable double bonds, hence the many configurations of elemental sulphur, its greater propensity to reaction and potential to form organic compounds, despite it being less electronegative and so reactive than oxygen.
  - **Sulphur Dioxide and Hydrogen Sulphide**—Sulphur dioxide is common in volcanic gas; hydrogen sulphide is often present in dissolved form in significant amounts in some geysers and hot springs [41]. In an environment with high concentrations of hydrogen sulphide and sulphur dioxide, they could have diffused into and out of vesicles simply based on the concentration gradients, without pumps or membrane pores.
  - **Sulphur Life Forms**—We know that anoxic metabolism of sulphur for energy is possible, because sulphur-metabolising bacteria exist in extreme terrestrial and oceanic environments. They create energy by the enzymatic metabolism of sulphur products including hydrogen sulphide, sulphates and sulphur dioxide.
  - **Chemical Origins of Life**—Prior to the development of enzymes could life's processes, in a low-oxygen, high-UVC environment have originated in simple vesicles, in volcanic-related aquatic pools, and within or with assistance from zeolite structures?
  - **UV Energy Source**—Hydrogen sulphide subjected to longer UVC, 253 nm [42], breaks down to hydrogen and sulphur ions without the need for enzymes, which process if contained in a simple vesicle could possibly have been used to produce energy substrates, and create the potential for proton gradients, before, and independent of, the development of protein-based enzymes, pores and product pumps. Hydrogen sulphide by absorbing UVC also acts as a protectant.
  - **Sulphur Driven Energy Sources**—The anoxic chemistry of interactions and interconversion of sulphides to sulphates and sulphur is complex, but importantly both the direction and rate of reaction are very sensitive to pH, temperature and mineral catalysts such as those found in zeolites. Intrusion, inclusion or the immediate vicinity of zeolite and its associated minerals and absorbed substrates, within the vesicle, would likely catalyse and support reactions. Importantly, the relevant sulphur reaction temperature and pH ranges are consistent with terrestrial life. The

existence of a simple vesicle would allow the control of pH within the membrane. The terrestrial diurnal cycle creates day/night, temperature and UV incidence fluctuation, energy change and membrane fluidity change, thus changing reaction conditions, leading in turn to potential for pH change, thus the determination of reaction type. Reaction determinants, including temperature, UV, pH and endothermicity or exothermicity, control whether sulphides are converted to sulphites/sulphates, or sulphites/sulphates to sulphur. Reactive changes within a vesicle, driven by the diurnal light energy cycle, through the changing, pH, UV exposure and temperature could create an energy-producing self-regulating anoxic diurnal endothermic/exothermic, reaction feedback cycle.

- **Modern evidence**—Consistent with this as a potential mechanism, a 'modern' light-based anoxic sulphur-metabolising lake-dwelling bacteria in a narrow anoxic water layer, accessible to both adequate UV and sulphide, appears to have an offset diurnal cycle, swapping between sulphide metabolism in the day and sulphate metabolism at night. This is reflected by low sulphide levels in the water during day and much higher levels at night, due to replenishment from below and/or return of sulphide by the bacteria to the water layer. Sulphide water concentration starts to rise in the afternoon. Strikingly sharp, probably light energy-related, changes in water sulphide concentrations were seen at what was likely dusk and dawn, with flat sections in both the middle of the day and night and a changeover period of about 4 h (Fig. 27.3), [43]. Sulphur using bacteria in a hot oxic-sulphidic spring in New Zealand [44] and oxic microbial mats [45] also appear to have diurnal rhythms, but with oxic-based reactions.
- **Diurnal driver?**—In early 'life forms', as in plants, diurnal cyclical energy systems would have made sense; use light energy for substrate production in the day; use stored substrate created in the day to provide energy at night. Are 'modern' anoxic diurnal energy systems in sulphur-based organisms, and oxic systems in plants, a reflection of the mechanisms and so conditions of existence that enabled the emergence of early 'life'?
- **Diurnal early membrane fluidity cycle**—Did changing membrane fluidity with diurnal temperature also play a part? Cold night-time and warmer day temperatures, as further influenced by membrane oxidation status, would restrict or increase membrane fluidity, diurnally moderating product entry or egress through membranes and contributing to the feasibility of a diurnal energy system.

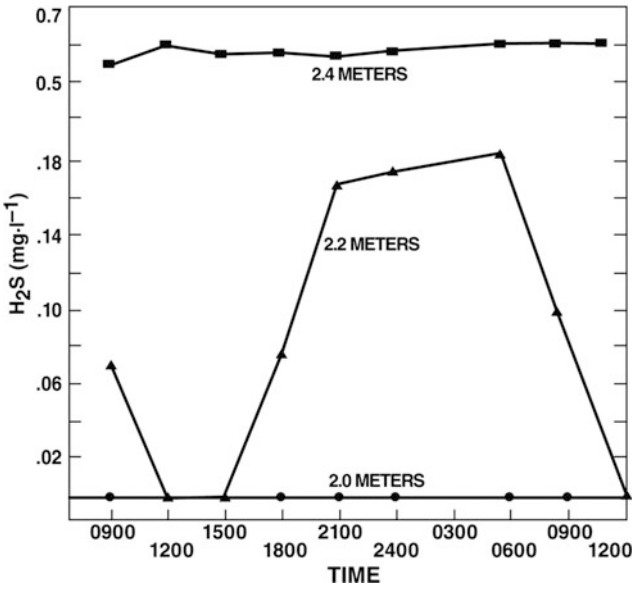

Fig. 27.3 In an anoxic thermocline water strata layer, of Knaack Lake during a 24-h summer period, water sulphide concentrations were mainly related to the activity of sulphur bacteria and were increased at night from stored sulphate or resupply from below. (The graph is from: 'The role of phototrophic bacteria in the sulphur cycle of a meromictic lake' by Parkin and Brock [43].) I am very grateful and thank them for their kind permission.)

- **Product transport into vesicle**—Hydrogen sulphide and sulphur dioxide are present in volcanic gases. Sulphur dioxide may be found in greater amounts than hydrogen sulphide, but hydrogen sulphide is less polar; so on a concentration gradient basis, it would cross a primitive membrane, much more easily than sulphur dioxide. Concentration gradient, hence intake, would be dependent on gaseous and/or dissolved emission concentrations, as well as conceivably local accretion and rerelease on saturation, by porous zeolite substrate, potentially following temperature change and/or release due to charge change in the structure of zeolite, consequent on the action of sunlight on photoelectric material and/or the effects of drying. As discussed, mineral ions, protons and/or electrons, could, independent of pores or transporters, be potentially taken across membranes by flip-flop, or related mechanisms.
- **Self-sustaining 24/7 cellular endothermic–exothermic energy cycle?**—Conceptually, an anoxic chemical energy cycle that was endothermic in the day, and exothermic at night, could provide the foundation for a 'living' energy production system. Arguably, diurnal UVC energy changes, combined with the existence of a closed vesicle, the interior of which can be accessed by hydrogen sulphide, sulphur dioxide, mineral and/or halide ions, could allow

sulphur reactions that are exothermic at night and endothermic in the day and lead to pH changes that both permit and control reaction feasibility by a number of mechanisms, in such a way as to comprise an anoxic light energy-based, 'self-sustaining', 24/7, exothermic/endothermic energy system.

Sulphur chemistry is clearly enormously complex, a specialist field, and much is unknown, but work on sulphur capture in chimney flues contains details of sulphur reactions that may meet these criteria in concept. It is assumed that flue gas research is a workable model for an anoxic system.

– **Experimental observations of sulphur reactions**—Experiments looking at sulphur reaction in flue gases showed that [46]:

pHs—pHs in the range 5–9 are both productive and reaction determinants for sulphur, hydrogen sulphide, sulphur dioxide, sulphite and/or thiosulphate/sulphate interactions and felicitously are within the PH bands consistent with the existence of life [47].

Reactions

- **(1) Acid exothermic—(night)**
  - Sulphide and thiosulphate react to produce elemental sulphur and water (acid conditions). ($S_2O_3^{2-}$ (aq) + $2H^+$ (aq) → SO_2 (g) + S (s) + H_2O) and/or ($2H_2S$ (aq) + $S_2O_3^{2-}$ + $2H^+$ → 4S (s) + $3H_2O$).
  - Hydrogen sulphide and sulphur dioxide in a ratio of 2:1 react to form elemental sulphur (acid conditions). ($2H_2S$ + SO_2 → 3S + $2H_2O$)
  - Over the night these reactions would reduce acidity. Further bromide ions might reduce daytime-produced stored SO_4^{2-} to SO_2 and water, assisting the system to turn alkaline.
- **(2) Alkaline endothermic + UVC—(day)**
  - Sulphite can be produced from volcanic sulphur dioxide (alkaline conditions). (SO_2 + H_2O ⇌ HSO_3^- + H^+) [48]
  - Volcanic sulphide and sulphite react to form thiosulphate, which is only stable in neutral or alkaline conditions (alkaline conditions). ($2HS^-$ + $4HS0_3^-$ → $3S_2O_3^{2-}$ + $3H_2O$)
  - Thiosulphate in the presence of halides is converted to sulphate, which would decrease alkalinity over the day driving pH back towards being acid. (alkaline conditions). ($S_2O_3^{2-}$ (aq) + $4Br_2$ (aq) + $5H_2O$ → $2SO_4^{2-}$ (aq) + $8Br^-$ (aq) + $10H^+$ (aq) [49]

Self-driving reaction cycle—A 24/7 cellular self-sustaining anoxic, endothermic alkaline daytime \ exothermic acid night-time energy cycle, in a simple vesicle, may be possible. The daytime production of sulphate and protons, and so acidification of the internal vesicle environment, could possibly provide a feedback control loop, slowing down the alkaline daytime reactions 2 as the day progressed. At night loss of UVC, so loss of the necessary photon power to drive endothermic day reactions 2, combined with rising acid conditions, falling temperatures and changing reactant concentrations, would likely result in night-time exothermic reactions 1 taking over. Over the course of the night, temperature falls would regulate reactivity and membrane fluidity, limiting substrate ingress and egress including of hydrogen sulphide, which is a potential night-time coreactant, and by restricting substrate egress leading to rising concentrations within the vesicle of the less membrane-permeable product substrates, sulphur and sulphur dioxide. Over the course of the night, production of sulphur by night reaction 1, by reducing proton numbers, combined with the removal of SO_4^{2-}, would likely reduce acidity and increase alkalinity in the vesicle, driving activity back towards day reaction 2. Once the PH had fallen back to being alkaline and with the reappearance of sufficient photon energy in the form of UVC, increased temperature and increased membrane permeability, endothermic day reaction 2 would likely recommence.

Postulate reaction 2 (day)—endothermic, anoxic, alkaline, + activation energy from UVC—On early earth, as daylight UVC returned, acidity fell, alkaline conditions resumed in the vesicle, temperatures rose and membrane fluidity increased, daytime endothermic reaction would resume. Sulphide and sulphur dioxide are present in volcanic gases and fluids; as membrane fluidity increased with temperature, they would likely enter a vesicle through the membrane on a concentration gradient. Reaction rates are sensitive to concentrations of sulphide and sulphite. Sulphur dioxide would be converted to sulphite. In the weak alkaline 8–9 pH range, the reaction of sulphide and sulphite produces thiosulphate and water, whose reaction increases significantly with temperature of 0–20 °C. Interestingly, and possibly of significance, thiosulphate demonstrates Rayleigh scattering; by widely scattering UVC and its energy throughout the vesicle, thiosulphate might help moderate the power of UVC, thus helping energise and propagate reactions. Thiosulphate in the presence of halides including bromide will form sulphate groups, which are inherently acid in solution. Further, the presence of halide ions and protons may open the door to other

organic reactions taking place and the existence of proton gradients. As the day progressed, the vesicle interior would become less alkaline, thus switching down the reaction rate.

Postulated reaction 1 (night)—exothermic anoxic acid—At the onset of sunset, as acidity rose, UVC energy input declined and then stopped, temperatures fell and the vesicle membrane became less fluid, the second reaction likely based on stored substrate, thiosulphate, would dramatically increase. In the acid 5–7 pH range, hydrogen sulphide [50], thiosulphate and/or potentially sulphate [51] are converted to elemental sulphur and water. The reaction is accelerated by silicon aluminium catalyst (also found in zeolites) and is exothermic, but becomes thermodynamically unfavourable and so will not happen at pH 7.6 and above. Siu notes a general reaction trend of more sulphur being formed as temperatures fell and sulphur dioxide rose; in addition to this, a fall in membrane fluidity may restrict egress of sulphur dioxide, thus possibly resulting in rising sulphur dioxide levels in the vesicle. As the sulphate and thiosulphate are exhausted, and sulphur is produced, the environment will return to being alkaline. After sunrise, once daytime UVC photon energy and temperature was sufficient to propagate endothermic reactions, and the membrane fluid enough to allow ingress and egress of necessary substrates, the daytime reactions will recommence, increasing in line with UVC availability, then flatten, and the fall as acidity again rises, so recommencing the night cycle. Consistent with this general direction of travel, acidification of freshwater lakes supports the reduction of sulphate and results in increased levels of sulphide [52].

Temperature—The sulphur reactions were very sensitive to temperature, a 1 K degree change translating into a 7–13 % yield change. A relatively small change in temperature could facilitate or terminate various reactions. In the relevant temperature range for life, 0 and 20 °C, large variations in reactivity were seen.

Mineral catalysts—Silicon oxide and aluminium (primary constituents of zeolites) increased the yield of sulphur from thiosulphate and hydrogen sulphide by a factor of 4–6 (night-time reaction).

Excess Sulphur—Any excess sulphur produced could possibly be exported by transmembrane excretion of elemental sulphur. Sulphur rings are relatively non-polar [53], and sulphur can sublime; so it could possibly cross a simple lipid membrane on a concentration gradient basis when the membrane became sufficiently fluid, or temporarily accumulate within the vesicle, until concentrations were sufficient to disrupt membrane function, thus allowing egress.

– **Feasibility of early sulphur metabolism**—Investigation of Archean sediments, including sulphur-based isotope experiments with UV ~ 193 nm and examination of related isotopes, suggests the then existence of life forms using UV-related sulphur metabolism and particularly sulphide and thiosulphate [54]. Interestingly indication of early sulphur life was found in volcanic shallow water caldera-type conditions, in a 'chert' [55] zone. Chert examined elsewhere included zeolites in its structure.

Based on the above, under a diurnal rythm, could hydrogen sulphide and sulphur dioxide of volcanic origin, crossing a lipid membrane on a concentration gradient into simple early vesicles, in an anoxic environment, be converted using a mix of daytime UVC energy, changing reaction energetics with diurnal temperature change, related pH fluctuation and membrane fluidity change, to sulphates in the day and elemental sulphur at night, creating an anoxic cyclical endothermic \ exothermic diurnal energy cycle, which in the day also provided surplus energy within the vesicle to fuel other, including organic, reactions. Minerals would catalyse reactions, as well creating potential for a mix of zinc and magnesium sulphates and organocomplexes in solution. Halides would further help catalyse organic activity, including elimination and substitution reactions. Self-evidently, but easy to lose sight of in our oxic world, reaction pathways in an anoxic world would have been very different.

If feasible, the suggested sulphur cycle or derivations thereof could form the building blocks of an anoxic sulphur-based metabolism and so provide a framework for the development of the reactions, on which early sulphur-based life was built. Other factors that may have played a part include the following:

- Once hydrogen sulphide is in the vesicle, could it have been used to reduce oxidised membrane polyunsaturated lipids that had been oxidised externally and flip-flopped across the membrane, or vice versa reducing lipids externally, so potentially moving protons or electrons into or out of a vesicle. Interestingly, in humans, hydrogen sulphide reduces LA lipid hydroperoxides in LDL to the less active HODE form.
- Thiols, the sulphur equivalent of alcohols, are also very powerful nucleophiles and redox agents, '*with a countless number of physiological functions*' [56] including the formation of glutathione. Thiols could have provided another anoxic pathway for reduction of oxidised LA and ALA in early vesicle membranes.

- Conceivably, substitution and elimination reactions could release ions and remake the double bonds and flip the reduced fat back across the membrane, or lead to a flip with an intermediate product attached, possibly halides and/or metal ions which complex with lipids, thus providing a possible cross-membrane transport system.

Organic reactions often involve halide ions. Volcanic vapours are significant sources of bromine and may contain more limited amounts of iodine. They may also contain significant amounts of hydrogen chloride, sulphur dioxide (1–50 %) and hydrogen sulphide (0–3 %) [57]. Further gases and reaction substrate will be dissolved in varied amounts in volcanic waters.

Organised Structure—Chirality and Non-bifurcated Chains

The next condition of life is organised structure [58]. Proteins can be produced from simple constituents through the application of ultraviolet light. The proteins created will be a mix of left- and right-handed forms, which if joined into chains would result in disorganised structures. Proteins of the same handedness when linked together to form gentle spirals, in the same way as Lego bricks form straight lines; it is a quality of their structure. It is the chirality, 'handedness' of organic molecules that allows, for example, the building of DNA, a spiral chain of chiral proteins, stabilised by chiral sugars. Oxidised lipids including LA and ALA also have chiral forms with different biological activities.

Circularly polarised UV leads to the production of polarised products including amino acids. Extraterrestrial rotationally polarised light could have been a factor; however, given that proteins and sugars do not all rotate light in the same direction, other factors may also be relevant to the evolution of terrestrial chiral products.

Simple lipid chains have different functions and constituents' properties to proteins, but like protein chains are non-bifurcated, begging the question why the production of single chains of molecules was preferentially selected over bifurcated organic structures; logically, there is no reason why side-chain products would not have formed equally, or indeed preferentially. Did the formation of life happen in zeolites, in their thin tubular longitudinal pores, the diameter of which would have restricted products formed to relatively simple non-branched chains? Were the fatty acid OOH, or OH groups, [59] a factor in their interaction with polar zeolites? Could polar tail groups have restricted the entrance of substrate to narrower pores of zeolite structures, creating a possible bond shear point, and mechanism by which a zeolite catalysed UVC-driven reaction could sever the polar group, allowing the chain remainder to enter the pore structure, providing a reaction point and a mechanism to facilitate elongation by addition of the next remnant that enters the pore?

Did planar ringed substances like cholesterol emerge from the planar fractures in the crystal structures? Further, the semiconductor status of some mineral crystals enclosed in the zeolite lattices may have enabled transfer of UVC energy from absorbed photons, into movable electrons, powering chemical change within the lattice.

How did simple non-bifurcated carbon chains, chiral forms of protein and sugar, and planar ring systems such as cholesterol, come to dominate key life processes, thus allowing the building of organised biological structures? There are a number of mechanisms [60, 61], which may concentrate particular chiral product forms, determine physical geometry including stearic properties and select for the formation of simple chains rather than bifurcated products. Potential mechanisms that have been demonstrated as at least feasible sources of chiral products and non-bifurcated chains include the following:

- Rotational polarised light, which could have originated from the universe including from neutron star bursts [62]; selective rotational polarisation of light by polarised mineral crystals as seen in quartz; and/or possibly water rich in polar product. Intriguingly, in manufacturing research, circularly polarised light induces nanostructures to form helical ribbons with chiral selectivity biased to the handedness of the polarised light source [63].
- Selective destruction of one enantiomer by polarised light leads to increased concentration, but not full chiral dominance of the unaffected product.
- Arrival of organic chiral seeding material in meteorites, after the formation through exposure in space to rotational polarised light or strong magnetised fields.
- Crystallisation and dissolution by similar or different solvents, on a repeated cyclical basis, concentrate chiral products in solution [64].
- Catalysed reaction including subject to temperature variations that may result in preferential production of isomers [65].
- Conceptually, within internal organised longitudinal structural pore configurations, substrate contact with polarised and or geometrical zeolite crystal lattices, thermal gradients [66], bound mineral and water lattice structures, could physically determine product outcome. In an anoxic volcanic scenario, hydrogen sulphide might also polarly bind with the crystal lattice in place of water, providing a whole new dimension of reactions. A system of microglass tubes which combined temperature gradients

with a flow recycling mechanism produced polymerised proteins, which self-promoted replication [67]. Pure industrially made zeolites are used extensively as catalysts for petrochemical molecular sieving including for non-bifurcated chains. They are also used in water purification systems and even in cat litter because they absorb ammonia, a key building block of life. There are about 40 natural zeolites, each with its own crystal structure, pore sizes and physical properties. They are often capable of forming clear crystals, so to varying extents will be transparent to visible light and UV, potentially could have 'chiral' forms and hence polarising properties. They are found in volcanic water-related natural rock outcrops. Some zeolites such as laumontite, which is found in large subterranean deposits, also can precipitate at the surface at modest temperatures 43–89 °C [68]; they require to be hydrated to maintain their crystal structure. Like many zeolites, laumontite contains significant amounts of water, which may be hydrogen-bonded and can be lost from the structure at room temperature, resulting in 'unit-cell' dimensions of the crystalline structure changing [69]. One could imagine as the mineral started to lose water, due to sun exposure and rising temperatures during the day; products such as strings of proteins, or short lipid chains, trapped in tubular and sometimes regularly interlinked pores, would be subject to significant heat, polar electron stresses and in addition to physical stress due to the deformation and shrinkage of pores. The range of potential output products possible would be limited both physically and electrochemically, by the structure of the crystalline lattice. Opportunities for product release would arise as the crystal lattice dehydrated; trapped product could be liberated, and fresh raw material be enclosed as the structure reformed on rehydration [70–72]. At least in thought concept, providing the necessary chiral protein and or other raw materials were present, and/or if the process itself was stearic, quite long lipid, protein, and/or peptide chains, as well as "RNA-like polymers" [73, 74], could thus be formed. Montmorillonite can also be found in volcanic material [75]. Zeolite pores by restricting by size, and by requiring interaction with a polar mineralised crystalline matrix structure, particularly if itself 'chiral', could also enhance the selection for chirality.

Vesicles—Evolutionary Structural Importance of LA and ALA

Given the enormous range of chemical energy transformations made possible by diurnal UVC energy provision, and that a vesicle-based self-regulating diurnal chemical sulphur energy cycle appears feasible, it is arguable that life with exothermic capacity likely originated on the terrestrial surface in thermal volcanic hypoxic waters subject to sunlight, rather than in ocean vents, where energy supply would be relatively constant. The development of 'life' in stable energy ocean vent conditions would not have required the development of both exothermic- and endothermic-based reactions, whereas a diurnal, day/night, sunlight, 'on/off', energy environment, would require the existence at least a minimal level of energy production at night for the development of any viable sophisticated life form and so predicate for development of life forms with both an endothermic and an exothermic energy mix capacity.

As discussed, the ability to create vesicles is central to the development of life. Simple lipids allow the development of basic vesicles, without the need for more sophisticated phospholipid polar head groups. LA and/or ALA are arguably critical structural components of prephospholipid vesicle lipid membranes, crucially providing mechanisms to import and export substrates of life, including; concentration gradient diffusion by creating sufficient 'flexibility' to provide membrane fluidity at temperature ranges consistent with life; structural symmetry allowing pi conjugation with themselves or other molecules, thus electron or proton transfer as likely seen in cardiolipin; an ability to conjugate with adjacent lipids encouraging the formation of lipid rafts and potential transfer of electrons or protons horizontal to the membrane plane; midline folding of lipid membrane members, thus substrate delivery potential; flip-flop mechanisms potentially allowing substrate or ion import/export; as well as being sacrificial protectors against external oxidative pressures including UVC and as chemical messengers when oxidised. LA and ALA arguably were the first mechanisms of sensory perception and reporters of external environment status. It has even been suggested that lipid membranes are the brains of bacteria in that they regulate their interaction with the environment [76].

Bacterial membranes generally utilise mainly monosaturated rather than polyunsaturated fats, making monosaturated fat-based membranes an evolutionary contender. However, a number of factors would arguably have restricted the early evolutionary prospects of surface sunlight monosaturated fat-utilising membranes developing diurnal energy systems [77]. Vesicles with membranes utilising monounsaturated fats would have been more susceptible to UVB and longer UVC damage, because they absorb UVC below rather than over 200 nm, thus lacking the protective ability of LA and ALA to absorb in the relevant UVC 200–290 nm range. UVC is damaging to life; modern-day experimental exposure to the UVC 250–270 nm wavelengths present on early earth is often fatal to bacteria [78]. In sea water (subject to photolysis and high in minerals), bacterial mortality is significantly increased by lower energy UVB. Further, prior to the development of active transport systems, a hypothetical early

earth monosaturated fat-utilising membrane, compared to membranes containing LA and ALA, would be insufficiently fluid throughout the relevant day/night range of temperatures, to allow passive concentration-based gradient diffusion of small non-polar molecules. To provide sufficient diurnal fluidity in monosaturated membranes by remodelling membrane composition, would have been needed rapid lipid compositional change, which would have required desaturation or enzymatic reverse elongation to shorten chains [79], but enzyme-based options were arguably not available to early life. In contrast, without the need for enzymes, LA, and more so ALA, significantly lowers the 18 carbon fat solidification temperature range towards and even below water freezing point, as well as providing a range of other characterises that may have assisted in substrate import/export and energy control. A combination of both monosaturated and polyunsaturated fats would allow the development of membranes that had a wider more nuanced membrane flexibility response to temperature change.

Interestingly, both 'modern' sulphur-based bacteria [80] and sandstone deposits believed to contain early sulphur-metabolising bacteria (Jiang), contain LA adding to evidence of the importance of LA in early surface terrestrial evolution. LA constituted 3–4 % of the lipids recovered from sandstone deposits, which interestingly is within the range found in wild animals today. The predominance of sandstone deposit fats was under 18 carbons, mainly even numbered, and a mix primarily of C:14, 15, 16, 16:1, 18, 18:1, and 18:2 fats, predominately C:16 and 18:1 n7.

Polyunsaturated fats are found in deep-sea thermal vent-related bacteria, but with a much greater variety of bond positions, which variety may be of particular relevance for their adaption to pressure and temperature. Protists, water-living organisms, so having the ability to shelter from UV, contain a wide range of polyunsaturated fats, notwithstanding which, LA or positional isomers, have been found at some level in almost all so far examined [81]. The emergence of LA and ALA as the dominant polyunsaturated fats in terrestrial so sunlit plants suggests their properties such as; structural bonding characteristics; shape making; internal hyperconjugation possibly through an ability to form transient cyclic pentene-type complexes; external hyperconjugation with adjacent fats to form lipid rafts; photoelectrical sensitivity; and potential down-chain and inter-chain electron and/or proton conductance; and messengering capabilities, differentiate these polyunsaturated fats, LA and ALA, as having particular significance and suitability as the predominant fats in sunlit terrestrial plant membrane vesicle systems; such properties include the following:

- **Amphipathic**—To form membranes, the components must be hydrophilic at one end and hydrophobic at the other; all fatty acids meet those criteria. However, at temperature ranges consistent with life, a membrane of saturated fats, for example palmitic acid with melting point 62.9 °C, would resemble a rigid film of tallow, being both inflexible and relatively inert; so would not allow ingress and egress of nutrient building blocks, nor respond/provide feedback as to external conditions. Further saturated fats, being transparent to UV would provide no protection against it. As discussed above, a membrane using monounsaturated fats rather than saturated fat, for example palmitoleic acid with melting point 31.82 °C, would also not be sufficiently porous to substrate in the temperature range that is consistent with common forms of terrestrial life of around −10 to +20 °C.
- **Length**—Vesicle membranes composed of short saturated fats are relatively permeable, permitting egress and ingress of ions and small molecules, but would not provide the impermeability required for sophisticated cell function. In contrast, longer and so less soluble 16 and 18 saturated carbon fats would provide adequate membrane impermeability, but not the capacity to allow cross-membrane substrate transport. The introduction of polyunsaturated fats into vesicle membranes would allow changes of membrane fluidity and permeability through rotational geometrical change characteristics, rafting abilities, hyperconjugation, oxidation and so polarity change and pi bond alteration and so interlipid attraction/repulsion changes. The 18 carbon multi-double bond containing LA and ALA would assist substrate import and export by facilitating concentration gradient diffusion; potentially via their ability to react with substrates external and internal to the membrane, including halogens and some metal ions, combined with possible flip-flop across the membrane, as well as the potential ability to transfer protons and/or electrons through structural shape change as discussed below and/or flip-flop of oxidised or attached products.
- **Flexibility**—To allow life, membranes need to be selectively porous and responsive to internal and external conditions, be that through chemical changes, and or alteration of shape or charge change through making pi electron orbital associations with themselves and/or neighbours. Polyunsaturated fats with cis bonds are said to be flexible. The use of the terms flexible is useful but somewhat misleading; the ability of cis polyunsaturated fats to change shape is due to the combination of the offset formed by a double bond; the ability of the adjacent single bond to rotate; and not being excessively inhibited from rotation by conjugated pi bonds or other physical constraints, together leading to a crank effect. Each additional double bond, so extra 'crank' pivot point, potentially imparts a wider range of possible

conformations, hence greater 'flexibility'. In contrast, so-called conjugated fats, where double bonds are only separated by single bonds, because of the formation of pi bonds down the chain, are relatively inflexible. For the chain to have the ability to take up different shapes, the double bonds must be separated by two single bonds in the cis formation. Further and importantly, LA and ALA do not have side chains or groups, such as found in squalene, which because of their side groups and bond configuration at the start of the chain form stable 6 ring cyclic products; squalene is the structural foundation of a number of cyclic products including cholesterol. In contrast, LA and ALA do not easily form cyclic products and so have relative structural stability, but cyclic pentene and hexene rings will form on heating during frying [82]. These LA and ALA products are bioactive [83], which, given the presence of such rings in the enzymatically formed, highly bioactive, AA-based prostaglandins, is probably to be expected.

Position of bond in chain—The position of the double bonds in the chain is also important. Omega 3 and 6 are descriptors of the position of the first bond in relation to the hydrophobic methyl end of the chain. The presence of three double bonds in ALA rather than two in LA, would increase the size of space that can be opened between adjacent membrane lipids and mean the first double bond is closer to the membrane bilayer interspace, together influencing fluidity, porosity and potential reactant access. 18 carbon LA and ALA have the shortest carbon chain that is; sufficiently long to provide adequate impermeability, divisible by both two and three, and demonstrates two single-bond separation between double bonds flexibility criteria, including at the ends of the chain. Divisibility of the number of carbons in the chain by three is important and particularly so for ALA and its relatives EPA and DHA containing greater numbers of double bonds; as discussed later, it is suggested the second and each additional double bond permits a conformational change into a transient pentene ionic resonant conjugated ring-type structure, which could domino down the chain, possibly allowing electron or proton transfer down the chain, or across to neighbours. Non-divisibility of the number of carbons by three would mean the ends would be conjugated but inflexible, and the symmetry of the geometry would be broken. Further, the existence of a double bond at the 9th carbon allows LA and ALA to 'fold' symmetrically in half, potentially providing opportunities to introduce reactants from the exterior to the interstitial space in a bilayer membrane (Fig. 27.4). Conceptually, folding could help open a space between and around the two arms of the chain in the membrane, which could conceivably act as a collecting and delivery 'arm' for substrate. The possibility that a central folding characteristic is of importance is also suggested by the predominance of C:14 n7 monounsaturated fats in early sulphur-based bacteria. The range of folding symmetries increases with the number of double bonds, providing greater hyperconjugation, electron and/or proton transfer potential, both within the fat itself and with neighbours. In Omega 3s uniquely, the pentene cyclic-type ionic resonant transient structure (see below) would sit almost adjacent to the interstitial bilayer membrane space, which may help explain the apparent electrical function of DHA in nervous systems. Omega 6s in contrast would be less effective, as is observed when DPA (n6) replaces DHA in nervous systems. If that is the case, other Omega 3s, such as ALA and EPA, may also be capable of similar but less powerful electrical physiological functions, greatly adding to the dietary importance of Omega 3s.

- **Formation of temporary conjugated charge transferring cyclic structures?**—The formation of internal pi bonds has been suggested to account for the hyperconjugation characteristics and so potential electrical conductivity of DHA and its consequent roles in both visual and neurological systems [84]. Further, conceptually, and potentially importantly, folding options may allow double bonds of LA and ALA at each 3rd carbon after the first, to form conjugated transient carbon pentene/pentadiene-type cyclic ionic resonant conjugated rings. A reaction or simple proton abstraction/electron acceptance at the carboxyl terminal of DHA (or later in the evolutionary process at the polar phospholipid head group) or the furthest double bond from the methyl terminal might create opportunities for transfer of electrons down, or protons up, the chain, by the sequential formation up or down the chain of transient carbon conjugated rings (Fig. 27.5). Say in DHA a proton was abstracted from the carboxyl group, which itself can be conjugated; transient closure of top ring would occur due to the transfer of a proton from the transient resonant ring, to the carboxyl group to replace the one lost; then, the ring being unstable would reopen and abstract a proton from adjacent carbons forming the next ring along, and it too would then transiently close and reopen abstracting a proton from its neighbours; the event would likely be repeated down the chain, leading to the abstraction of a proton from the interstitial bilayer membrane space. Consistent with this, Baldwin's rule seems to suggest that 5 carbons rings would not permanently form in this scenario. So abstraction of a proton would lead to a scenario where the ring tries to close, and the point of closure would become an ionic charge centre, a potential electric switch; a cyclic ionic pi bond resonant cyclopentene/cyclopentadiene conjugated ring

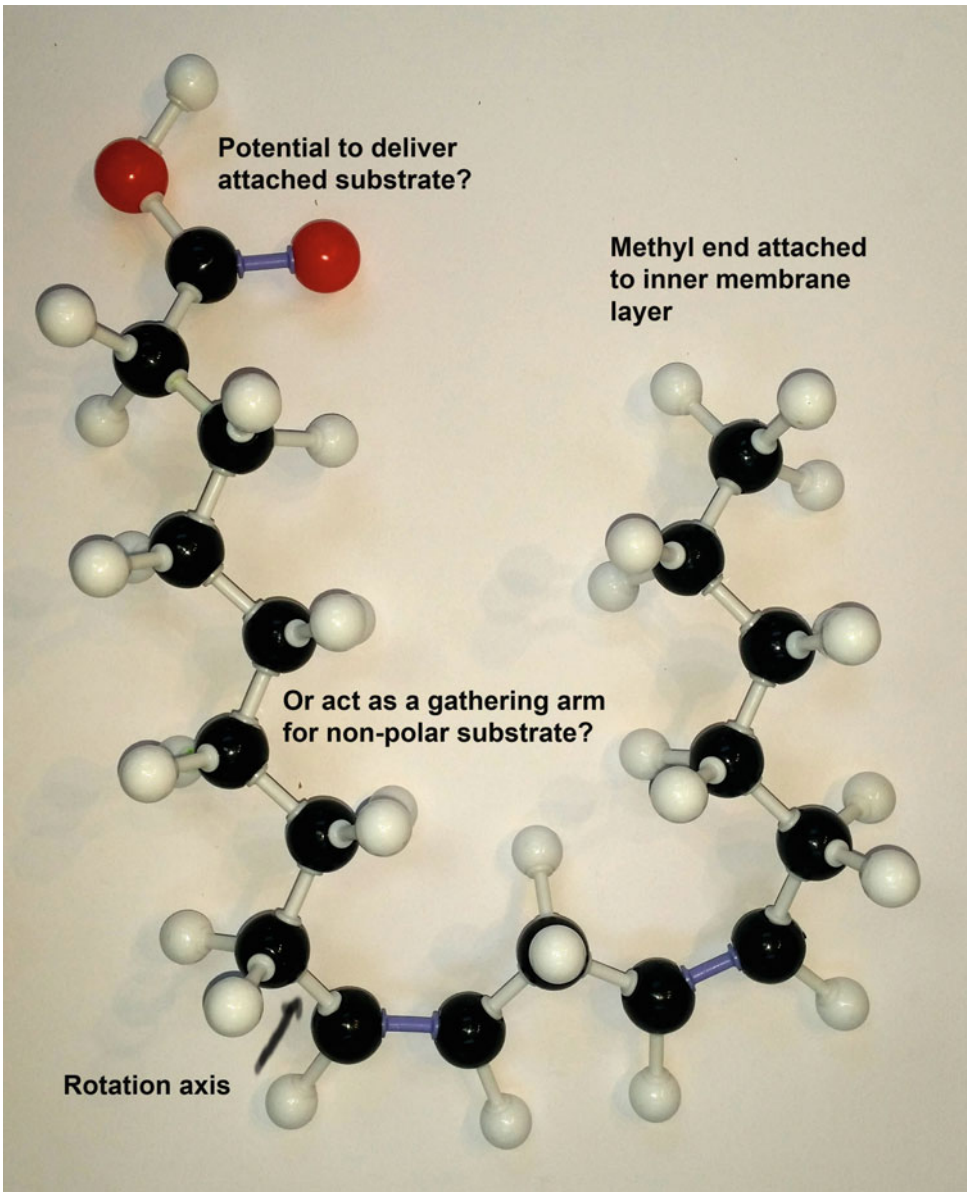

Fig. 27.4 Linoleic acid; one possible folding configuration. In early simple vesicle membranes, prior to the development of phospholipid head groups, the ability to 'fold' might have assisted transport of substrate from the membrane surface to the inner intermembrane space

could conceivably form allowing electron or proton transfer down the chain; the cyclic cyclopentene/cyclopentadiene would prove unstable [85] and so seek to reacquire a proton to allow return to a simple chain structure. Cyclopentadiene spontaneously and reversibly dimerises at room temperature and also on deprotonation becomes conjugated, adding support to the idea the adjacent transient rings would interact to form conjugated pi orbitals, which in turn would assist the movement of electrons and so areas of charge density [86, 87]. The effect could domino down the chain, or horizontally through adjacent Omega 3 or 6 lipids, as each ring formed and broke. In the case of DHA, with 6 double bonds, plus the conjugation effects of oxygen in the carboxyl group, a proton could travel from the interstitial bilayer membrane space to replace that lost by the carboxyl group. It is conceivable that similar interaction could occur with horizontally adjacent DHA in membrane rafts, as seen in the retina; for example allowing horizontal charge transfer between individual DHAs stacked in the DHA-rich section of retinal rods and cones. The mechanism conceptually, through physical constraints and atomic structural properties, could act as a series of gated switches down the length of and between DHAs, allowing transfer of charge in a controlled manner, digitising moderating smoothing voltage and current load, and so assisting visual and neural processes. Small rotations at single-bond connections would allow the 'switch' points, the ring connection locations, to sequentially open and close down the chain;

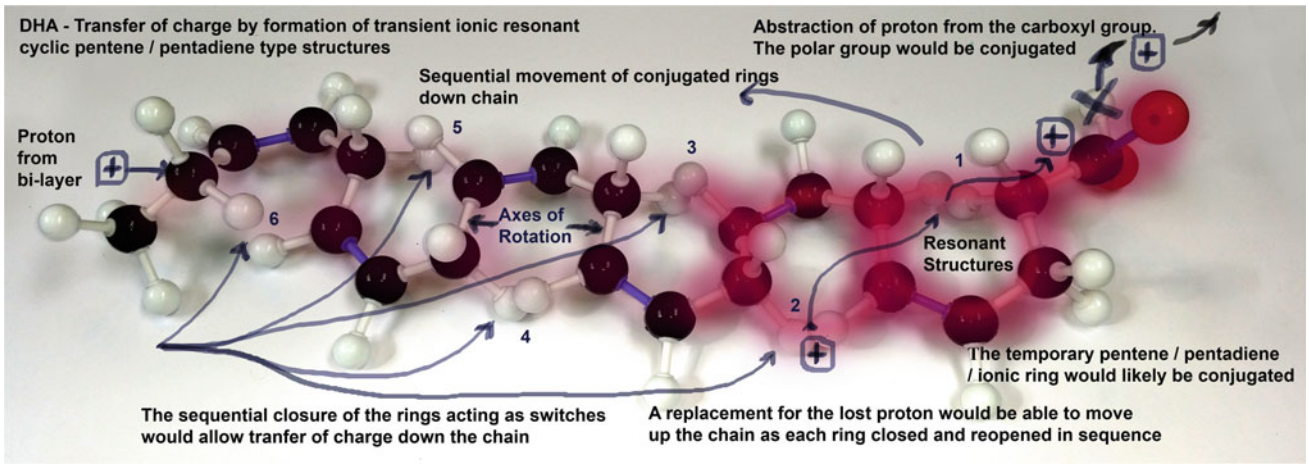

Fig. 27.5 DHA: a possible structural arrangement—a transient conjugated carbon pentene/pentadiene-type cyclic ionic resonant structure; conjugated ring pairs forming in sequence down the chain, potentially allowing the transfer of protons/electrons from/to the inner membrane space, following abstraction of a proton from the carboxyl group. ALA would have similar but more truncated cyclic ring features and a less clear route for the initial proton abstraction; however, the existence of isoprostanes confirms the feasibility of proton abstraction from a variety of double-bond positions and not just the carboxyl group

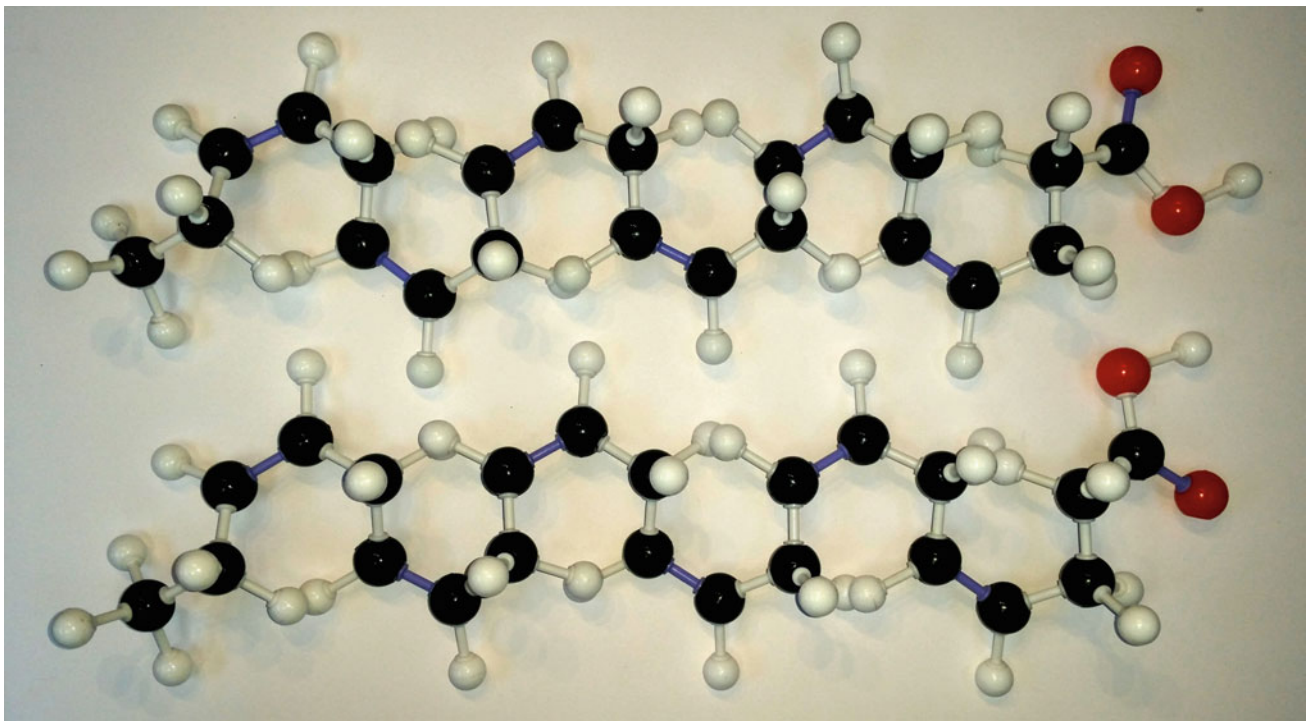

Fig. 27.6 DHA; the possibility of an organised pentene-type cyclic ring lattice lipid raft of DHAs, for example in the retina, which could conceptually interact, passing charge horizontally through the membrane; in effect becoming a conduction medium regulated by a series of digital switches and current controllers. A similar but more 'diluted' effect might be seen with ALA

it may be that DHA and other Omegas spend considerable amounts of time in this conformation during movement of charge, because conjugation potentially allows greater stability and minimises membrane depth irregularity due to chain length differential. This could also possibly work on a three-dimensional basis, creating a switch grid lattice between multiple DHAs (Fig. 27.6). The conformational change [88] during switching across a DHA 'grid' would also cause organised structural change and ripples in the membrane, particularly in DHA-rich visual and neurological tissues. Whilst superficially a more obvious choice, the creation of

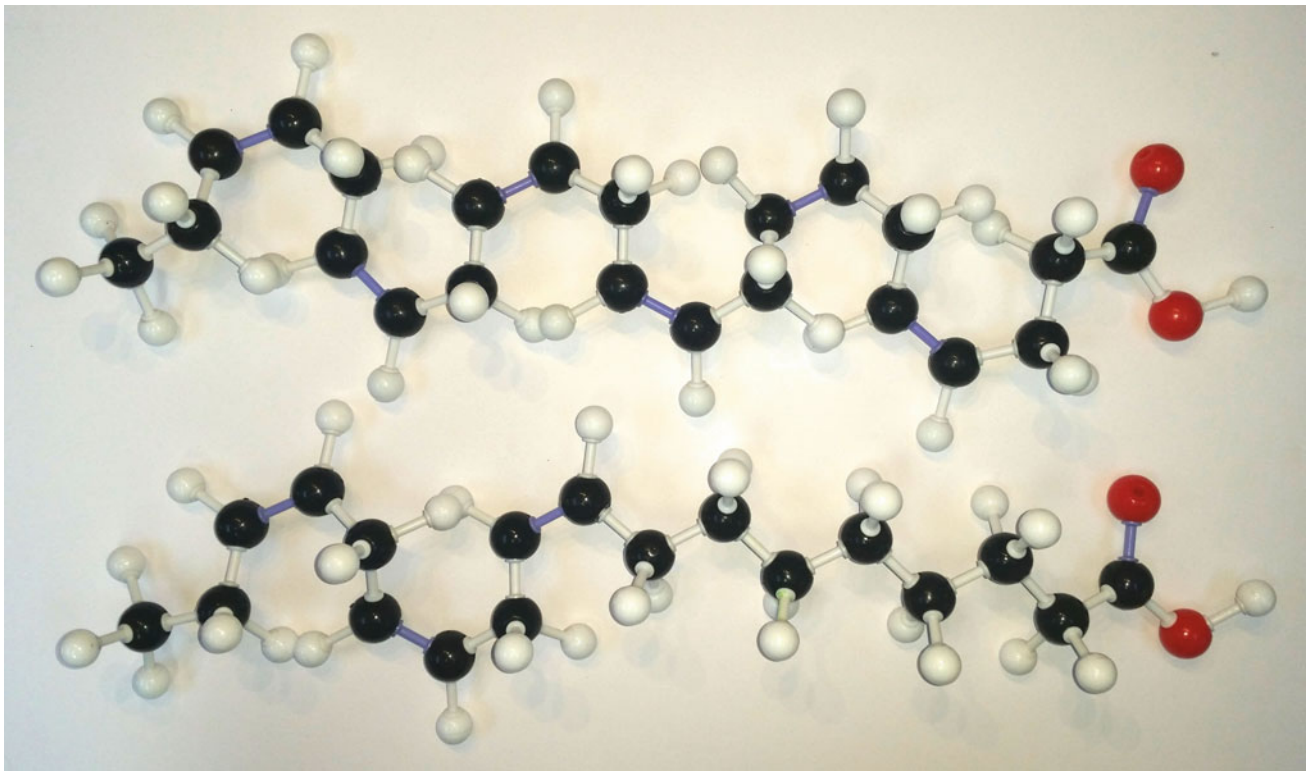

Fig. 27.7 DHA; formation of a pentene ring-type chain structure would shorten DHA, making it a similar chain length to the more prevalent 18 carbon fats, so potentially creating a more ordered membrane, consistent depth and so even surface, as well as reducing risk of exposure of the DHA containing polar head group above the membrane surface, thus increased risk of oxidation

cyclohexene rings does not work, allow the same neatness of structure and does not have the same potential to transfer charge, or the same structural instability. Whilst in humans, electron rather than proton transfer may be viewed as central to vision; proton transfer [89] forms part of the 'visual' systems of bacteria, algae and plants [90, 91]. Interestingly, in humans, proton pump inhibitors have in some instances been noted to cause blurred vision [92]. Proton abstraction from AA at a variety of double-bond positions is a mechanism for formation of isoprostanes and reaction products of random, including peroxide-related oxidation, which interestingly form cyclopentene-type rings as part of their structure. The potential transient cyclic ionic pi bond resonant cyclopentene/cyclopentadiene-type conjugated configuration of Omega 3s and 6s; resulting in a chained domino repeated pivot switched, electrical charge, likely proton, transfer mechanism; as well as an ability to regulate membrane thickness to around 18 carbons, arguably the most common length, has a structural simplicity symmetry economy and neatness about it, which is why I cautiously propose it.

- **Requirement of conjugation and symmetry for raft formation**—The ability and possible tendency of polyunsaturated fats to congregate in lipid rafts might be the result of formation of electron associations between pi bonds, including through temporary planar cyclic pentene rings in adjacently situated Omega 3 and 6 lipids. The formation of a cyclic ring-type chain conformation would also shorten the DHA chain; as a result, DHA, ALA and LA would be of similar lengths, which may facilitate a more organised membrane and ensure that DHA is not exposed to greater oxidation risk by virtue of protruding from the membrane (Fig. 27.7). If the double bonds in polyunsaturated fats were at different positions relative to the neighbouring fat, because say double bonds were randomly ordered, for example at carbons 12 and 15, and the neighbour at carbons 10 and 13, the dynamics between them and so structural qualities and behaviours of lipid rafts would change.
- **Reactivity**—The introduction of double bonds into saturated lipids increases their oxidisability. When a pi double polyunsaturated bond is oxidised, its bonding orbital structure and polar qualities alter, changing its relationship with adjacent membrane lipids, thus changing the structural qualities of the membrane. Bond position changes will also alter rigidity. Oxidation of LA has been observed inter alia to increase flip-flop rates in more sophisticated membranes. Oxidation of polyunsaturated fats in simple membranes, by altering membrane

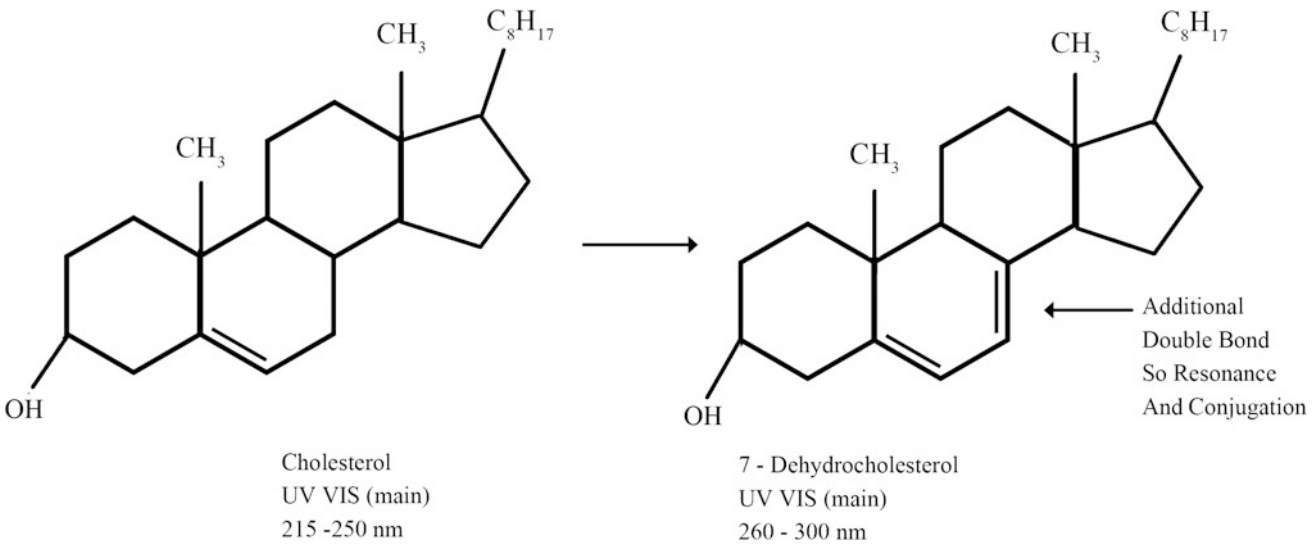

Fig. 27.8 The different wavelength range sensitivity of cholesterol and 7-dehydrocholesterol suggests that in conjoined ring structures, two double bonds and a polar group are sufficient to lead to some conjugation, which is not seen with a single double bond and charged group

qualities, may provide an additional mechanism for the control of ingress and egress of nutrients, as well as a rudimentary sensory signalling mechanism of external environmental conditions including temperature and light status. Polyunsaturated fats could also provide a vehicle for bonding to other molecules, including through halide-related mechanisms and through flip-flop, importing or exporting them to or from the interior of the vesicle, as well as potentially allowing import or export of charge through a reduction oxidation flip-flop. The structural properties of polyunsaturated double bonds allow shape change and so more intimate contact with other substrates including proteins and by definition greater reaction capacity.

- **Permeability**—Subject to temperature fluidity change effects, and the strength of osmotic gradients, simple polyunsaturated fat containing vesicle membranes would likely be permeable to small relatively uncharged molecular structures, hence the necessary building blocks of early life, including those found both in volcanic waters and in gaseous emissions, such as hydrogen sulphide, sulphur dioxide, cyanide, ammonia, water and carbon dioxide.
- **Saturated but not polyunsaturated fats are transparent to UV over 200 nm**—Importantly, saturated and monosaturated membrane fats are relatively UV transparent; they would not absorb and provide membrane protection from the early earth UVC spectrum of 200 nm and upwards [93]. C–C and C–H single bonds absorb in the non-relevant short UVC spectra at under 180 nm. Double bonds in isolation, as in monounsaturated fats, also absorb at below the important 200 nm UVC threshold [94].
- **Variable photosensitivity response to UV in the critical 200–280 nm range**—Double bonds increase the wavelength of absorption. In contrast to saturated fats, LA, ALA and derivatives absorb light between 200 and 280 nm, thus providing UVC membrane protection, as well as potential for reactive change. Lipid rafts might migrate to higher energy areas of the membrane, so to the face of the vesicle exposed to the sun. The ability of polyunsaturated fats to form pi bonds either with themselves, or with adjacent polyunsaturated fats, by aligning otherwise separate double bonds by structural rotation, would further change the UV absorption characteristics increasing the range of wavelengths absorbed, as would oxidation of the membrane lipids. By traditional measures, LA and ALA would not be regarded as conjugated structures. The wavelengths absorbed by 'non-conjugated' LA (217 nm) and ALA compared to 'conjugated' $H2C{=}CH{-}CH{=}CH_2$ (217 nm) [95] suggest that on more nuanced consideration, complex conjugation of some sort is taking place in LA and ALA. Many other simple biological products found in membranes are also active in these wavelengths including cholesterol and squalene [96]. The difference in UV absorption between cholesterol and its derivative 7-dehydrocholesterol, the vitamin D precursor [97], adds evidence that even two double bonds in a hex ring possibly subject to the additional influence of a polar OH group lead to partial conjugation sufficient to change light absorption properties (Fig. 27.8).

Summary—Evolutionary Role of LA and ALA

The likely existence of conditions that lead to the fabrication of LA and ALA, and subsequent creation of a desaturase enzyme capable of inserting double bonds to form LA and ALA were fundamental points in life's evolution. The membrane related and wider properties of LA and or ALA and their potential for formation of transient ionic conjugated pi bonded resonant pentene/pentadiene-type cyclic rings, internally or with neighbouring polyunsaturated fats; the potential for UV protection; membrane flexibility; transport of rudimentary substrate, ions, electrons and/or protons; the ability to form lipid rafts; and for chemical redox change so signalling and messengering, were arguably collectively fundamental to vesicle formation and function, so life.

The plethora of oxylipins of LA and ALA developed from being light-sensing and UV protection systems, into ubiquitous non-light-dependent core corporal messaging systems and became inter alia the basis of immune, defence, interspecies signalling and reproductive control systems, which are key to modern plant and mammalian function. With the appearance of the desaturases necessary to make DHA, the light-sensing and likely proton and/or electron-shifting capacity of LA and ALA had evolved into the necessary substrate to support full visual and nervous systems.

Role of LA and ALA in Plants

Conservation of Gene Pathways in Eukaryotes—Evolutionary Importance

The genetic systems of multicellular organisms, the building blocks of bacterial, algal, fungal, plant, and mammalian life, whilst differing between species, often exhibit broad interspecies functional similarities, as seen in cardiolipin, haemoglobin, the LOX enzymes and the mitochondrial enzyme cytochrome-C [98].

The roles of genes and the biological substrates they orchestrate are closely bound. LA and ALA are building block substrates, enablers, energy sources and controllers, storage mediums and regulators, protection systems and messengers, so fundamental in plants, bacteria, algae and likely fungi. It is therefore unsurprising that these significant functional evolutionary roles of LA and ALA have transferred to mammals including humans giving LA and ALA much greater functional importance in humans than is generally recognised.

Plant systems conserved and adapted in mammals, including peroxisomes and mitochondria, originated in plants and their precursors, and so were based on 18 carbon polyunsaturated fats consequently the preferred substrates of such systems, and related organelles, derived from 'plant precursors', are likely to lean towards LA and ALA. Arguably, that is what we see in humans; LA and ALA, structurally and when oxidised, have special relevance in those processes humans share with 'plant precursors' including mitochondrial function, peroxisomal beta-oxidation, reproduction, metabolism, immune defence, pheromone systems, cellular repair and renewal systems, fat deposition and other systems, including as discussed above UV protection and signalling.

UVC, and temperature gradient and range, were arguably key factors in the early evolutionary differentiation between terrestrial and ocean life forms, and likely resulted in the selection of LA and ALA because of their ability to respond to UVC and to impart membrane fluidity in the relevant temperature range, as the key polyunsaturated elements in terrestrial sunlit lipid membranes. The essential properties and characteristics of LA and ALA including; absorbance spectra, solidification temperature, bond arrangement, chain symmetry, hyper-conjugation ability, and likely ability for 'switched' membrane controlled electron and/or proton transfer through shape shifting, foreshadowed the functional characteristics and so importance of DHA, which in turn made possible the development of our very sophisticated visual and nervous systems.

Consistent with the importance of the UVC, and later UVB, in the development and maintenance of life, oceanographic ozone research suggests that our ecosystem is sensitive to even small changes of atmospheric UV penetration [99]. Crucially, in evolutionary terms, in contrast with terrestrial plants, many water-based life forms could choose and regulate their UVB exposure, simply by moving up or down in the water column [100], or into shade; indeed, young fish exhibit such behaviour. In consequence, with the exception of those permanently occupying the very upper surface levels [101], oceanic life forms have a less critical need for UVB protection systems than plants. Terrestrial plants and immobile life forms generally had limited options to variably control the light to which they are exposed, such as leaf structure and folding, so required more complex and sophisticated redox antioxidant systems, including membrane protection systems, to enable them to control UV exposure yet harvest light longer light, and so maintain vitality. Arguably, and importantly in terms of the relevance of plants in the diet to human health, plant eaters utilise, even depend on, those very sophisticated plentiful plant antioxidants as part of their redox control systems.

In this review, consideration of the wide range of metabolic, structural and UV protection roles of ALA and LA focuses on plants rather than photosynthetic bacteria, because they are the basis of the terrestrial food chain and like us developed and exist in a diurnal oxygen-rich

high-light environment. Nature conserves building blocks of systems that work. By considering the depth breadth and importance of LA and ALA in plant function, we arguably can learn much about their importance and potential relevance to human metabolic pathways.

Plant—Photosynthesis and UV Protection

Photosynthesis is fundamental to the development and maintenance of terrestrial life and indeed modern civilisation. Stored sunlight created the fossil fuels that power the scientific advances, communications, agriculture and transport that modern life depends on. Oxidised LA and ALA became the basis of light-sensing systems, wider messaging systems and early UV protection, and ultimately came to control many fundamental aspects of plant and related life.

ALA and/or LA are the predominant fats in photosynthetic chloroplast membranes, so clearly functionally important, although their role in photosynthesis and UV protection is not fully determined. The formation of conjugated and oxidised downstream products of ALA and other later evolutionary products such as squalene and cholesterol would increase UVC absorbance in the critical early 200–290 nm range. Following the introduction of atmospheric oxygen, the 200–290 nm UVC range became non-relevant, but UVB still penetrated the atmosphere and remained an oxidative driver for genetic change. To maintain sufficiently stable DNA to sustain long-term replication, emerging terrestrial plants developed additional UVB-specific protection absorption mechanisms such as those conferred by anthocyanin, flavonoid and phenolic compounds [102, 103]. Interestingly, plant tissues subject to short-term UVC stress also increase antioxidant output, such as transresveratrol in grapevines [104]. Such complexes may not have been present in quantity very early on and bind to phospholipid-based membranes, which again were likely not present at the outset, but may have developed whilst there was still some UVC incidence on earth.

The likely increase of LA and ALA with altitude might be in part a response to UV exposure, as well as to temperature falls. Interestingly, in cyanobacterium delta 12 (Omega 6) desaturation of membrane lipids is increased under high-light stress and confers increased light stress tolerance [105]. UV exposure also increases the content of Omega 3s in farmed salmon dorsal skin [106].

Experiments suggest the involvement of LA and/or ALA in photosynthesis in both algae and plants, but exact effects and mechanisms are yet to be determined. Under artificial light (likely low in UV), a mutant plant lacking the desaturase enzyme to make ALA did not show growth inhibition. Depleted ALA was replaced by LA, so conceivably only one or other is required for photosynthesis [107]. ALA levels rise in leaves of germinating plants only once exposed to light. LA is a key component of cardiolipin which also has a role in photosynthesis [108]. LA and ALA are also likely UV protectants in plants.

In humans, LA the most common polyunsaturated fat in the skin, also has light-responsive roles; it is oxidised, likely with catalytic assistance, by UVB, to 13HODE, which signals pain and promotes inflammation, thus providing a direct sensory link to sunlight. LA is present in the cornea, thus protecting the retina from UV [109] and potentially providing a UV-based signalling messaging system to the eye; oxidised LA is a factor in cataract formation [110].

Plants—Galactolipids and Phosphatidylcholine

ALA primarily, followed by LA and then palmitic acid are the predominant fats in green leaves [111, 112]; LA is the predominant fat in seeds and nuts; jointly LA and ALA are consequentially the primary fats in the plant-based terrestrial food chain, so of particular importance in the metabolic and signalling pathways of all creatures that subsist directly or indirectly on plants. LA is present in seeds and nuts mainly in the form of triglycerides. ALA in green material is present mainly as galactolipids, which are the most abundant lipids on earth [113], making gut galactolipid absorption mechanisms potentially physiologically important. Phosphatidylcholine is also important in plant metabolism, contains significant amounts of ALA as well as choline, and is present in the chloroplast, but it is a minor component in comparison with galactolipids. It is not fully known how monogastric mammals including humans metabolise galactolipids, or if they are absorbed similarly to phospholipids, hence potential suppliers of LA and ALA to the brain. Arguably, the form in which fats are ingested is of importance to the way they are supplied to the wider body, and their consequent end usage, to which point the next chapter returns.

Plants—Peroxisomes

The biological multifunctionality and relevance of peroxisomes [114], their role in mammalian pathways including; energy production, detoxification, substrate creation including of cholesterol, production of peroxide and catalase, with consequential roles in oxidative tone and messengering which impact; immune function, internal oxidative stress levels, pheromone communication, and defence functions, as well as cell repair renewal and maintenance, is underappreciated.

Given the crucial importance of LA in; germination, related messengering and energy provision; the role of ALA in the wider energy pathways in plants; and their joint predominance as plant fats, it is unsurprising ALA and then

GLA followed by LA are the preferred substrates of peroxisomes [115], or that the oxidised products of LA and ALA act as feedback control mechanisms for peroxisome activation. The outlines of these functionally important pathways have transferred in large part to humans with considerable metabolic implications. Many of the functions performed by peroxisomes in plants have a counterpart in humans, crucially including in energy pathways.

Peroxisomes provide the primary beta-oxidation pathway in plants, little beta-oxidation takes place in plant mitochondria. Peroxisomes are directly or indirectly essential to photosynthesis [116], immune defence, protection against predators, pheromone signalling, energetics and production of oxidised messengers, and catalase and peroxide production. Peroxisomes by being a source of thermogenesis might also assist plants, and likely humans, to maintain necessary operational temperatures.

Peroxisomes contain multiple enzymes, have different functions in different tissues and vary by species. They are considered very plastic performing a wide range of functions; for example, in plants, they morph from a form used in photosynthetic tissue, to one used in glycosomes during germination of seeds [117]. In humans too, they come in different forms, shapes and sizes, with different functional effects dependent on in which cells they are located. In humans, LA and ALA remain the preferred substrates of the peroxisomes, and in oxidised form are important activators of peroxisomes. Indeed, peroxisomes are life crucial and fundamental metabolic components in human physiology. Consequently, the intake, amount and balance of dietary LA and ALA have considerable metabolic ramifications in humans, the implications of which are largely unappreciated outside relevant research spheres.

Plants—Energy Production and Storage

Plants can completely peroxisomally beta-oxidise fats using an ACOX short fat oxidation enzyme; in contrast and importantly, mammalian peroxisomal enzymes can only shorten fats down to a minimum of 8 carbons [118]. Plants primarily use glucose for fuel, but in darkness and after exhaustion of carbohydrate reserves, peroxisomally beta-oxidise lipids, mainly ALA, for general energy provision [119]. In contrast, peroxisomal beta-oxidation of LA is mainly used to power germination and immune function. Taking a simplistic overview, in humans as in plants, peroxisomal beta-oxidation of ALA is primarily used for daily energy provision; in energy sufficient situations peroxisomal beta-oxidation of LA in humans, as in plants, is associated primarily with reproduction, tissue creation, repair and immune function. It is crucial to appreciate that LA and ALA, in the general scheme of things, each have distinct and different roles in relation to the peroxisome-related metabolic pathways, and their oxidised products also have very different feedback control activation loops and wider signalling roles.

Plants—Enzymes LOX and COX

LOX is an enzyme that oxidises polyunsaturated fats at specific points in the carbon chain creating hydroperoxides. Different LOX versions are active in both humans and plants and have considerable physiological influence in both. Given LOX originated in plant precursors, it is unsurprising the 18 carbon polyunsaturated fats ALA and LA are the preferred substrates of the LOX enzyme. Crucially, the preference of the LOX enzyme for ALA and LA is conserved in humans. LOX Omega 6 pathways tend to be inflammation-related, and LOX Omega 3 pathways are associated with resolution. LOX is more activated by Omega 3s than 6s, so more by ALA than LA, two reasons of many as to why dietary imbalances of LA and ALA have important physiological ramifications in humans.

As well as acting on ALA and LA, LOX acts on AA, which is of significant functional relevance in humans, but not plants; hence, LOX nomenclature is confusing. Historically, naming was based on the actions of LOX in mammals on the 20 carbon polyunsaturated fat AA, hence the naming of LOX products in mammalian physiology as 5, 12 and 15 LOX, based on the addition of oxygen to AA carbons adjacent to the double-bond position at 5, 12 and 15 by reference to the carboxyl end of AA, whereas in 18 carbon LA and ALA, the carbons are inserted at carbons 9 and 13, and in reference to plant physiology called 9 and 13 LOX [120].

In mammals, the COX enzymes like the LOX enzymes produce complex physiologically important influential families of polyunsaturated oxylipins, including from AA, ALA and LA, but in contrast to LOX, the COX enzymes are not found in plants. The preferred COX substrate AA [121] is not present or is a minor component in plant species [122]. However, LA and ALA are still about 40 % as COX active as AA. LA and ALA activate COX nearly equally with a possible small preference for ALA [123]. LOX is arguably much more physiologically important in oxidative stress pathways than COX, because it can oxidise polyunsaturated fats in situ in the lipid membrane, whereas COX can only oxidise lipids once released from the membrane by phospholipase enzymes, again giving the human dietary intake levels, and imbalances, of ALA and LA in particular and under-recognised importance.

Plants—Oxidised Derivatives and Signalling Systems

More is known about the wider families of plant LA and ALA oxylipins than mammal oxylipins; they assume greater functional importance in plants in the absence of the 20 and 22 carbon polyunsaturated fats AA, EPA and DHA. Oxylipins are often more water soluble [124], so active in the aqueous compartments of cells, as well as attaching to phospholipids in lipid membranes, or lipids in transit. Plants use oxylipins, as signalling mechanisms to control fundamental aspects of existence such as energy production, reproduction, germination, immune function, competitor and predator defence, pheromone production, and as sensors for environmental conditions including sunshine, temperature and moisture [125].

Plant oxylipin oxidation formation pathways include free radical, photo, auto, enzymatic including plant-based LOX and P450. Peroxisomes are a significant source of peroxide; so peroxisomal activity has a role in determining oxylipin production. In plants, LA and ALA oxidation products 9 and 13 hydroperoxide sit at the top of an extensive family tree of 7 oxidised product lines (The ACOS Lipid Library—plant oxylipins). Products include the 9 and 13 hydroperoxy derivatives of ALA jasmonate and jasmonic acid, which are important in plant reproduction, immune function and germination [126]. Adding to the complexities, the plant CYP450 enzyme family can produce hydroxy, epoxide and dicarboxylic fats from LA- and ALA-oxidised products [127]; the CYP450 enzymes are also very influential in human physiology.

Downstream products of LA derivative 13 HODE have important roles in plants, and include 4HNE, an exclusive LA product, and MDA, a non-exclusive ALA and LA product. 4HNE damages plant mitochondrial proteins [128] and signals plant wounding [129]. MDA is produced by wheat seedlings under oxidative temperature stress [130], from ALA [131] and LA following photo-oxidation [132]. 13HODE is also essential for germination [133] and is a possible peroxisomal energy substrate pathway. 13HODE, MDA, 4HNE and other related products also have similar fundamentally important very extensive signalling roles in humans, at low levels are likely protective, at high levels are damaging and are associated with many 'Western' non-communicable conditions [134].

Plants—Immune System and Predator Defence

Plants use oxidative stress as a defence mechanism against pathogens [135]. A wide range of oxylipins are produced from LA and ALA by a variety of oxidative mechanisms [136, 137]. They are used in plant signalling, to indicate they are under attack, to advise neighbours to prepare for attack, as pheromones to attract the predators of pests [138], to directly attack or destabilise pathogens and likely to encourage or discourage other species from growing nearby [139]. They also deter insects, can be toxic and can induce leaf necrosis [140]. Given the physiological importance of LA and ALA oxylipins, and a common need for defence in both plants and humans, even if against different pests and pathogens, it would be surprising if some of these mechanisms have not been adapted and adopted in humans; indeed, 13HODE and other oxylipin derivatives are the basis of multiple signalling mechanisms in humans including being central messengers in the immune system [141], key to tissue creation maintenance and repair, factors in the pain pathways, signallers for inflammation and have several other roles.

Plants—Reproduction and Fat Storage

Plants store LA, and sometimes ALA, in quantity as triglyceride in seeds and nuts to fuel germination and early growth. LA is the predominant storage fat in plant reproductive material, exceptions being some tropical fruits such as palm nuts and coconuts. ALA-rich seeds exist such as flax and perillia, but are limited in number in comparison with those rich in LA [142]. Oxylipins generally, particularly LA LOX product 13HODE, and ALA product jasmonate and jasmonic acid, have important roles in facilitating plant reproduction, and it appears are obligate factors in germination and pollen function, respectively. Interestingly, the importance of Omega 3s to fertilisation in plants is reflected in the requirement for Omega 3s for human sperm functionality. The crucial wider importance of LA in plant reproduction and fat storage has also transferred into humans. In humans, oxidised Omega 6 LA and AA products are key controllers in appetite and fat deposition; oxidised products of AA, and the availability of them, are crucial determinants and controllers of sex hormone production; importantly the presence of LA- and AA-oxidised products are ultimately obligatory for successful human reproduction, which is discussed in more detail in the accompanying chapter.

Plants—Cardiolipin

Cardiolipin, a double phospholipid carrying 4 fats, is a significant constituent of the inner mitochondrial membrane and found in many life forms including sulphur-based bacteria. It is closely physically associated with cytochrome-C and believed to assist in moving protons across the mitochondrial membrane. Given cardiolipin is a factor in the

control of complexes I, III, IV and V of the human electron chain, cardiolipin composition clearly has a fundamental role in energy production.

Plant mitochondria contain and use cardiolipin [143], which is also present in the photosynthesis compartment [144]. The primary cardiolipin species in plants mainly contains LA on all four phospholipid arms, which trait to a lesser and varied extent has been conserved in mammals. Interestingly, cardiolipin content varies between species and indeed tissues. Cardiolipin in rat testes contains mainly C:16; human lymphoblasts C18:1; and human heart C18:2 LA [145] which differences beg many questions, particularly in respect of the role of the phospholipid composition of cardiolipin in energetics. In plants, ALA is present in significant amounts in the wider mitochondrial phospholipids, but not in cardiolipin [146]. In humans, ALA in cardiolipin appears similarly rare, but DHA can be present in appreciable amounts although LA generally is the dominant fat in cardiolipin. Cardiolipin lipid compostion impacts mitochondrial function including energetics and apoptosis. In humans, the amount of DHA and LA in cardiolipin is significantly, but not wholly, dietary intake dependent, thus creating a direct link between environmental fecundity, lipid intake and mitochondrial function including energetics and apoptosis.

Plants—Light Sensing a Precursor to Vision

UV photo-oxidation of LA and ALA provided very basic signalling mechanisms by which simple early evolutionary vesicles could sense light changes, hence day and night, shadow and fluid depth. As discussed, the pi orbitals of double bonds are susceptible to photoexcitation by UVC and UVB. The structural symmetry of the conjugated arrangement of regularly spaced double bonds, sometimes in association with ionic structural atoms, or when part of a pi orbital compatible ring structure, result in the formation of pi electron clouds on both sides and parallel to the conjugated chain or ring, which likely increases the probability of electron interaction with photons, either as discrete particles or waveforms.

The wavelength optimally trapped increases with the number of pi interlinked double bonds [147], or number of interconnected cyclic pi rings, but is also dependent on other factors such as the presence of iodine and ions. The longer visible wavelengths are trapped by conjugated long-chain plant pigments such as beta-carotene (9 double bonds), or conjugated ring systems such as chlorophyll, whereas UV is effectively trapped by LA (2 double bonds) and its oxidised products. The wavelength trapped by LA is longer than that would be conferred by a single carbon-to-carbon double bond, suggesting a hyperconjugation effect is present. As discussed previously, do polyunsaturated fats, including DHA particularly, with symmetrical double-bond spacing, two single bonds between each double bond, and an acid carboxyl group prone to proton donation, flip between ionic pentene related resonant conjugated cyclic ring structure(s), which are inherently relatively unstable and a chain configuration; the ring structure possibly moving in domino sequence down the chain, which would allow a 'switched' transfer of the proton released upon formation of a transient carbon–carbon bond on closure of the ring, so replacement of the proton lost by the carboxyl group or other abstraction route, and overall a mechanism to move protons. The methyl end would act as a point of anchor and a transfer point to or from the interior of the membrane.

Interestingly, DHA (6 double bonds) is essential to retinal function, suggesting either or both interaction with visible wavelengths, or a role in proton/electron transfer, and potentially switching, 'digitisation', current control and/or 'current' regulation characteristics.

Particular structures preferentially absorb certain wavelengths and so likely became messengers for individual processes dependent on selective wavelengths, such as UVB-specific oxidation of a cholesterol derivative, to form the vitamin D precursor [148], part of an ancient hormone system common to plants and humans; interestingly, UVB conversion (likely assisted) of LA to 13HODE in humans signals pain and inflammation. After excess exposure to UV, these sun exposure control systems also induce formation of UVB-absorbing products such melanin and vitamin D. Photo-oxidised structures were the first environmental signalling systems; they arguably evolved into more sophisticated photo-oxidised product messengering systems, providing a direct messenger response to sun exposure and shadow; this was arguably honed over time in higher life forms using longer polyunsaturated fats including DHA, to a variety of 'visual' systems. Humans use the visible spectrum for vision; infrared-sensing systems have been developed including by snakes [149] and bats and UV visual systems by cats, dogs, caribou and bees [150]. Through oxidised messengering systems, light links terrestrial life to environmental changes, for example, controlling reproductive timing in some species and diurnal activity in others, and is linked to mood and behaviour including in humans [151]. In humans, as well as their pets and livestock, the modern-day lack of non-burning exposure to sunshine of all wavelengths from UVB to infrared may have much greater biological functional significance, and effect, than is currently appreciated.

Arguably what started as a simple polyunsaturated based UV light dependent messengering system, ultimately became the basis of both; a photo-oxidation based messengering system as seen in the skin; and a universal non-light dependent, inter and intra cellular oxidised messengering

systems based on a mix of enzymatic, including COX, LOX, CPY450, and non-enzymatic oxidation, of polyunsaturated fats.

Chiral Lipid Forms, Enzymes, and Oxidation: Relevance to Messengering

Organic enzymatic oxidative processes selectively produce specific enantiomers. Non-enzymatic oxidation produces racemic products, a mix of all the possible enantiomers. Selective incorporation of chiral product into structure, appears to distinguish life forms from inert organic matter, defining organic structural shape such as the chirality of proteins and sugars, and direction of coil of DNA [152].

Two different chiral enantiomers, S and R, exist even for simple oxidised fats, including the LA product 13 HODE. Free radical oxidation of 13HODE produces S and R in equal amounts, whereas enzyme products tend to be of specific chirality. Chiral preferences are seen in plant processes (Feussner), but most research into enantiomers of oxylipin products appears to have been done in humans rather than plants. In humans, 13HODE produced by the LOX enzyme is in the S form. Action of COX on LA will produce the S and R forms, depending on substrate, as discussed in greater depth in the accompanying chapter.

Relevance to Mammals and Humans

The fundamental importance of ALA and LA in plants to peroxisomal function, energy control, energy storage, immune function, reproduction, fat deposition, UVB sensing and protection, pheromone signalling, likely proton transfer and other crucial functional pathways is conserved in humans to varying extents. It is generally accepted LA [153] and ALA, and/or the Omega 3 and 6 families as groups are essential nutrients. Mammals including humans do not possess the necessary desaturase to make or moderate their levels of LA and ALA, and so can only obtain them and moderate their physiological effects, by regulating intake of dietary LA and ALA. No matter what the food source, in the natural world, the LA and ALA in human and mammal systems will have originated from plants and algae.

In humans, excess dietary intakes of Omega 6 LA, deficit of Omega 3 ALA, and consequential imbalances, magnified by; the preoxidation of theses fats during processing, exacerbated by rising internal cellular oxidative stress levels consequent on falling intakes of natural plant and other antioxidants, and changing phospholipids/triglycerides intake patterns, including rising Omega 6 LA oil intake; together arguably significantly contribute to the huge increase in incidences of human non-communicable 'western' diseases, which proposition is the subject of the related chapters, which follow in this publication, 'Omega-3 Fatty Acids'.

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Bioactive Oxidised Products of Omega-6 and Omega-3, Excess Oxidative Stress, Oxidised Dietary Intake and Antioxidant Nutrient Deficiencies, in the Context of a Modern Diet

28

Robert Andrew Brown

Terms	
AA	Arachidonic acid (omega-6 20-carbon derivative of LA)
ACOX1	Acyl-CoA oxidase (first step in peroxisomal beta-oxidation)
ACoA	Acetyl coenzyme A (raw material for the energy/cholesterol pathways)
AGE	Advanced glycation end-product (non-enzymatic covalently bound sugar to protein or lipid)
ALA	Alpha linolenic acid (omega-3 18-carbon plant-based polyunsaturated fat)
APOE	Apolipoprotein E (lipid transport signature protein)
ATP	Adenosine triphosphate (enzyme used as an energy carrier)
BBB	Blood–brain barrier (barrier between blood stream and brain)
CD36	Cluster of differentiation 36 (fatty acid translocase receptor)
COX	Cyclooxygenase (enzyme catalysing oxidation of fatty acids)
CoQ10	Ubiquinol (fat-soluble component of mitochondrial electron transport)
CPT1	Carnitine palmitoyl transferase (acts as shuttle mainly for long-chain fats C:16-18 into mitochondria)
DHA	Docosahexaenoic acid (omega-3 22-carbon derivative of ALA)
eNOS	Endothelial nitric oxide synthase 3 (constitutively expressed enzyme produces nitric oxide)
EPA	Eicosapentaenoic acid (omega-3 fatty acid C20:5)
FABP	Fatty acid binding protein (family of transport proteins)
FAS	Fatty acid synthase (enzyme system to make palmitate)
FAT1	FAT1 transgenic mouse (inserted desaturase converts omega-6 to omega-3s)
FGF	Fibroblast growth factors (involved in angiogenesis and repair)
GSH	Glutathione(s) (a very important antioxidant family)
GLA	Gamma linoleic acid (omega-6 fatty acid C18:3)
HAEC	Human aortic endothelial cells (human aortic endothelial cells)
HbA1c	Glycated haemoglobin (non-enzymatic glycated haemoglobin)
HMGCoA	3-Hydroxy-3-methylglutaryl-CoA (found in two forms reductase and synthase. Reductase regulates the cholesterol production. Synthase regulates HMGCoA production. HMGCoA is substrate for ketones or cholesterol)

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HSL	Hormone-sensitive lipase (different forms mobilise lipids from triglycerides and esters also from the adipose tissue)
iNOS	Inducible nitric oxide synthase (inducible isoform involved in stress response in macrophages microglia and other tissues)
LA	Linoleic acid (omega-6 18-carbon plant-based polyunsaturated fat)
LOX5	Lipoxygenase (enzyme catalysing oxidation, including AA and EPA)
LOX12/15	Lipoxygenases (enzymes catalysing the oxidation of multiple lipid-based substrates)
LDLR	Low-density lipoprotein (LDL) receptor (LDL receptor for minimally oxidised LDL)
LPL	Lipoprotein lipase (mobilises the lipids from chylomicrons, VLDL, LDL, both at the vascular face and intercellularly)
Lp-PLA2	Lipoprotein-associated (hydrolyses phospholipids with phospholipase A2)
LTB4	Leukotriene B4 (product of LOX5 action on AA, a messenger)
MCAD	Medium-chain acyl-coenzyme A (dehydrogenation of fats C6-12 in mitochondria and present in inner mitochondrial membrane)
MDA	Malondialdehyde (non-exclusive oxidation product of omega-6)
MCT	Medium-chain triglyceride (triglyceride containing fats between C6 and C12)
NAPDH	NAPDH oxidase (generates superoxide in phagocytes including macrophages and potentially microglia)
NAFLD	Non-alcoholic fatty liver disease (fat deposition in the liver not due to alcohol)
NEFA	Non-esterified fatty acid (fatty acids not attached to a carrier)
NO	Nitric oxide (an important signalling messenger and oxidant)
OA	Oleic acid (omega-9 monosaturated fat C18:1)
OLR1	Oxidised LDL receptor 1 (receptor for oxidised LDL, sometimes called LOX1)
4ONE	Trans-4-oxo-2-nonenal (omega-6 product common in the adipose tissue)
Oxo-HODE	Oxooctadecadienoic acid (oxidation products of HODEs, also called KODEs)
PA	Palmitic acid (saturated fat C:16)
PGE2	Prostaglandin E2 (primary COX2 product of AA)
PPAR	Peroxisome proliferator-activated receptor (3 forms alpha, gamma and delta)
P450	Cytochromes P450 (family of often oxidative enzymes)
ROS	Reactive oxygen species (reactive molecules containing oxygen)
RXR	Retinoid X receptor (receptor that hosts PPARs in conjunction with other activators including retinoids)
SA	Stearic acid (saturated fat C:18)
SCD1	Stearoyl-CoA desaturase (delta-9-desaturase, a key to the formation of OA)
SOD	Superoxide dismutase (reduces superoxide to oxygen or peroxide)
SN2	SN2 position (the location of fat in triglycerides or phospholipids)
SREBP-1c	Sterol regulatory element-binding transcription factor 1 (a protein regulating lipid and glucose metabolism)
TRPV1	Capsaicin receptor (pain- and temperature-related receptor)
VEGF	Vascular endothelial growth factor (a protein signalling angiogenesis)
Wy14643	PPAR alpha activator (activates PPAR alpha-related peroxisomes)
4HNE	4-Hydroxynonenal (exclusive omega-6 fats peroxidation aldehyde)
4HHE	4-Hydroxy hexenal (exclusive omega-3 fats peroxidation aldehyde)
4HPNE	4-Hydroperoxy 2-nonenal (oxidation product of omega-6 LA and likely AA)
9HODE	9-Hydroxy-10E, 12Z-octadecadienoic acid (major LA oxidation product of LOX12/15, COX, photo-oxidation and autoxidation)
13HODE	13-Hydroxy-9Z, 11E-octadecadienoic acid (major LA oxidation product of LOX12/15, COX photo-oxidation and autoxidation)

13HOTE	13-Hydroxy-9Z, 11E, 15Z-octadecatrienoic acid (major ALA oxidation equivalent of LA product 13HODE)
15HETE	15-Hydroxy-eicosatetraenoic acid (major AA LOX15 oxidation product)
15d-PGJ2	15-Deoxy-Δ12, 14-prostaglandin J2 (downstream AA COX2 oxidation product and PPAR gamma activator)
Ptd-cho	Phosphatidylcholine (A major phospholipid in mammals and plants)

LA and ALA: The Most Common Terrestrial Fats

LA and ALA are the two most common fats in terrestrial plant material [1, 2]; as a result, they constitute key components of the food chain of land-based surface life forms including humans. They are also essential nutrients with fundamentally important biological roles in cellular membranes, messaging systems and energy pathways.

LA: Enabler and Controller of Reproductive Capacity and Related Processes

It is quite possible that emergence of LA-rich seeding plants permitted the evolution of mammalian placental reproductive systems [3]. LA and ALA are diet-derived hence controllers of multiple and fundamentally important biological reproductive mechanisms, and systems; likely environmental fecundity through plant based LA availability including in plant reproductive material via these pathways ultimately switches on and off human capacity to reproduce [4].

LA and ALA: 'Essential Nutrients'

Unlike plants, humans cannot make LA or ALA; they are 'essential' externally derived nutrients crucial to human function at many levels [5]. It is accepted that ALA downstream product DHA is irreplaceable for the development of the nervous system, visual capacity and the brain, and so higher human function. Some would argue that it is the longer-chain omega-3s including DHA rather than ALA, which are 'essential nutrients'. However, it is indisputable that omega-3s and omega-6s are both 'essential nutrients' for human existence, and ALA is the source material for DHA and EPA.

The Crucial Point

The current historically unprecedented excess dietary intake of LA, combined with very low ALA intake, in the **context** of partially pre-oxidised, nutrient-damaged and antioxidant-diminished Western diets, by causing oxidative, structural, signalling and energy imbalances, through a number of mechanisms including overstimulation of LA oxylipin-related signalling, and diminished mitochondrial function, will have substantial negative physiological health outcomes.

Roles of ALA, LA and Their Oxidised Products in Humans

Fundamental roles of ALA, LA and their oxidised products in humans include the following:

- They are oxidised directly or indirectly by UV light, so LA and ALA have functions in visual and other sensory systems including skin photo-sensitivity.
- LA and ALA are key to organised structural, cellular and lipoprotein 'membranes'.
- They assist temperature adaptation through membrane fluidity control and peroxisomal thermogenesis.
- The extensive oxidised downstream LA products, including HODEs, are predominant plasma oxylipins, fundamental to intracellular signalling, the regulation of lipid storage, immune function and related tissue creation and repair, including during reproduction.
- They are the primary and preferred substrate of the LOX12/15 oxidative enzymes, and secondary COX enzyme metabolites.
- The 18-carbon plant-based fats ALA, GLA, LA, in that order, are the preferred substrates for peroxisomal beta-oxidation.
- Their peroxisomal beta-oxidation products include the following:
  - peroxide, which can be used as a source of oxidative stress to support immune function, related tissue creation and repair;
  - short-chain fats, which can be utilised by mitochondria for energy;
  - and ACoA, which can be used as a substrate for tissue repair-related activities, or directed to energy pathways.
- ACoA, the product of peroxisomal beta-oxidation of LA, is directed through PPAR gamma, and related LDL receptor CD36 and OLR1 pathways, to tissue creation maintenance and repair-related activity including cholesterol and lipid production.

- Excess PPAR gamma-related peroxisomal beta-oxidation of LA increases oxidative stress-based signalling, via the net production of peroxide in excess of catalase [6]. PPAR gamma-influenced macrophages and microglia may use their significant iNOS capacity [7], in combination with the ability of cytokines and other factors including peroxide to activate iNOS, to produce NO and so to deactivate the haem-based peroxisomal catalase enzyme, allowing pathway regulation, including increase in net peroxide-based oxidative stress production, to meet immune function, tissue destruction and tissue repair requirements.
- Oxidised products of LA, including the HODEs, are the primary endogenous activators of PPAR gamma, which is a '*master metabolic controller*', with roles in immune function and inflammation regulation, lipid and cholesterol production, adipogenesis and increased adipose and intracellular fat deposition. Further LA products uprate co-related gene pathways.
- LA is usually the predominant lipid species in mitochondrial cardiolipin; and present in proportion to dietary LA intake. Unoxidised and oxidised LA as a cardiolipin component, and unoxidised LA as beta-oxidation substrate, feature in mitochondrial energetics control, the initiation of apoptosis and the regulation of consequent oxylipin cascades.
- In contrast to PPAR gamma pathways, PPAR alpha has significant roles in uprating energy production. Energy deficit strongly, and omega-3 surpluses and high protein intake to a lesser extent, activate peroxisomal alpha and delta beta-oxidation pathways. During energy deficit, available LA and ALA are likely diverted to PPAR alpha and delta pathways for peroxisomal beta-oxidation to short-chain fats and ACoA, which are then utilised as mitochondrial fuel substrates. Concomitantly, gene pathways are activated that increase energy of creation activity and antioxidant production, including catalase.
- Excess, deficiency or imbalance of LA/ALA and their oxidised products have very significant widespread and known physiological impacts on human cellular function including on human reproduction and health.

The Centrality of LA to the Control of Reproductive Capacity and Function

LA through its oxidised products—via PPAR gamma RXR receptors, AA prostaglandins, 9, 13HODE and Oxo-HODE and related reactive oxygen species, nitric oxide and moderating antioxidant systems—controls sex hormone production; provides unique regulatory signalling and feedback mechanisms to synchronise energy regulation, appetite, metabolic rate, fat and related nutrient deposition, cellular creation and repair including angiogenesis, placental and foetal development, parturition, immune function and defence; hence the presence or absence of LA in the environment and in the diet likely ultimately facilitates or inhibits the reproductive function capacity.

The Physiological Consequences of LA/ALA Imbalances, Excesses and Increased Oxidative Stress

Western diets are imbalanced in dietary LA and ALA, which when combined with increased oxidative, stress consequent on nutrient imbalances and deficiencies has a significant impact on redox-driven fertility, reproductive and derived pathways including immune function, tissue creation and repair, with multiple consequences including for; fertility, infant health, age of menarche, brain development and behaviour as well as wider inflammatory- and oxidative stress-related dysbiosis.

Oxidative stress-driven PPAR gamma-related peroxisomal activity influences; breakdown, recycling, maintenance and development of tissues, and multitude of related pathways including angiogenesis [8], implantation [9], cell division, migration, inflammation, placentation, maturation, parturition and foetal development [10, 11]. Synergistically peroxisomal PPAR gamma related beta-oxidation activation provides substrate and materials required for tissue growth, including fats and cholesterol.

Omega-6 oxylipins, particularly HODEs, through the activation of PPAR gamma-related peroxisomes [12–14] indirectly regulate macrophages [15] and microglia, which as well as having immune- and repair-related roles are central to early [16] embryonic development [17, 18], development of vascular system and nervous tissue formation [19, 20], including in the brain [21].

Possibly because of its importance to reproduction, priority is given to storing LA in fat tissue. Synergistically, LA oxidised HODE products including 13HODE, through the activation of PPAR gamma, are master signallers for fat deposition. A few of the common downstream oxidised products, 'oxylipins' [22], of LA and ALA that are referred to in this overview are set out below (Fig. 28.1).

In addition to the gender-based sex hormone production by the testes and ovaries, adipose tissue fabricates sex hormones in significant amount on a gender-independent basis, providing a background tone level of sex hormones, which possibly explains why female fertility is conditional on adequate fat stores. In the obese, adipose sex hormone production results in hormonal imbalances, due to higher than expected levels of testosterone in women and oestrogen in men.

ALA and LA, in that order, are the preferred substrates of the LOX 12/15 enzymes, which also have important roles in the fertility cycle. The ALA/LA balance and their oxidised

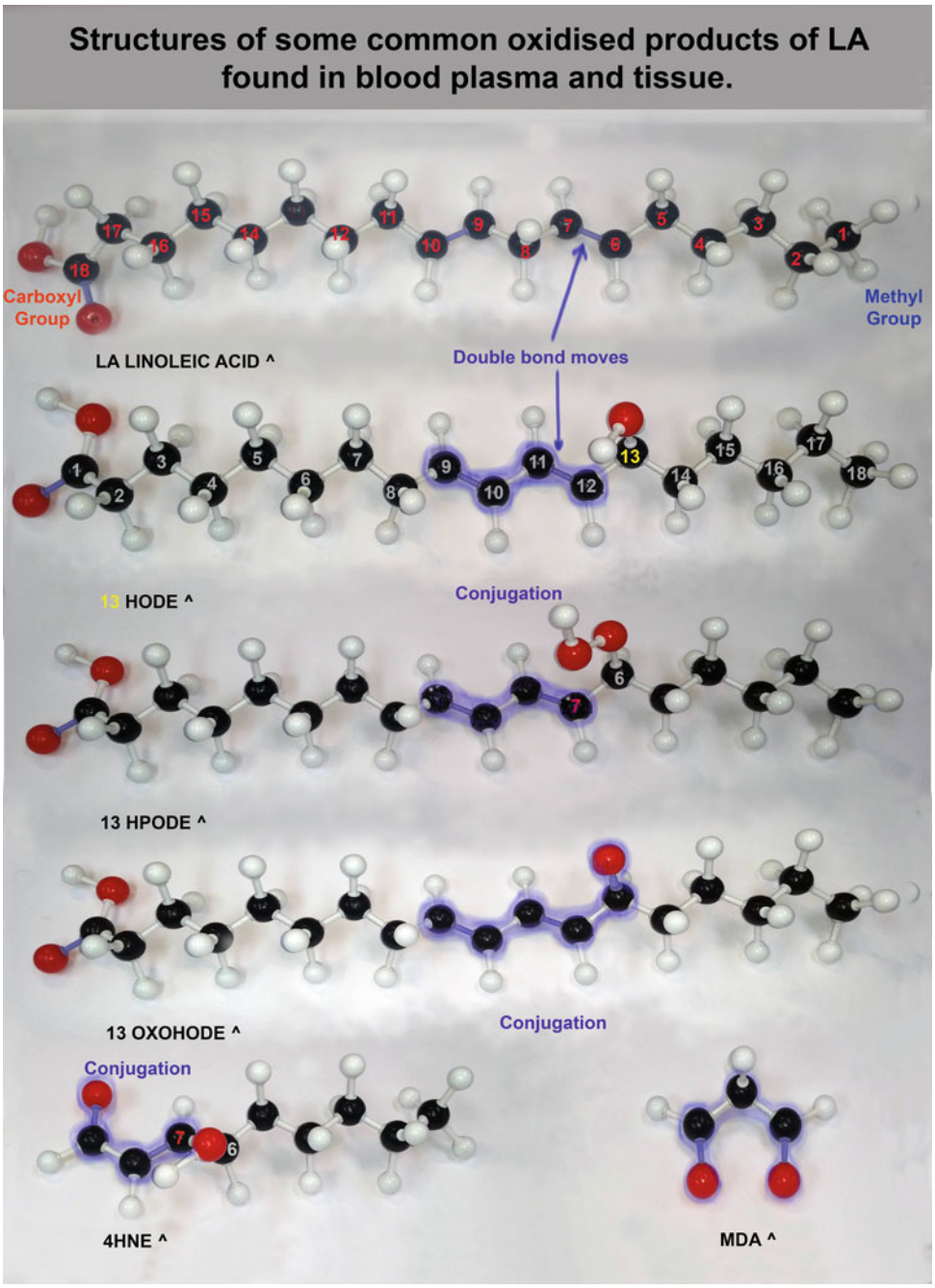


Fig. 28.1 Structures of some common oxidised products of LA and ALA found in the blood plasma and tissue. On oxidation, the movement of the double bond and so the creation of a conjugated segment and the addition of an oxygen 'group' will change the charge profile, the reactivity, and so the potential for interaction with other substrates and also reduce the 'flexibility' of the chain, thus making membranes less fluid. Oxidation will also change UV absorption. The Human Serum Metabolome database [93] lists and shows structures for over 4500 wider blood products. The body is self-evidently a huge complex and interdependent organism. LA and ALA are part of the relatively limited list of externally derived, rather than internally made, nutrients that are essential to life; these are external generally dietary factors that determine our existence and function, over which we individually and as a species have domain, and so the power to optimise our capacity for health and optimal neurological function. The external origin, seasonal availability, structural properties and relatively high presence of LA and ALA in the plasma and tissue give their oxidised products a combination of evolutionary and functional importance

downstream products have underrated roles in infertility, menstrual and related conditions, including implantation. Strikingly, '*At implantation, maximal induction of 12/15-LOX enzymes was observed, with a parallel increase in the eicosanoid metabolites 12-HETE, 15-HETE and 13-HODE (13-(S)-hydroxyoctadecadienoic acid) in the uterus. Furthermore, leucocyte 12/15-LOX null mice exhibited impaired implantation*' [23]; a LOX blocker had the same effect, and a PPAR gamma promoter (so bypassing the need for the activation of PPAR gamma by HODEs) reversed that inhibition [24]. Congruently, hydroperoxides promote both LOX12/15 and COX activities [25], creating a feedforward loop; conversely, LOX12/15 pathway activity is moderated by antioxidants including glutathione and vitamin E [26].

LA elongation product AA, through COX derivative PGE2, is a major controller of hormone production, including both testosterone and oestrogen. Inhibition of PGE2 blocks or limits hormone production, and stimulation of PGE2 increases hormone production.

AA in competitive conjunction with omega-3 DHA has fundamental roles in reproduction, including positive feed-forward and feedback mechanisms controlling the fertility

cycle and facilitating reproduction, through self-reinforcing mechanisms such as; AA increasing PGE2; PGE2 increasing oestrogen; oestrogen in turn increasing desaturase function and so AA production from LA, and to a lesser extent DHA production; available DHA is likely taken up by the cells of the uterine lining until failure to implant, when rising DHA will compete for COX enzymes, thus moderating the effect of surplus AA, assisting closedown of the fertility cycle. Interestingly, 4HNE also increases COX activation and so PGE2 production.

In contrast omega-3 activation of PPAR alpha energy-related peroxisomes would help support energy and antioxidant requirements in the womb. ALA also would compete with LA for LOX12/15 and COX enzymes. Consequential oxidised products of omega-3s would moderate the tissue repair and related inflammatory activities of omega-6 oxylipins, including during thickening of the uterine lining, implantation or shedding. Antioxidants such as catalase and glutathione would further moderate the processes.

Peroxisomes also have a number of other LA related roles that are central to reproduction, and neural development, including the production of DHA, cholesterol and plasmalogens.

Dietary LA and its consequential oxidised products, including through AA product PGE2 as competitively moderated by ALA and longer Omega 3 derivatives, alter hormonal and corticosteroid production with multiple reproductive consequences, including logically being factors in behavioural change in animals during the mating season, e.g. increased aggression and territoriality, reduced co-operation in males and greater predisposition to mating in females. Consequently, dietary changes likely also alter human behaviour on an individual, national and even global basis.

LA: Age of Menarche, Puberty and Consequent Effects on Brain Maturation

Excess LA and its oxidised products combined with the loss of pituitary melatonin production are factors in early menarche [27]. The age of puberty has dropped from mid- to late teens in the late 1800s, to pre-teens today. Some non-Westernised groups such as Inuit still had later onset of puberty into the 1900s.

Omega-3:6 imbalances, excess LA, combined with increased net oxidative stress due to industrialised diets, including pre-oxidised dietary LA, not only signal for increased sex hormone production generally via AA and PGE2, but also encourage weight gain so gender-independent adipose hormone production, together promoting early menarche. Surprisingly, even feeding rat dams high dietary LA during pregnancy results in early puberty in offspring fed a standard laboratory diet from birth [28].

Dietary-based factors including low DHA and increased oxidative stress accelerate pineal gland shrinkage seen at the onset of puberty, so reduce melatonin production. Melatonin is a very powerful multiuse lipophilic antioxidant, which also uprates antioxidant function, including SOD and glutathione, and downgrades nitric oxide synthase [29] and NO-based catalase inhibition. The antioxidant function of melatonin delays puberty and mammary formation [30]. Increased oxidative stress, the loss of melatonin function, improved COX2 activity and thus PGE2 production which is a controlling factor in sex hormone production and synergistically oxidative stress, and LA oxylipins, signal for adipose tissue deposition.

Inadequate nutrients and lack of dietary antioxidants, high LA and low omega-3 DHA, consequent high levels of oxidative stress, magnified by early loss of pineal-related melatonin antioxidant capacity, would increase the risks of impaired, or early termination of, brain development and maturation. The effect of LA-related oxidative stress combined with DHA deficiency on brain development will be exacerbated in males compared to females, due to the lack of compensatory increase in DHA (assuming ALA dietary availability) seen in females consequent on desaturase function rising with increasing oestrogen production post-menarche. Oestrogen increases desaturase function, and may have antioxidant functions.

In summary, early menarche may be due to a nutrient-depleted, antioxidant-poor, pre-oxidised Western diet, excess calories combined with oxidised LA, inadequate ALA/DHA intake and/or impaired desaturase function due to diet and/or desaturase polymorphisms, and lifestyle factors, including lack of exercise, sleep and adequate time overnight between meals for 'fasting' related activation of PPAR alpha-related peroxisomal pathways to occur. Early puberty and impaired brain formation have significant wider societal implications.

Desaturase Function: Dietary and Genetic Differences

Unlike some other life forms, mammals including humans cannot insert the double bonds into carbon chains at the correct position necessary to make LA and ALA. Whilst humans cannot make LA and ALA, they can elongate and desaturate LA and ALA to create longer-chain fats of their respective families using the elongase and desaturase enzymes, which is self-evidently of considerable significance and particularly in those who are vegetarians, who do not eat preformed dietary animal or marine food sources of long-chain omega-3s and omega-6s.

Vegetarians are at greater risk of omega-3 EPA and DHA insufficiencies, and particularly so in pregnancy. Impaired

conversion, which may be due to lower desaturase function, dietary imbalances or genetic factors, can lead to insufficient EPA, DHA and AA, before, during and post-pregnancy during breast feeding.

Even if ALA is available in the diet, conversion to DHA is not guaranteed, due to the risks of impaired conversion, consequent on both dietary factors and genetic polymorphisms. The desaturation processes are inhibited by a whole range of dietary factors including LA/ALA intake balances; mineral and other nutritional deficiencies; other dietary factors; and wider health imbalances including in; insulin, glucagon, adrenaline, glucocorticoids and thyroxin pathways, as well as by genetic desaturase variation [31]. Evidence suggests that conversion [32] of omega-3 ALA to EPA and DHA is much higher where intake of both LA and ALA is relatively low and balanced [33] and where diets are relatively unprocessed.

The efficiency of desaturase function varies significantly, up to 40 %, between different gene variants. The ability of early man to move away from dietary shoreline sources of long-chain omega-3s to areas without access to fish may have been as the result of the development of a more efficient desaturase variant that became fixed in the population [34]. Subsequent migration and settlement again in marine areas, where the metabolic expense of conversion was no longer warranted due to marine dietary DHA, EPA and AA availability, likely lead to the loss of the variant.

Higher conversion, both because of better less processed, more balanced, nutrient-rich diets, and genetic influences, may explain the ability of some populations to thrive with limited dietary intake of long-chain omega-3s. Where ALA is lacking in the diet, those with higher desaturase function may experience negative consequences of excess AA production, which brings focus back to the evolutionary importance of omega-3s including dietary ALA [35].

In women, desaturase function and so long-chain fat levels are increased by oestrogen, tying desaturase function to fertility status including to the fertility cycle and pregnancy; rising oestrogen through the fertility cycle would increase AA, EPA and DHA output; rising DHA availability through its oxidised products and by substrate competition is likely a controlling factor in termination of the fertility cycle following non-fertilisation of the egg.

The link between oestrogen and desaturase function would assist mothers to provide the EPA and DHA required by infants for neural development. Further, greater desaturation function in females arguably helps define the characteristics of women, including; brain structure, neuronal outgrowth sophistication, sensory perception capacity; and on a functional level, oxidative status including oxidised lipid product balances, which combined with likely greater antioxidant production potential may be factors in behavioural attributes including patterns of thought, responses to stress and abilities such as multitasking. Desaturase function in women is arguably both impacted by and a controller of the fertility hormone cycle.

Interestingly, FAT1 mice, which are genetically modified to contain the necessary desaturase to convert omega-6 to omega-3, when fed LA rebalance their omega-3:6 ratio to 1:1, which may be an indication of optimal tissue levels, and so provide a basis for determining optimal ALA and long-chain omega-3 intakes.

For those with optimal desaturase function, optimal polymorphisms and perfect diets, downstream internal conversion of ALA to DHA and EPA, may provide adequate long-chain fats; but with current trends of LA/ALA imbalances and nutrient insufficiencies, for most a dietary source of long-chain fats including EPA and DHA is probably prudent, particularly given the existence in some populations of polymorphisms that significantly reduce desaturase function.

Western diets high in LA and low in ALA increase the requirement for preformed DHA. Enormous possibilities exist to improve the omega-3:6 status of our diet through food selection and better livestock nutrition [36].

Oxidative Overload: The Importance of Nutrients

LA/ALA imbalances; [37] protein, sugar and lipid oxidation; crosslinking; nutrient damage or depletion; the loss of antioxidant function so oxidative stress overload, lead to imbalances in oxidative signalling systems and ultimately cause cellular malfunction.

Declining health due to nutrient deficiencies, imbalances, oxidative damage in food preparation and storage are exacerbated by; caloric excess, impaired gut microbiota and function and related physiological changes including increased stress and appetite.

As recognised by Hippocrates, plant- and higher food-based nutrients are the building blocks of life. Conversely, insufficiencies due to; nutrient-reduced crops, refining out of nutrients, pre-oxidation, crosslinking, reduced bioavailability, failure to access pre-made plant antioxidants and imbalances including LA, ALA and/or DHA, may be foundation stones of non-communicable oxidative stress-related ‘Western diseases’, including declining neurological health and impaired brain formation in utero.

The brain is the most nutrient-dependent and sensitive organ. The effects of nutrient deficiencies and imbalances start at conception and are magnified in pregnancy leading to;

failure to implant; early term; irreversible suboptimal brain development including the loss of function, size [38, 39] and IQ; diminished capacity for abstract thought and empathy; and consequent behavioural and developmental issues [40].

Western diets, imbalanced in easily oxidisable LA and ALA, combined with high levels of dietary-related oxidative stress, restricted internal antioxidant capacity due to mineral and other deficiencies, and lacking episodes of internal energy deficit stress, must result in oxidative stress-related suboptimal cellular function, which when combined with individual genetic susceptibilities are logical foundations for 'Western' non-communicable 'diseases' [41] including greater incidence of perinatal development issues [42].

LA/ALA Imbalance; Overprocessed Food; Current Agriculture; 'Western' Disease

The change from an ALA-rich green-centric diet to an LA-rich grain-centric food chain (including indirectly through the livestock), high in LA-rich triglycerides, has changed the dietary ratio of triglycerides to phospholipids, as well as their lipid contents and availability, with significant consequences for human health and behaviour.

Oxidised and elongated products of LA, the primary endogenous activators of PPAR gamma; are necessary for reproduction; key to fat deposition; and essential to the related processes of tissue destruction, inflammation, creation and repair, including oxidative stress-based cytokine, prostaglandin, peroxide and oxylipin signalling; through interaction with epithelial related tissues, macrophages and microglia.

Rising Oxidation of LA and ALA in Foods Combined with the Loss of Antioxidants

Dietary oxylipins, and antioxidant loss, are significantly increased by multiple and cumulative processes, including; biocide application to grain and other foods in storage [43]; separation from protective antioxidant co-content; extraction, refining, fine milling, exposure to air and bleaching [44]; mechanical recovery and fine mincing of meats including exposure to air, iron-rich marrow and metal-containing proteins [45, 46]; biocides including bleaching agents to prevent bacteria in processing and packing [47]; irradiation; pasteurisation; fumigants; extrusion; enzyme addition; foaming; cooking particularly frying; wider storage; and cook chill.

For example, '*refined wheat flour loses 83 % of total phenolic acids, 79 % of total flavonoids, 93 % of ferulic acid, 78 % of total zeaxanthin, 51 % of total lutein and 42 % of total β-cryptoxanthin compared with whole wheat flour*' [48].

Overconsumption of LA and Its Oxidised Products Combined with Heavily Processed Foods—Relevance to 'Western' Disease

In a 'Western' low ALA dietary setting, overconsumption of LA and its oxidised products has the capacity to be relevant to most 'Western' oxidative stress so inflammation-based, non-communicable diseases including obesity, diabetes, infertility and cardiovascular disease [49].

These LA oxylipin-related oxidative pathways are amplified by 'Western' food overprocessing, through pre-oxidation and crosslinking of lipids, sugars and proteins and consequential nutrient, mineral and antioxidant, unavailability and/or deficiencies, as magnified by current farming and crop breeding practices, marketing and food chain mechanisms, which processes from soil to seed to supermarket, as a generality focus on everything but nutritive value.

Limited Capacity of Plant-Based Antioxidants to Negate Effects of Omega-6 Oxylipins

Evidence suggests that increasing omega-3 s, or antioxidant-rich plant-based foods, cannot biologically fully moderate the effect of excess LA [50] (see Figs. 28.2 and 28.3), more so when pre-oxidised and set in the context of a nutrient-insufficient diet.

The common focus on high intakes of long-chain omega-3 s EPA and DHA to mitigate the effects of omega-6, whilst somewhat effective, ignores the physiological importance of the oxidised products of omega-6 LA (and relevance of ALA).

This was neatly demonstrated by Ramsden; his team in a 12-week human study, mainly female study, found that the addition of omega-3, EPA and DHA to the low omega-6 diet, 2.42 % compared to 6.74 % baseline, made little difference to the reduction seen in the oxidised products of LA in fasting blood plasma [51].

In contrast, reducing dietary LA intake significantly reduced both total LA and HODEs, as illustrated below (see Fig. 28.4), which is also interesting for the analysis of the partitioning of LA between the various plasma fatty acid pools; the strong presence of LA in cholesterol esters and phospholipids adds to evidence of the importance of LDL as a transporter of LA.

A similar trend was seen in mice, where lowering LA and balancing ALA were very effective at reducing obesity, whereas DHA significantly reduced but was unable to fully reverse the obesogenic effect of an LA-rich diet [52] (see Fig. 28.5). Supplementation with DHA did not significantly change ALA or LA levels in plasma. Reduction in the trend

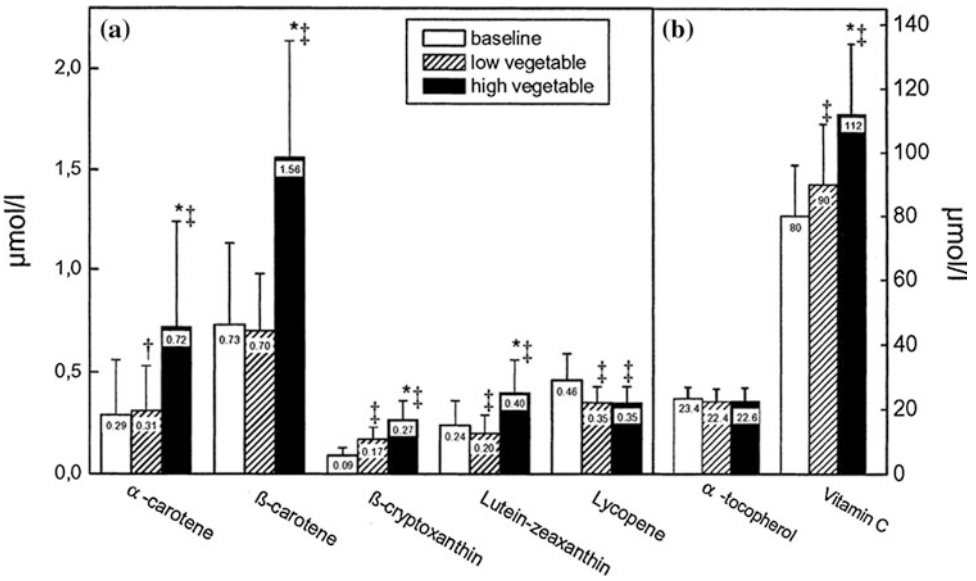


Fig. 28.2 '*Plasma concentrations of carotenoids, vitamin C and alpha-tocopherol of all the study subjects* ($n = 37$) *during the study diets. Values are mean ± SD. *P < 0.001; statistical significance for the difference between the low-fat, low-vegetable diet and the low-fat, high-vegetable diet (Wilcoxon signed ranks test and Student t test for paired samples). †P < 0.01; statistical significance of the difference between the baseline diet period and the study diet period (Wilcoxon signed ranks test). ‡P < 0.001; statistical significance of the difference between the baseline diet period and the study diet period (Wilcoxon signed ranks test)*'. From '*Changes in Dietary Fat Intake Alter Plasma Levels of Oxidised Low-Density Lipoprotein and Lipoprotein(a)*' with very grateful thanks to the authors for permission: Silaste et al. [50]

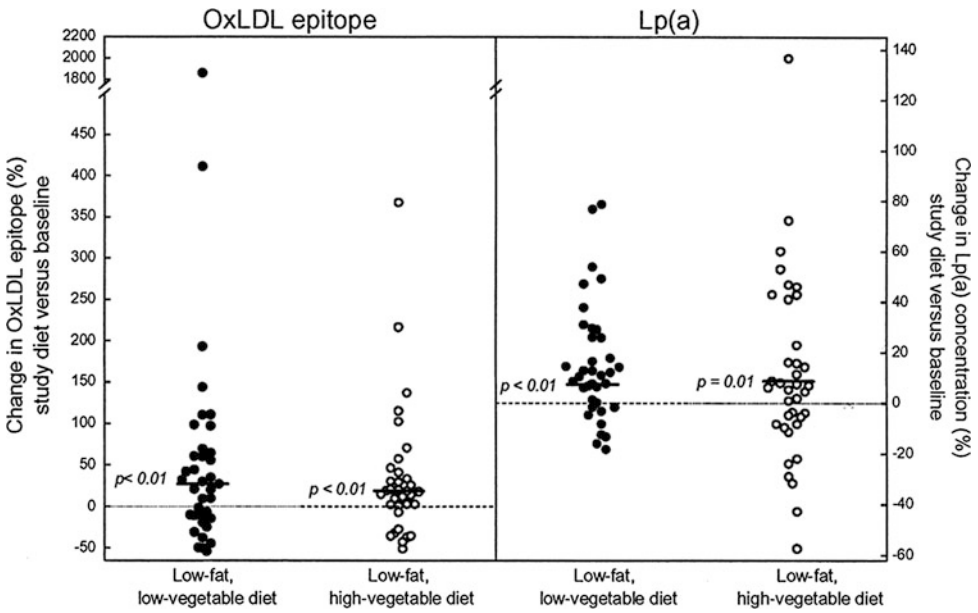


Fig. 28.3 '*Relative median changes in the plasma concentrations of oxidised LDL* (*left*) *and lipoprotein(a)* (*right*) *of all the study subjects* ($n = 37$) *from baseline to low-fat, low-vegetable diet and from baseline to the low-fat, high-vegetable diet'*. From '*Changes in Dietary Fat Intake Alter Plasma Levels of Oxidised Low-Density Lipoprotein and Lipoprotein(a)*' with very grateful thanks to the authors for permission: Silaste et al. [50]

to obesity due to dietary DHA is presumed to be due to a mix of competitive blocking of LA oxylipins, potential reduction in hunger and food intake, and likely activation of the PPAR alpha pathways.

In the context of an oxidised Western diet, where there is excess LA intake and LA intake is not reduced; the lack of significant impact of plant antioxidants on oxidised plasma content, or long-chain omega-3s on LA oxylipins, but conversely as noted improvement in blood LA HODEs with reduced omega-6 intake, suggests that the only truly effective strategy to reduce LA oxidative stress-related health impairment is to remove excess LA from the diet and in addition to ensure adequate ALA intake; in the words of Professor Lands 'Nix the Six'.

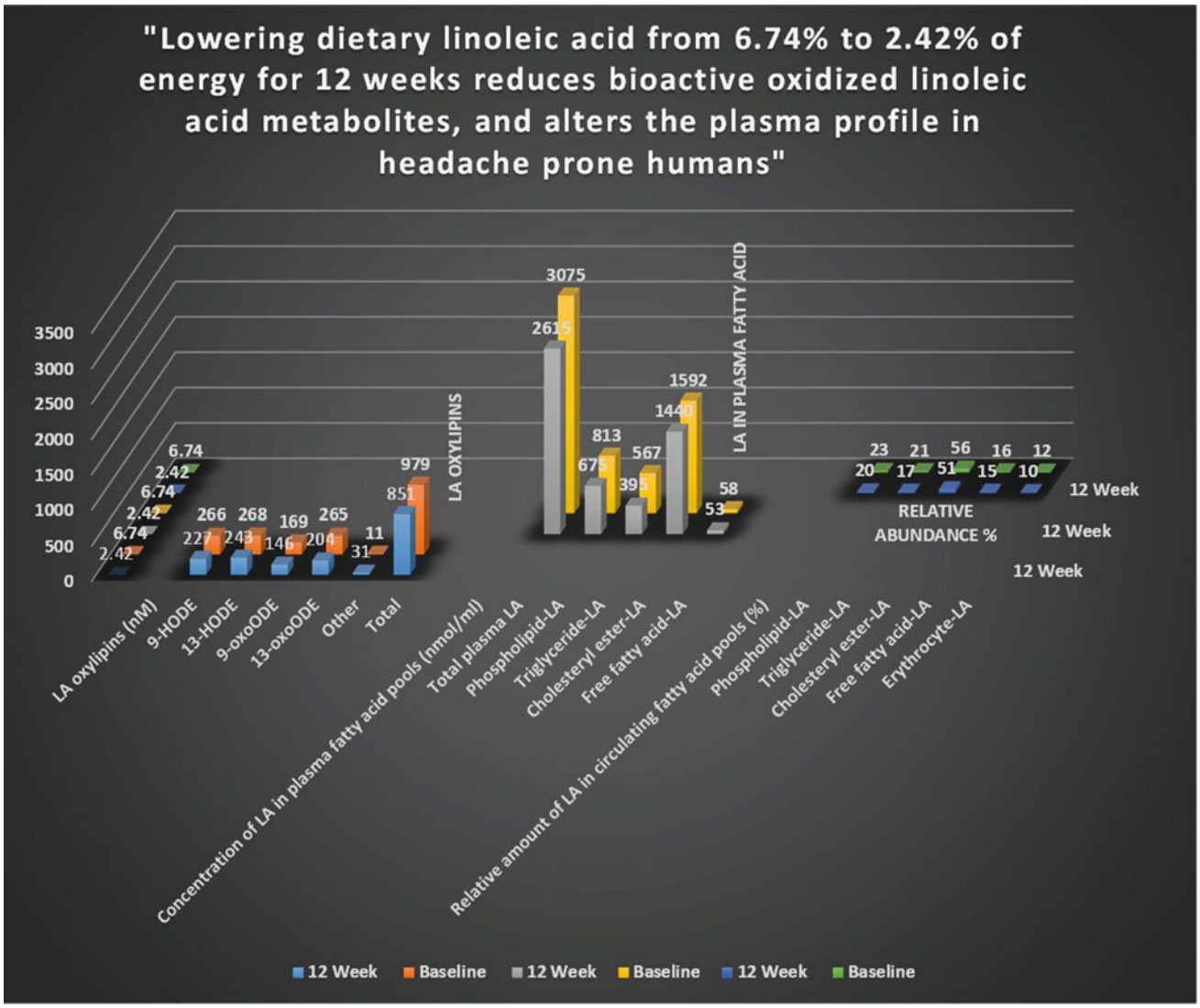


Fig. 28.4 Reducing dietary LA significantly reduced plasma LA and HODE oxylipins. The data were abstracted from Table 2 of '*Lowering dietary linoleic acid reduces bioactive oxidised linoleic acid metabolites in humans'* with very grateful thanks to the authors: Ramsden et al. [51]

Physiological Relevance of Oxidised Products of LA and ALA

Sensitivity of ALA and LA to Oxidation, the Variety and Different Physiological Properties of Their Oxidised Products

The capacity for photo-oxidation, autoxidation and enzymatic oxidation of LA and ALA into a wide range of oxidised products that act as key messaging systems, is fundamental to plant and mammal biology.

It is the sensitivity of ALA and LA to oxidation, the variety and different physiological properties of their oxidised products, including incidental peroxide; reactivity, reaction time, and ability or not to travel across cell membranes [53], which makes them good substrates for oxidative product-based signalling systems.

Multiple Pathways for Oxidation

There are multiple pathways for the oxidation of polyunsaturated fats, including enzymatic, LOX12/15, COX, P450, NAPDH oxidase, as well as nitrous-related, photo-oxidation, autoxidation and free radical oxidation.

Excess, imbalance and conditions of oxidative stress lead to the formation of peroxide, hydroxyl radicals, hydroperoxides and consequent ALA- and LA-related downstream oxidised products, primarily through a mix of enzymatic activation and free radical propagation.

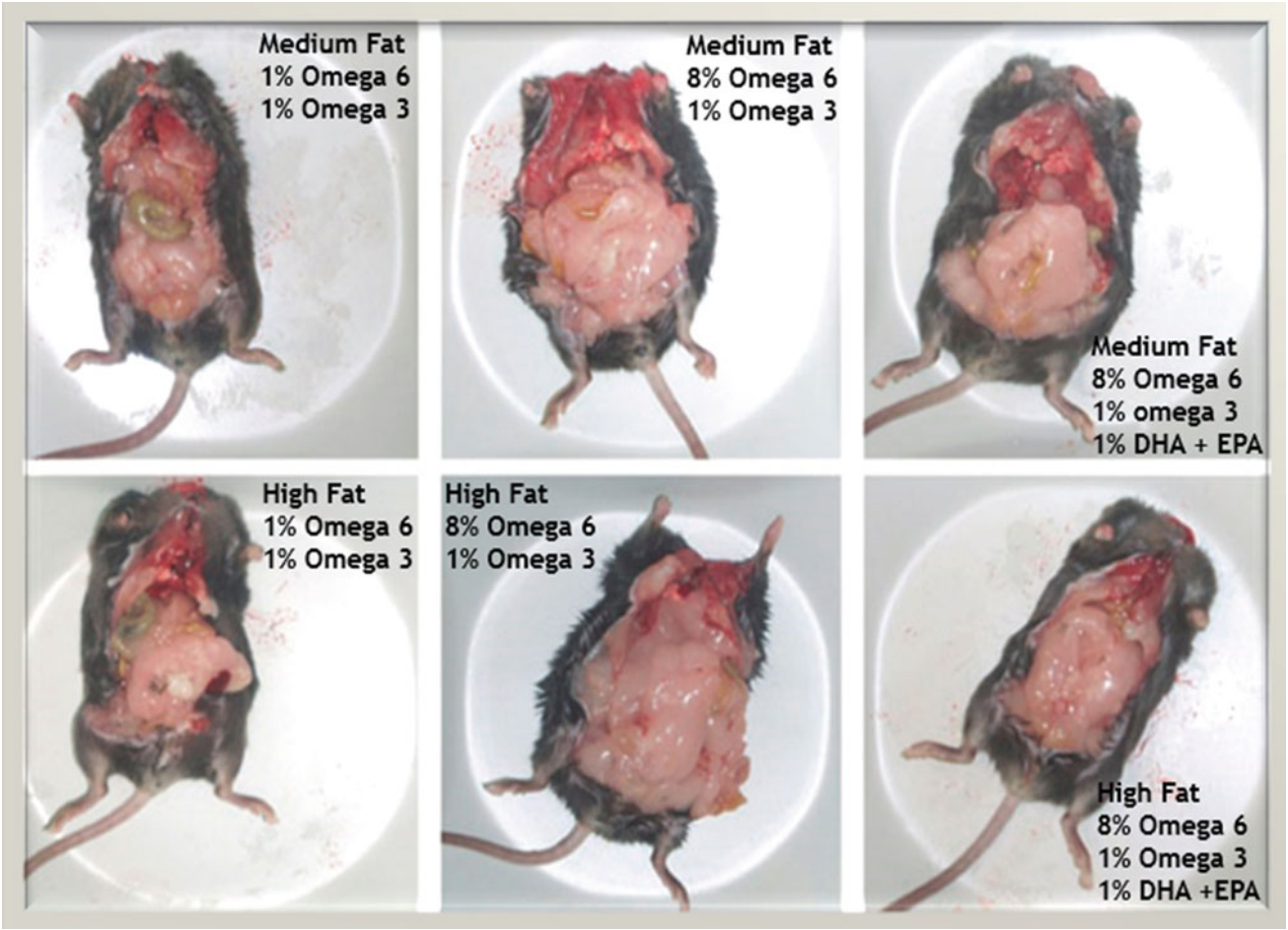


Fig. 28.5 '*Reducing dietary LA to 1 en% prevents adipose tissue accumulation and reverses the obesogenic effects of a high-fat (60 en %) diet. Animals fed 8 en% LA accumulated more fat than animals fed 1 en% LA. The addition of 1 en% n-3 EPA/DHA to 8 en% LA diets prevented the increase in adipose tissue seen in animals fed 8 % energy LA. The obesogenic properties of a high-fat diet (60 en% fat) were reversed by selective reduction of LA from 8 en% to 1 en% and replacement by greater saturated fat. The animals shown are representative for the animals in each dietary treatment. Upper row; isocaloric medium-fat diets of 35 en% fat, lower row; isocaloric high-fat diets of 60 en% fat. The fatty acid composition, not total fat calories, determined the obesogenic properties of the diets. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid'*. From 'dietary *linoleic acid elevates endogenous 2-AG and anandamide and induces obesity*' with very grateful thanks for permission to the authors: Alvheim et al. [52]

From an LA and ALA perspective, the activity of the LOX12/15 enzyme is particularly crucial, because its preferred substrates are ALA and LA in that order. Furthermore, LOX12/15 does not appear to require induction in the same way as the COX enzymes and can oxidise the lipid component of phospholipids in situ in membranes [54], where COX enzymes require the release of the lipid from the membrane.

Lipid peroxides and hydroxyl radicals in excess have great influence, not only because they are highly damaging, but also because there is only a limited range of antioxidants factors, including glutathione, found in both the aqueous and lipid compartments that are capable of preventing wider hydroxyl and hydroperoxide reaction, and thus damage. The hydroxyl radical and hydroperoxides can also oxidise phospholipids in situ, causing changes in the shape, fluidity and function of membranes, including altering the flip-flop ability of lipids and thus changing the function of cells.

9 and 13HODE

Oxidised LA products have a significant functional influence. 9 and 13HODE and their related products [55] including Epode's and DiHome's together constitute

possibly as much as two-thirds of the total oxylipin content of plasma and have far bigger presence than the AA-derived oxylipins including prostaglandins.

13HODE is endogenously formed in many cells including "*polymorphonuclear leucocytes, eosinophils, bronchiolar cells, breast carcinoma cells, Syrian hamster embryo fibroblasts and human dermal fibroblasts*" and macrophages.

Importantly, 4HNE, peroxide and 13HODE can cross membranes, including in the gut and brain, and travel significant distances, and so they have widespread oxidative and signalling effects including on cell and tissue growth, differentiation, neutrophil adhesion and apoptosis.

Phosphatidylcholine, often bearing LA, is a major membrane component, is a predominant component of lipoprotein shells and is very susceptible to oxidation. LA is peroxidised to 9 and 13HODE, 4HNE and other products whilst still attached to phospholipids, including mitochondrial cardiolipin [56], which on release are a major source of free LA oxylipins. Oxidation of mitochondrial cardiolipin alters its function, including of the electron transport chain leading to the disruption of ATP production and ultimately factoring in apoptosis.

LA Oxylipins in Food Actively Cross the Gut Membrane

LA oxylipins including 13HODE and related products can be formed in significant amounts during food processing, particularly during, but not restricted to, frying in LA-rich vegetable oils. Significant amounts can cross the gut membrane; in excess of 50 % of an oral dose of radiolabelled autooxidised LA was absorbed and excreted in urine, or as CO_2 [57].

Industrialised production of food together with changed methods of preparation has vastly amplified the amount of dietary oxidised LA products crossing the gut, arguably with significant physiological effects, which cannot be fully moderated, for example, by increasing plant-based antioxidant intake, but only by their removal from the diet.

ALA and Wider Oxylipins Including Conjugated Lipids—Current Knowledge

Oxidised downstream products of LA, ALA and other polyunsaturated fats are diverse and numerous. Given their wide-ranging and crucial signalling roles in other species including plants, they are all likely to be of physiological relevance of varying degrees in humans.

However, knowledge on omega-3 ALA oxylipins is limited. There is little research on conjugated ALA oxylipins [58]. Some research has been done into long-chain omega-3 oxylipins including DHA, mainly in relation to the brain [59, 60]. GLA has not been included in this overview.

Photo-Oxidation Products

LA is the most common polyunsaturated fat in and essential to healthy skin [61]. UVB is more effective than UVA at oxidising substrate including lipids by direct or catalysed oxidation, because it has greater energy. However, it penetrates tissues less deeply than UVA [62].

UVB photo-oxidation of LA in the skin, direct or catalysed, through oxidised LA derivatives, including 13HODE, Oxo-HODE and 4HNE, as well as through secondary oxidation leads to cellular stress, inflammation, and activation of TRPV1 [63, 64] pain pathways [65, 66].

ALA intake moderates the effect of LA oxylipins. Rats with ALA/LA intake of between 1:3.5 and 1:5 showed reduced pain, better retention and learning, and temperature change tolerance [67]. Rats fed ALA are much less susceptible to sunburn [68]; UV effects are multi-factoral.

Cox and Lox 12/15 Enzymes—Functional Differences and Relevance to LA and ALA

ALA followed by LA (in animal models) are the preferred LOX 12/15 substrates (possibly an evolutionary reflection of plant biology [69]), and interestingly, ALA and LA are preferred to EPA as COX enzyme substrates.

Equally significantly, in contrast to the COX [70] enzymes, LOX12/15 as well as free hydroxyl radicals can oxidise phospholipids in situ, without the need for lipid release from the cell membrane. Additionally, LOX12/15 activation appears to require a lower induction threshold than COX.

LOX12/15 is activated by random oxidative stress factors such as peroxide, so is part of a self-reinforcing oxidative stress propagation system. COX in comparison with LOX12/15 appears much more regulated, because its activity is dependent on prior activation of phospholipase to cleave the fat from the membrane, as well as the activation of the COX enzymes.

In the context of the differing functions of COX and LOX, the modern dietary ALA/LA imbalances, and ALA deficits, are of particular physiological significance because:

- LOX12/15 is active in, and expression is associated with diseases in a number of tissues including; pancreatic islets, microvasculature, nervous system, adipose, kidney, brain; furthermore, LOX12/15 is a factor including in diabetes, vascular disease, kidney disease, Alzheimer's disease and Parkinson's disease [71]. LOX 12/15

knockouts are protected against insulin resistance, fatty liver [72], atherosclerosis, obesity [73], axon degeneration [74] and diabetes type 1 [75].

- LOX and COX omega-3 and 6 products have very different physiological effects.
- 18-carbon fats ALA followed by LA are the preferred target for the LOX enzymes. In rats, an excess of LA over ALA more than doubled liver inflammatory-related oxylipin products 9 and 13HODE [76].
- When LA, ALA and AA were individually oxidised with reticulocyte-derived LOX enzyme, the products were respectively
  - LA: 13HODE 75 %, 9HODE 25 %,
  - ALA: 13HOTE 82 %, 16HOTE 11 %, 9HOTE 5 %, 12HOTE 2 %,
  - AA: 15HETE 71 %, 11HETE 19 % and 12HETE 16 %.
- Where human plasma cholesterol esters were oxidised with LOX, the related products were 13HODE 56 %, 9HODE 41 %, 15HETE 3 % in a mixture of chiral forms in differing ratios [77]. ALA products will form if the substrate is present, but LA 9 and 13HODE and related products tend to dominate in human plasma. It is not known to what extent ALA supplementation would alter plasma oxylipin mix.
- As a generality, omega-3 LOX and COX products tend to be anti-inflammatory, whereas omega-6 products tend to be inflammatory. 13HOTE, the omega-3 equivalent of 13HODE, has been shown to be potentially strongly anti-inflammatory [78]. In humans, there is very limited research into HOTE; as of 2013, possibly there is just one paper but there is much more research in plants.
- Whilst AA is the preferred target for COX enzymes, COX will also target LA and ALA equally and in preference to EPA. So an excess of LA over AA will result in more COX LA products, but there will be a compensatory corresponding reduction in COX AA products; the net effect of a drop in COX AA products and increase in LOX and COX LA products will be complex and not necessarily of net benefit.
- Lipids attached to phospholipids including cardiolipin do not require cleaving for oxidation by LOX12/15 and are well oxidised in situ whilst still esterified. LA hydroperoxides including 9 and 13HODE [79] are found in cardiolipin. In a paper looking at LOX12/15 oxidation in rabbit membranes, 85 % of lipids were found to have been oxidised in situ [80]. This gives phospholipids and the fats they carry particular relevance.
- Oxidised phospholipids are cellular messengers in their own right, recognised by oxidised LDL receptors, and other factors including VEGF and FGF [81].
- Oxidised phospholipids are readily taken up by cells.
- Oxidised phospholipids alter the membrane structure and function. They are important in tissue formation, wound healing and tumour formation, as well as effecting the expression of 1000 genes, but in excess they contribute to inflammation and immune overactivation.
- Lysophosphatidylcholine, often produced in tissue by the cleavage of oxidised LA from the SN2 position, is associated with oxidised LDL and in excess is pro-inflammatory, although some species are reported to be anti-inflammatory [82]. Lysophosphatidylcholine is associated with oxidative stress and intima thickening in smokers [83]. 12/15LOX also acts on lysophosphatidylcholine.
- In excess, oxidised products of LA including 9 and 13HODE and Oxo-HODE are primary activators of oxidative stress-related PPAR gamma peroxisomes; PPAR gamma is also activated by the products of AA, such as LTB4 and other prostaglandins, but compared to AA product 15d-PGJ2, 9 and 13HODE bind PPAR gamma by an order of magnitude more powerfully and over twice as effectively as 15-HETE.
- 'Excess' PPAR gamma-related peroxisome activation likely produces peroxide in excess of catalase, through the concurrent cytokine- and peroxide-based iNOS activation, and thus NO production and consequent catalase enzyme inhibition, increasing LOX12/15 activity, background oxidative stress levels and hydroxyl radical production, thereby creating self-reinforcing oxidative cascade loops.

There is also a 15LOX 2; it is of limited relevance to this overview because arachidonic acid is the preferred substrate. It is found in particular tissues, including skin, prostate, lung and the cornea. The main products are HPETEs. Similarly, LOX5 is not considered in this overview as not active against 18-carbon fats.

Significant physiological effects of excess LA, and ALA imbalances or deficits, can be expected from the displacement of COX AA [84] and EPA products by COX LA and ALA products. Such changes will alter the nature of immune and inflammatory responses, and could be detrimental if resulting in responses outside normal physiological parameters, in scenarios such as pregnancy.

Adding to the complexities, intermediate oxidised LA product 13HPODE, precursor to 13HODE, may competitively inhibit prostaglandin formation from AA [85]. Further outcomes will alter as balances between chiral S and R forms change.

The above hints generally at the huge physiological importance of the balance between LA and ALA as the preferred substrate of LOX12/15 enzymes in plasma, adipose tissue, wider tissue triglycerides and phospholipids.

The balance between LA and ALA, and their oxidised products in plasma and tissue membranes, will in turn significantly depend on dietary intake. The addition of modest levels of ALA to the diet, through the alternation of membrane composition, preferential production of LOX12/15, likely anti-inflammatory ALA oxylipins about which limited amounts are known, and co-reduction of the amount of LA oxylipins, will give rise to significant physiological effects.

The pathways are immensely complex interlinked and interdependent; much is still to be learnt. However no matter the complexities, oxidative stress is inevitable [86] and, in combination with excess of LA and lack of ALA, has significant roles in vascular and related inflammatory non-communicable Western diseases.

In contrast, biological mechanisms to explain the sometimes claimed central role of 'saturated fats' in vascular disease are limited (this comment is in respect of pure saturated fats, as against what are commonly imprecisely referred to as 'saturated fats', namely linoleic-rich (up to 25–30 %) heavily oxidised non-ruminant animal fat, such as found in industrially produced chicken, and to a lesser lard, the primary fat in many rodent high-fat laboratory diets).

CYP450 Enzymes

It would be surprising if LA and ALA were not also the preferred substrate of P450 enzymes, because they too have plant origins. P450 products include oxylipins [87]. They have been shown to have roles in inflammatory conditions, early infection, respiratory distress, cardiac failure, induction and resolution of infections and in the pain pathways.

P450 LA products can form a significant proportion of plasma oxylipins and, in numeric terms, generally comprise a much greater proportion of plasma oxylipins than AA products. Although P450 products of AA are also biologically important in immune function, and might stimulate tumour proliferation, migration and invasion, they can be cardioprotective [88].

A fascinating study inter alia examined the lipodomic profile of "*human nasopharyngeal lavages obtained during the 2009–2011 influenza seasons*" in low, medium and high responders. LA oxylipins were significantly raised in humans with low symptoms, as shown in comparatively stacked bar graphs and heat map (4) of their paper, which also considers COX, LOX, CYP450, non-enzymatic, linoleic, linolenic, DHA and EPA oxylipins.

LA oxylipins had both inflammatory and anti-inflammatory effects, which proportionately changed through infection and resolution phases. The balance between and the predominance of 5, 12 and 15LOX, DHA and, to a lesser degree, P450 oxylipins were strikingly different between the low-, medium- and high-response groups. The paper highlights the very complex interrelationship between the oxylipin products pathways, and the significant relevance of LA oxylipins to immune function and resolution [89].

Oxylipins Generally

LA and ALA are oxidised as free fats, directly in phospholipids [76], and found in oxidised form in cardiolipin, cholesterol esters [90], triglycerides and LDL. Effects of oxylipins are likely rapid and systemic [91]. LA oxylipins are both made within the tissues and delivered to them by LDL. LA is converted including via 9 and 13HODE to an array of downstream oxidised products.

Overall, LA and ALA oxylipins have a significant influence on reproductive, vascular, cardiac, lung, liver and brain tissues. Importantly, oxidised LA products including HODEs, 4HNE, MDA in excess give rise to mitochondrial dysfunction, a significant factor in 'Western diseases', as well as neurological conditions including dementias such as Alzheimer's disease.

Conversely at 'normal' maintenance levels, 13HODE and related oxylipins help regulate physiological 'housekeeping' processes including the suppression of platelet activation and aggregation [92].

In a human study looking at oxidised blood lipid metabolites, LA products dominated and were raised in hyperlipidaemic individuals. The concentration of different oxylipins was '$LA > AA = ALA \geq DHA > EPA$', which '*correlates well with the abundance of the PUFA in the human organism*'. The concentration of serum hydroxy fats, in decreasing order, was '$LA > AA > EPA > ALA$', and epoxides '$LA > ALA > AA > DHA > EPA$' [55]. Whilst the general trends appear fairly consistent, the levels of oxylipins vary significantly between individuals and groups; lifestyle including activities such as smoking will alter profiles. In addition, different studies may focus on different oxidation products. The '*Human Serum Metabolome*' paper usefully sets out a number of blood parameters, including the analyses of LA oxylipins; 13HODE is by far the predominant component [93], but the P450 product 9,10 DiHOME level is also high (see Fig. 28.6).

DiHOMEs, products of P450-related pathways, are significantly implicated in inflammatory conditions, including lung damage from smoking, and in a mouse model rose by much greater amounts than AA products [94]. DiHOMEs are also '*potent chemoattractants for neutrophils*' [89].

LA plasma oxylipins generally significantly outnumber ALA oxylipins; the balance is somewhat modulated by the dietary intake of the longer-chain omega-3 and omega-6 fats.

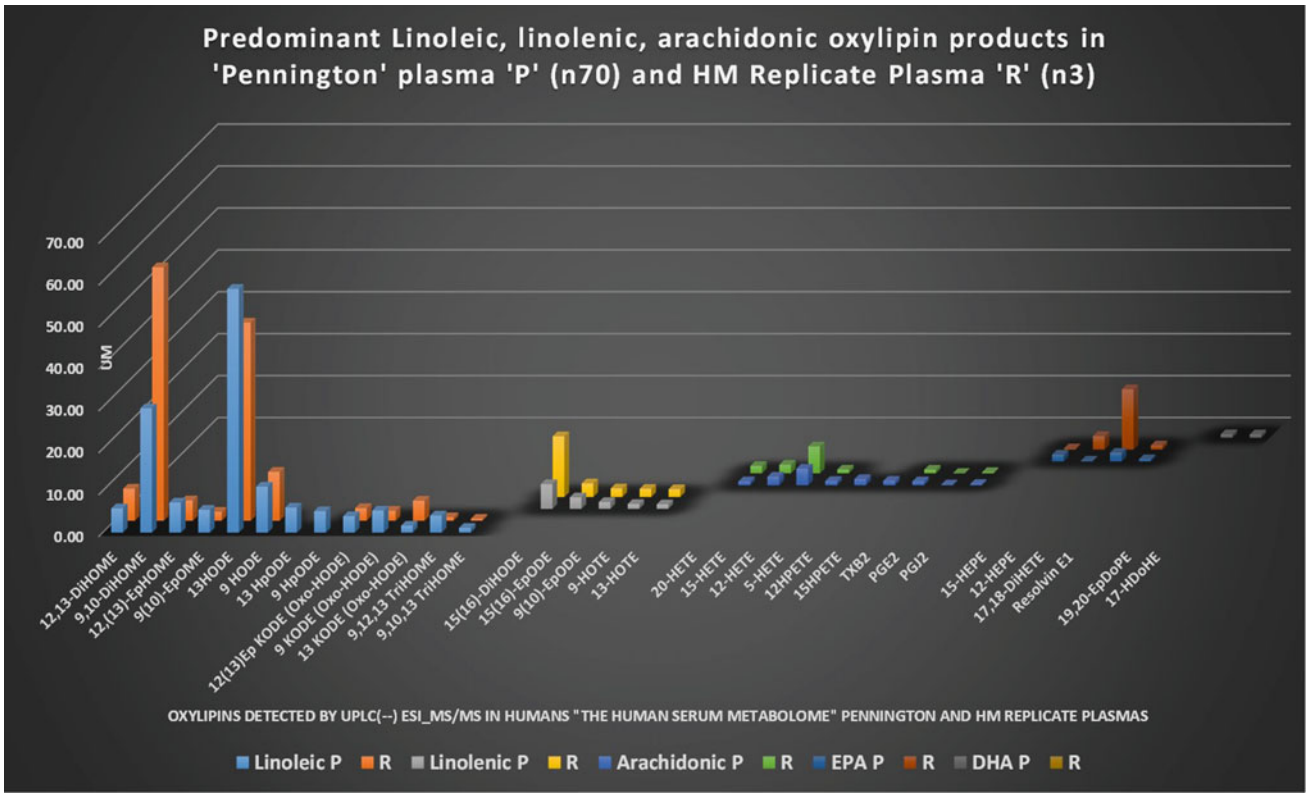


Fig. 28.6 This graph of the predominant oxylipins in plasma is illustrative of the dominance of LA oxylipins over the oxidised products of AA, EPA and DHA, including the COX2 AA prostaglandins. The list only selects the major oxylipins recorded. The figures for PGE2 and PGJ2 have been included to give an indication of comparative concentrations; they were not in the predominant group. The differences between the groups are interesting and particularly the significant disparities of levels of omega-3 oxylipins. The 9,10 DiHOME level is high in comparison with other groups. DiHOMEs may inhibit mitochondrial respiration and increase vascular permeability. Comparison is difficult because ratios of oxylipins differ significantly between groups, as can be seen by comparing these data to the Schuchardt et al. [55], Ramsden data [51] and Spath [22] data, but the general trend that LA oxylipins are the largest group of oxidised lipids in plasma is clear. Omega-3 supplementation by Schuchardt [91] (1008 mg EPA + 672 mg DHA) significantly increased EPA oxylipins compared to baseline, peaking at about 6 h and remaining a little above baseline at 48 h. The graphed data are selectively abstracted from Tables 7, 8 and 9 of the '*The Human Serum Metabolome*' paper with very grateful thanks to the authors: Psychogios et al. [93]

For example, dietary intake of longer-chain omega-3 fats, including mainly EPA and DHA (4 grams Lovaza), increased omega-3 oxylipins two- to fivefold and decreased omega-6 oxylipins including 9 and 13HODE by around 20 %. Competition for LOX12/15 and COX may have contributed to the changes.

In total parenteral nutrition patients, significant changes are seen in the ALA/LA balance of plasma phospholipids [95]. Changes in the ALA/LA content, and so oxidised content of the phospholipid shells, would influence their interaction with LDL receptors, activation of the peroxisome pathways, thus moderating macrophage activation.

Clearly, dietary and endogenous oxidised products of LA, which will be present in plasma as well as in vascular and other cells, play significant physiological roles. Little research has been done on the physiological changes consequent on the competitive effects of ALA and its oxidised products on the metabolism of oxidised products of LA.

4HNE

4HNE is a stable product and an extraordinarily reactive oxidant with a concentration-dependent effect, an '*LDL magnet*' and '*extraordinary*' compound [96], which favours interaction with phospholipids and proteins [97], including cysteine and lysine, so impacting enzyme function and protein folding [98], as well as glutathione production.

4HNE is made exclusively from omega-6 LA and downstream derivative AA. Mechanisms are still not fully understood. It can migrate by diffusion through cell membranes including in the brain. Phospholipid-derived LA including from biphospholipid cardiolipin appears to be a significant source of 4HNE [99].

4HNE is capable of oxidising DNA, and proteins, including commonly attacking ApoB100 in phospholipids, binding to as many as 486 sites [100]. It binds to DNA, so modulating gene function, and in excess damages genes.

4HNE formed from cardiolipin is a significant factor in mitochondrial damage [101] and so has a role in metabolic disorders including obesity. Glutathione is a major detoxification pathway of 4HNE, and depleted by it, so an important metabolic regulator.

Other related products 4HPNE and ONE are likely even more reactive, so more damaging, but in consequence will have a lower ability to travel. 4ONE is reported to bind more powerfully than 4HNE, possibly being '*>100-fold more reactive*' and so capable of significant impact on mitochondrial function [102]. Spinal cord material from ALS patients showed significantly higher ONE activity than in controls [103].

There is an omega-3 equivalent 4HHE (see below). 4-HDDE rather than 4HNE is the primary equivalent product of AA, but whilst highly reactive [104] is only present in plasma in very small amounts and so not considered in detail.

Excess 4HNE and 13HODE together have been strongly implicated in; cancer; cardiovascular disease; pulmonary disease including COPD; endothelial dysfunction; sepsis; shock; ischaemia/reperfusion injury; mitochondrial dysbiosis; glucose transporter inactivation; Parkinson's disease and Alzheimer's disease; alcohol-related conditions; hepatitis; Crohn's disease; deep vein thrombosis; lupus; lung disease; rheumatoid arthritis; and diabetes [105].

MDA

Whilst MDA can be made from a variety of substrates, much is likely to originate from LA, because LA predominates in plasma, LDL, cardiolipin, some phospholipids and is replenished by Western diets and/or from LA stores in adipose tissue. Like 4HNE, MDA is also widely linked with cellular malfunction and disease and particularly protein and DNA damage.

Omega-3 Oxylipins

Like omega-6s, omega-3s form oxylipins, which will also be found in LDL plasma and wider tissue. ALA oxidised products 9 and 13 (S) and (R) HOTE will likely have different downstream roles to LA, and be present generally in much lower amounts. In vivo 15,16-DiHODE and 15,16EpODE, likely P450 products, are the two most common ALA oxylipins in human plasma [55, 93].

To date, limited research has been done on omega-3 oxylipins and particularly those of ALA. Omega-3 oxylipins are generally found to have effects that counterbalance those of omega-6, and so 'protective' roles, but very little is known about the impact of dietary ALA/LA balances on LA and ALA oxylipin production.

4HHE, a product of omega-3s, mainly of DHA but also potentially of ALA, whilst less reactive than 4HNE, is present in plasma, capable of causing oxidative stress, and may have a role in apoptosis [106]. Like omega-6 oxylipins, omega-3s are 'housekeeping' signalling messengers at low concentrations, but can cause damage at high concentrations. 4HHE has been associated with Alzheimer's disease, and in mice with retinal and liver damage, highlighting the importance of determining optimal EPA and DHA intakes, minimising oxidative stress and optimising enzymatic and wider antioxidant function [107], although omega-3 oxylipins may in quantity have medicinal qualities, possibly in part, with vitamin D and iodine, accounting for the benefits of oxidised fish oil seen in treatment in the 1800's of tuberculosis of the lymph nodes, 'Scrofula'.

TRVP1 Pain Pathways

The LA HODE family are key activators of pain pathways. UVB photo-oxidised products of linoleic acid 13HODE and 4HNE signal COX and TRPV1 activation, so resulting in inflammation and pain [63]. Besides COX and LOX, CYP 450 enzymes may also result in the production of LA oxylipins that trigger TRPV1. A P450 pathway blocker moderated oxidised LA-invoked inflamed tissue TRPV1-based pain response [87].

TRPV1 is also activated by PGE2, a potent mediator of pain [108, 109]. TRPV1 is a factor in breast pain and tenderness [110]; oxidative stress as measured by hydroperoxides peaks at the time of ovular maturation and possible implantation [111]. Oestrogen peaks around ovulation; oestrogen increases desaturase function, so providing AA, the substrate for the production of PGE2; in a feedforward cycle, PGE2 will increase oestrogen production, which would fit with oestrogen rising until non-fertilisation leads to failure to implant.

HODEs and 4HNE will increase COX2 activity and so PGE2 production, as well as LOX12/15 activity. During inflammation, PPAR gamma will also be increased by 13HODE, creating a feedforward loop through increased peroxide and 13HODE.

In the absence of competing and/or moderating factors, including omega-3 s and antioxidant capacity, it is likely that the 13HODE-related oxidative stress pathways will tend to positively and overly reinforce themselves. Intriguingly, TRPV1 and 13HODE are also overexpressed in the lungs of human asthmatics and related to mitochondrial dysfunction [112].

A Western diet high in pre-oxidised foods including vegetable oils, imbalanced in omega-3 and omega-6, together with a lack of selenium and cysteine and so glutathione, may be responsible for increased dysmenorrhoea and other

fertility-related conditions such as PCOS that are treated with COX inhibitors.

If ALA was present, oestrogen-promoted desaturase would increase the production of protective DHA, much of which would likely be utilised to support uterine and ovular tissue requirements; ALA and DHA would compete for COX and LOX substrate, and particularly so when an unfertilised egg does not implant, DHA being liberated and/or available once no longer required to support new tissue production.

Omega-3s also bind TRPV1 but with much lower affinity [113] and may by competition for, and blocking of COX LOX and other pathways, reduce the effect of omega-6 oxidised products in promoting pain pathways. Consistent with this, omega-3 intake, and/or reduced omega-6 intake, has been associated with reduced pain sensitivity including headaches.

Oestrogen Desaturase and Cardiovascular Disease

Several lines of evidence suggest that oestrogen increases desaturate function. In the absence of dietary omega-3s, higher desaturase function would increase AA production, which combined with low dietary antioxidants may lead to increased COX2, and LOX12/15 action on AA as well as LA, and so increased oxylipins including prostaglandins, which might conceivably have a role in the reported increases in risk of cardiovascular disease in those on oral contraceptives.

Interestingly, oral contraceptives variously increased lipid oxidation [114], possibly through the iNOS and NO pathways [115], but also in some instances increased catalase production. Different omega-3:6 plasma balances could theoretically also account for some of the different outcomes seen in studies of LOX12/15 including in cardiovascular disease.

Pathways are as ever complicated and differ between oestrogen types. In addition to catalase production, serum oestrogen increases glutathione and SOD which mechanism is postulated to be a factor in the greater longevity of women [116]. Oestrogen correlates in a linear fashion with plasma antioxidants through the menstrual cycle [117]. Antioxidant levels decline at menopause.

This again brings us back to the limited pathways including action by HDL, to 'detoxify' dietary or endogenous plasma LA/AA oxylipins; the above may suggest as a generality that females despite better antioxidant capacity are at risk of oxidative lipid-based stress, if they lack the necessary nutrients to support antioxidant function, or systems are overwhelmed by excess of omega-6 oxylipins, and/or the lack of omega-3 s.

Both omega-3 s and 6 s are central to healthy reproduction. Omega-6 related oxidative stress is essential to the reproductive processes, but delicately balanced, and dependent on good antioxidant function, and the presence of competition for enzymes by Omega 3 s, to prevent oxidative stress moving up to damaging as against facilitating levels.

Oxidised Lipids: PPAR Gamma and Alpha Activators

Oxylipins also have significant effect through the activation of peroxisomal activity. HODEs are the primary endogenous activator of PPAR gamma. In contrast, PPAR alpha is primarily activated by energy deficit stress, but there are pointers towards oxidised ALA products, and possibly, high protein intake acting as lessor activator of PPAR alpha. A high-protein diet is lower in accessible energy substrate and may lead to ketosis, which pathways have links to PPAR alpha activity.

It would make sense for excess DHA in fish, and ALA in non-ruminants, to have the capacity to promote beta-oxidation to moderate excess lipid build-up of highly oxidisable, antioxidant resource 'expensive' polyunsaturates, in adipose and non-adipose cells.

Chiral Oxylipin Products

Oxylipins take chiral S and R forms [118–122]. Dietary chiral oxidised products, in addition to raising oxidative stress in crucial body systems, may contribute or even help explain the even mix of 13HODE S and R enantiomers noted in vascular plaque. The apparent inflammatory effect of the S form of LA 13HODE, its activation of PPAR gamma [123] and its production through LOX12/15 activity again emphasise the need for and importance of moderation of LA in the diet, and the inclusion of adequate ALA.

Nitrogen-Related (Nitrous) Oxidation Products

Nitrous oxides are active in the lipid membranes, acting as powerful antioxidants as well as oxidants [124]. NO is also a messenger; '*NO has emerged as a fundamental signalling device regulating virtually every critical cellular function, as well as a potent mediator of cellular damage in a wide range of conditions*' [125]; the subject is extensive and complex [126, 127].

Importantly in tissues where NO is readily expressed, it may regulate the activity of the catalase enzyme [128]. At times of high levels of PPAR gamma-induced peroxisomal beta-oxidation, NO produced by iNOS may amplify oxidative stress by inhibiting the activity of the catalase enzyme, as in vascular tissues including in macrophages during immune- and damage-related stress. At low peroxisomal beta-oxidation levels, NO may assist in the reduction of peroxide.

Lipid peroxides and peroxide may enhance NO formation, which in turn may help, either promote or inhibit lipid oxidation creating feedback loops [98]. NO production has been associated with increased 4HNE production [129], COX activation and PGE2 interaction [130].

Nitrous products in common with oxidised polyunsaturated fats, and particularly LA, play important roles in endometrial receptivity, implantation and menstruation [131], and reproduction, as well as in cardiovascular disease [132], suggesting the likelihood of extensive direct and indirect interaction between nitrous products and lipid pathways, be that in an oxidant or antioxidant capacity. NO production will ultimately also reflect dietary availability of arginine and other amino acids.

Metabolic Sensing and Pathway Preferences for Oxidised Product

Of wider metabolic interest, including in the consideration of evolutionary signalling pathways and their development and purpose, priority appears to be given to the uptake and hydrolysis by HSL and LPL of 9 and 13HODE; they formed by far the largest proportion of oxylipins released by LPL from triglyceride-rich lipoproteins [133, 134]. HSL may have a concentration-dependent preference for oxidised cholesterol esters [134, 135]. Further, LPL enhances 15LOX activity [136], which may help promote oxidative stress, and LA HODE-related feedforward mechanisms, potentially providing mechanisms to magnify the effects of environmental and endogenous LA oxylipin availability.

Wider Implications of Excess Oxidative Stress

At normal physiological levels, oxidised products are part of the body's control signalling systems, but in long-term excess they become an issue. At low concentrations, oxidised LA, 13HODE, 4HNE, MDA and other pro-oxidant products signal for antioxidant production, but at high levels magnify oxidative stress leading to DNA and protein damage, and so enzyme and mitochondrial dysfunction, cellular malfunction and ultimately cell death.

Oxidative stress once out of control fuels oxidative stress cascades; it draws on diminishing antioxidant resources and pathways, arguably overtime leading to self-propagating downward health spirals. These spirals can only be reversed by addressing the underlying dietary causes, including reducing the intake of foods that are heavily processed, pre-oxidised and depleted of antioxidants and other nutrients.

Deficiency of ALA in the Diet—Oxidative Stress Implications

Arguably, nature has compensated for lower ALA tissue and blood levels relative to LA, through the LOX12/15 (1) enzyme preference for omega-3 fatty acids 18-carbon fats. For ALA to compete with LA for available LOX12/15, COX and P450 enzymes, ALA needs to be present in the diet in adequate and proportionate quantities; if it is missing from the diet, self-evidently it cannot compete with LA for conversion to bioactive and potentially protective ALA oxylipins.

Phosphatidylcholine and Cardiolipin: Their Particular Relevance

Phosphatidylcholine is only one of several phospholipids, but of particular relevance because it is easily oxidised, and the primary phospholipid in the shells of chylomicrons and LDL, as well as a significant cell membrane component. Polyunsaturated fats preferentially bind at the SN2 position of phosphatidylcholine, so LA and/or ALA if present in the diet will be commonly attached phosphatidylcholine passengers in lipoprotein shells.

Oxidised phosphatidylcholine products are a factor in vascular fatty streak initiation [137], are found in plasma, LDL and comprise a major component in mature plaque [138]. They are also capable of gene activation.

Phosphatidylcholine is a zwitter ion carrying both a negative and positive charge in its head group. One can envisage that this would allow phosphatidylcholines in lipid vesicles, to bind next to each other positive to negative, in a staggered fashion, creating a slightly polar external positive charge, which would be consistent with most LDL exhibiting slight positive charge, although oxidised content [139] and the presence of lysophosphatidylcholine may change the shell polarity from positive to negative.

LDL receptor OLR1 (also called LOX1) has a greater affinity for negatively charged LDL [140]. Negative LDL is associated with oxidative stress-related conditions including cardiovascular disease, metabolic syndrome and diabetes [141].

Oxidised phosphatidylcholine is recognised by oxidised LDL receptors, likely by altering LDL membrane characteristics, for example protruding into the aqueous compartment [142], or if attached to oxidised matter, or proteins, for example the C reactive protein [143, 144] (CRP is significantly

increased in conditions such as diabetes and metabolic syndrome (CRP: 0.54 mg/l control; 3.61 mg/l metabolic syndrome; 4.82 mg/l diabetes 2)) [145]. Oxidised phospholipids can also crosslink with amino acids including lysine and likely cysteine to form amino-phospholipids [146]. Recognition is suggested to depend entirely on the nature of the oxidised SN2 residue, rather than the changes in the head group [147].

Consistent with the importance of oxidised lipids in signalling systems, oxidised but not unoxidised phosphatidylcholine promotes macrophage activity, platelet-activating factor, reduced vascular relaxation, ischaemia, inflammation, radiation stress, multiple sclerosis and lung injury [148].

In addition, structural membrane changes may facilitate the metabolic regulation including by promoting flip-flop activity [149], which would potentially allow the export of oxidised lipids such as 13HODE [150] from cells including mitochondria. The removal of an oxidised lipid and the potential subsequent replacement with a new unoxidised polyunsaturate, and flip-flop back across the membrane, would provide a mechanism to help regulate mitochondrial oxidative stress.

Phosphatidylcholine as a sacrificial 'antioxidant', a major structural component of shells of chylomicrons, VLDL and LDL, is an important part of a coordinated regulatory system to monitor, control and 'sweep' the blood of oxidised material and pathogens for delivery by endocytosis of LDL to the interior of vascular membranes and adipose tissue for temporary storage and/or reprocessing; the endocytosed oxidised product and pathogen content synergistically initiate both LDL receptor activity and immune and repair responses in vascular and related adipose tissues, and also deliver unoxidised polyunsaturated fats cholesterol and lipid-soluble antioxidants to cell membranes as substrate for maintenance renewal and/or energy substrate.

Lysophosphatidylcholine

Lysophosphatidylcholines are of physiological relevance, recognised by some receptors, chemoattractants for phagocytes, and present in increased amounts in inflammatory-related conditions, including smoking [83], cardiovascular disease [151], diabetes, pre-eclampsia and hypertension. They may play key roles in a number of '*chronic inflammatory processes where oxidised phospholipids are known to be present*' [152]. In smokers, lysophosphatidylcholine and oxidised product levels were well correlated with intimal thickening and an increased risk of atherosclerosis. Oxidation of LDL increases lysophosphatidylcholine content as a proportion of phosphatidylcholine from 1 to 5 % to 40–50 % [153].

Cardiolipin—LA a Primary Component and Likely Metabolic Regulator

Cardiolipin is a biphospholipid primarily found in the inner mitochondrial membrane that differs in composition between tissues. Outside the brain, LA is generally the predominant fat in cardiolipin, 80 %; this is partly a reflection of Western diet and may be a distant genetic nod to cardiolipin species in plants. Almost no ALA is incorporated in cardiolipin [154], although DHA is.

Dietary DHA is incorporated in preference to LA in cardiolipin in the liver and heart, amounts plateauing off at about 10 and 20 %, respectively [155]. Replacing 2 double bonds (LA) with 6 double bonds (DHA) will have significant functional consequences for membrane properties, including impacting apoptosis, as well as proton and/or electron transport via the cytochrome C pathways.

In contrast with wider tissues, brain tissue cardiolipin LA content was much lower approximately 17 % [156], which may be because lipid uptake by the brain is highly regulated, and ALA and LA entering the brain are almost all metabolised, likely in peroxisomes for a mix of energy and substrate for cholesterol and other fats.

The lipid composition of cardiolipin reflects the dietary fat intake particularly during extreme dietary change such as famine. Induced essential fatty acid deficiency produced a fall in cardiolipin LA content from 79 to 19.6 % after 66 days, which recovered quickly on the transfer to a non-deficient diet. LA was replaced by OA [157].

Conversely, increased dietary LA raised LA content in cardiac and brain tissue cardiolipin (in rats). LA rose and DHA, AA and OA fell. These changes start to happen quickly; significant effects were seen in two weeks in the brain and one in the heart; these surprisingly rapid metabolic and neurological responses suggest that seasonal changes in dietary availability of LA and DHA could provide direct mechanisms to moderate metabolism and behaviour [158] including reproduction according to seasonal environmental changes, so optimising species survival.

Excess industrial dietary season-independent 365 availability of LA, excesses and deficits of key nutrients including LA and DHA, via cardiolipin in the brain and wider body, and through oxidised signalling including raised PGE2 hence altered metabolism including steroid hormones production, will result in subtle shifts in population-wide behaviour likely towards those associated with reproduction and transmission of genetic material, including 'mating-related' behaviours such as increased territoriality, aggression and display of 'self-interest'.

LA Species in Cardiolipin Are Very Susceptible to Oxidation Impacting Cytochrome C Function and so Energy Production

LA in cardiolipin is both a major target for 13HODE and 4HNE [159] and a source of them. Hydroperoxides [160, 161] appear to trigger oxidative reactions between cardiolipin and cytochrome C. Oxidisation of LA in situ to 9 and 13HODE inhibits ATP production, giving oxidised LA availability a role in regulating metabolic rate.

Oxidation of LA in cardiolipin can take place in situ; significantly only limited mechanisms can oxidise phospholipids in situ including LOX12/15 (but not COX enzymes), hydroxyl radicals, related radicals including 4HNE and the action of the mitochondrial enzyme cytochrome C.

LA is a preferred substrate for the LOX enzymes and a preferred beta-oxidation substrate of peroxisomes. Peroxide, a substrate for the hydroxyl radical [162] and a primary activator of LOX12/15, is produced inter alia by peroxisomes. LOX 12/15 and peroxide [163] appear central to mitochondrial dysfunction [164].

The primary oxidised products of lung irradiation were 9 and 13HODEs and derivatives. Oxidation of phospholipids other than cardiolipin and phosphatidylserine was limited [165]. Irradiated saturated phospholipids did not significantly undergo oxidation.

As seen in Barth syndrome [166], cardiolipin oxidation results in the degradation of cytochrome C function, including in the heart, brain [167] and lungs [168, 169]. In a mouse model of Barth syndrome, 13HODE and 9 Oxo-HODE were by far the most common oxidised products in cardiolipin [170].

LOX12/15 is required for the high-fat diet induction of early adipose tissue inflammation and insulin resistance in mice; in contrast, 12/15 LOX-knockout mice were protected from these effects [171], pointing to lipid-induced mitochondrial dysfunction and so reduced energy function having a role in obesity as well as in energetics generally.

ALA if present is preferred to LA as a LOX substrate, but not a structural component of cardiolipin. These factors give LA, generally the predominant fat in both cardiolipin and LDL, particular physiological relevance.

Oxidation of cardiolipin and the consequent increase in omega-6 oxidative products, including 13HODE and related products including MDA and 4HNE, are associated with a large number of Western conditions. Oxidation products are often water soluble, so if released from the membrane can travel and will promulgate oxidative stress-based signalling, mitochondrial genesis and cell autophagy, and when present in excess, oxidative cascades, mitochondrial and ultimately cell death. Cardiolipin deficiency, or malfunction due to oxidative stress, may have a role in diabetes, ischaemia, hyperthyroidism, brain trauma [172], ageing and cancer.

Lack of antioxidants capable of reducing hydroperoxides, including melatonin, glutathione, CoQ10 [173], vitamin K1 and/or antioxidants with similar double bond-rich structures, would significantly contribute to cardiolipin oxidation. Glutathione production has been linked to oestrogen production and would moderate oxidative stress, but the required nutrients for glutathione production must be present. Melatonin, a powerful antioxidant [174], may also support glutathione function [175] and helped prevent age-associated decline in mitochondrial function [176], emphasising the importance of both sleep and lipid-protective antioxidants to health.

Adequate mitochondrial energy production is central to cellular function and so health and metabolic weight control. Oxidised LA increases oxidative stress, diminishes mitochondrial function and induces PPAR gamma-related peroxisomal activity and so intracellular lipid and cholesterol deposition; in contrast, omega-3 ALA and fasting reduces oxidative damage by inducing PPAR alpha-related peroxisomal activity, increases energy output, increases antioxidant status and reduces stored adipose deposits.

The opposing effects of PPAR alpha and gamma, and the potential for improvement by DHA of energetics through mitochondrial cardiolipin species and wider mitochondrial membrane changes, or alternatively reduction in energy production capacity by LA-related oxidation of cardiolipin; as well as the production during peroxisomal beta-oxidation of heat rather than energy; emphasise that energetics including weight control, and wider health, is about more than simply calories.

Oxidation of Foods; Crosslinking; Nutrient Loss

Ironically, the attractive savour and smell of cooking foods including meats is a consequence of oxidised products including linoleic acid. Protein degradation including cysteine, and protein–sugar crosslinks, also add to ‘meaty’ flavours and roasting odours. Some oxidised linoleic acid products have very desirable flavour.

A downstream oxidised product of 9HODE called 2,4-decadienal is an important factor in the attractive flavour of fried foods [177]. The most important flavour in bread crust is from the autoxidation products of LA [178], and they appear to be a factor in the flavour of dry cured ham [179].

There is a deep irony that, as a consequence of the industrialised processing of foods including the use of designed flavourings, we are strongly drawn to pre-oxidised, antioxidant- and cysteine-diminished, LA-rich foods that arguably in excess speed the onset of debilitating ‘Western diseases’.

Our attraction to these flavours might be an evolutionary consequence of the effect of moderate heating of some food products, such as those based on grains, in that heating makes them more digestible and so capable of supporting human life; bush fires would have been an early source of cooked grains and meats. Further heating reduces pathogens. Without processing of some sort, in time many of our dietary staples would kill us [180].

Conversely, excessive heat, processing and storage make nutrients, including vitamins and proteins, less available and in some cases by significant amounts. Crucial dietary components such as ALA, and cysteine, are particularly sensitive to oxidation and so prone to damage from heat and wider processing. Traditional cultures use fermentation as an alternative to render foods more digestible and nutritious.

General Processing

Food processing, as well as oxidation, can cause protein–sugar and lipid crosslinks, including in baby 'formula' breast-milk substitute and enteral feed [181–185], resulting in the loss of particularly lysine, cysteine and methionine, other proteins and antioxidants including glutathione, CuSOD and Zn-SOD [186]. Polyunsaturated fats are particularly susceptible to crosslinking and oxidation. Thought-provokingly baby milk 'formula', which is generally formulated to be rich in LA, contains lower antioxidants and greater amounts of oxidised products including MDA, 4HNE and 4HHE, compared to breast milk [187, 188].

Pottenger (cats) [189], McCarrison (rats) [190] and Western Price (mice) [191] all demonstrated that animal models fed highly processed foods exhibited severe health decline and behavioural change including aggression; by the 60th day, McCarrison's rats fed a 1930's British diet began to kill and eat the weaker amongst them.

Despite research results, we continue to 'design' and process food with ever more determination and expertise for optimal crunch, savour, texture and mouth sensation, with insufficient focus on the most important quality of food, its nutritional value.

Protein–Sugar–Lipid Crosslinks Including DNA Damage

Foods rich in polyunsaturated oils, particularly when combined with easily oxidised fructose and glucose, and/or proteins susceptible to oxidation including cysteine and lysine [192], when cooked, sprayed, dried or stored at temperature, are likely to both oxidise and form a wealth of glycation products, many of which have been shown to be bioactive at multiple levels, including; enzyme activation and deactivation, recognition by oxidised LDL receptors [193], causing DNA damage [194], and in excess even potentially have roles in cancer activation.

Exogenously derived or endogenously created oxidised components of dietary plant reproductive material including polyunsaturated fats, fructose and glucose, in nature signal environmental fecundity, but in Western dietary excess lead to dysbiosis.

Glycated haemoglobin HbA1c levels are a strong predictor of all-cause mortality [195]. AGEs lead to and are recognised markers for ageing in almost all tissues [196, 197]. ApoB100 glycation in diabetics was approximately twice controls; MDA was double; 9 and 13HODE were ~50 % higher; and 15HETE was ~80 % higher [145], as were AA and LA. AGEs attached to the signature LDL protein ApoB100, and AGE oxidised LDL, are higher in diabetics [198].

Damage to Lipids and Use of Lysophosphatidylcholine in Food Processing

Industrial preparation of food oils, including bleaching and heating, may result in the presence of some oxidised products, including some lysophosphatidylcholine products. Antioxidants are sometimes added back to industrially refined oils, but it is unlikely that they will deactivate all oxylipins generated in processing.

Frying and cooking will result in a wide range of oxidised products. About 25 to 50 % of the LA content of heavily used high omega-6 frying oil may be oxidised [199], containing a basket of oxylipins [200] and other products [201]. Heavily processed foods contain oxidised products of LA including 13HODE, 4HNE and MDA [202], as well as AGEs and other crosslinking products, which can cross the gut membrane. Oxidation products also potentially bind with minerals and reduce availability [203].

Phospholipids breakdown at over 150 °C [204] and are removed from processed vegetable oil as far as possible because they are less stable [205]; but their inherent instability and their presence in virgin cold-pressed oils may protect other antioxidant factors such as phenols, potentially helping explain the observed health benefits of such products [206].

As discussed elsewhere, plant phenols such as those found in aromatic leaves, seeds and unprocessed oils such as olive and unrefined red palm oil may protect against the formation of crosslinked oxidation products [207] and oxidation of lipids, although at the cost of reducing their bioavailability.

Oxidative DNA Damage

Peroxide-based hydroxyl radicals are potent causes of DNA damage [208], as are HPODEs, oxylipin aldehyde products

including MDA, 4HNE, and the more powerful oxylipins 4HPNE and ONE [209]. Vitamin E may reduce wider oxidation, but has limited capacity in vivo to reduce hydroxyl radicals [210], and it is not clear whether vitamin E has any significant direct role in protecting DNA [211] or histones. Glutathione is reported to be effective at reducing hydroperoxides and related products including 4HNE. Other antioxidants such as vitamin C play a supporting role, but functionally they cannot replace glutathione.

Internal Antioxidants for Hydroxyl Radicals and Oxylipins Are Limited

Oxidative processes are moderated by the antioxidant product pathways. Antioxidants that can best prevent highly damaging peroxide-derived oxidative hydroxyl and oxylipin cascades are of particular physiological relevance and include glutathione; multiple double bond structures such as those produced in the cholesterol pathway from squalene; photoactive pigments; plant compounds found in whole plant food generally such as phenols [212], flavonoids and anthocyanins [213]; and substances used to protect the lipids in seeds, including resveratrol, quercetin and interestingly salicylates [214]. Further carotenoids compete for and are metabolised by LOX12/15 enzymes and, in a concentration-dependent fashion, inhibit LA hydroperoxide formation [215].

Plants have had around 2500 million years to perfect antioxidant products for protection against UVC- and UVB-related oxidation, a number of which are know to maintain human health, but self-evidently will be inaccessible if not in the diet, and reduced in overly processed foods. Much remains unknown about the potential benefits and wider biological impact of many plant products on human cellular pathways. Plants grown under plastic or glass, deprived of UV, may contain lower levels of related antioxidants. Oxidation pathways are complex and best addressed holistically [216].

Glutathione

Absence of glutathione production allows the accumulation of oxylipins and its consequential effects, the subject of this overview. Cysteine and selenium are key to glutathione production, which in turn is possibly the most important protective antioxidant pathway against the propagation of oxidation of lipid products (Awasthi p. 181); importantly cysteine is also a key factor in protein folding [217].

Glutathione is '*uniquely*' [218] important, is recyclable and has the rare ability to not just prevent, but repair initial oxidative damage to some extent. Glutathione enzymes comprise a substantial portion of cellular protein in major metabolic organs, such as the liver and kidneys, and are present in high amounts in other organs including the brain, testes and ovaries [25] (Awasthip p. 180).

Vitamins C and E do not centrally address the issue of lipid oxidation; they support the regeneration, for example, of glutathione and diminish antioxidant initiation, thus helping prevent hydroxyl and hydroxy oxidative initiation [219], but could be of limited benefit or indeed act as pro-oxidants if other required substrates of glutathione such as cysteine and selenium are missing.

Glutathione is a primary and major detoxification system for oxidised polyunsaturated fats and their oxylipins, including of in situ membrane phospholipids, and aqueous compartment products where lipid-based antioxidants such as vitamin E and beta-carotene are likely not effective. Glutathione may downregulate LOX15 and acts on oxidation products including 9 and 13HODE, hydroxyl radicals [220, 221], aldehydes such as MDA and 4HNE and nitrous products, giving it particular direct physiological relevance.

Glutathione is a sulphur based system; interestingly given the role of glutathione as an antioxidant, sulphur and oxygen are in the same periodic table family, so both form two covalent bonds, but sulphur is less electronegative than oxygen and thus bonds and disassociates with much greater ease [222], which is an essential characteristic for a recyclable antioxidant. Selenium, also essential for glutathione function, is also in the same family in the periodic table.

Glutathione production is dependent on cysteine, and indirectly methionine, which can be converted to cysteine via homocysteine. Methionine, cysteine, taurine and homocysteine are the four common sulphur-bearing amino acids; only methionine and cysteine are incorporated into proteins. Methionine is classed as an essential nutrient, and cysteine a semi-essential nutrient. The precursor for glutathione, cysteine, along with methionine, is the most susceptible amino acid to oxidative stress [223] and indeed an antioxidant in its own right [224]. Processing including chlorine bleaching as well as cooking at high temperature will reduce the cysteine content of food and make the protein less available [225].

Glutathione deficiency is not uncommon in the elderly [226], is seen in diabetics [227] and may be an issue in wider populations due to the loss of dietary glutathione and/or its precursors such as cysteine in processed foods including pasteurised milk, stored breast milk, cooked spinach, fruit and fruit juices [228]. Cysteine and glycine supplementation reversed glutathione deficiency.

Selenium is essential for glutathione production, but selenium is deficient in many soils and may also be removed during refining; those living in low-selenium areas consuming significantly processed food are at greater risk. Benefits of supplementation are more likely to be seen in groups low in selenium rather than in the wider population.

A diet rich in highly processed foods, low in accessible cysteine and methionine, compounded by low selenium and reduced glutathione intake, will impair glutathione function, which in concert increases the risk of lipid-derived oxidative cascades, protein misfolding, cellular damage including of mitochondrial DNA, and ultimately ill-health.

Catalase; PPAR Alpha, Delta and Gamma; Nitric Oxide and INOS Expression

PPAR gamma-type peroxisomes are activated during repair and immune function. Besides producing peroxide, peroxisomes are the major producers of catalase, the primary antioxidant for peroxide. Catalase is an enzyme and activated independently of peroxide production, which is a by-product of the peroxisomal beta-oxidation pathway. Restraining antioxidant capacity is important in tissue repair, because oxidative processes are overridingly-important to tissue destruction repair and creation; hence arguably antioxidant function is suppressed during PPAR gamma repair related activity, and in particular through uprating of iNOS based NO production so suppression of catalase activity, with a consequential increase in the availability of net peroxide produced as the by-product of peroxisomal beta-oxidation of lipids including LA and OA.

Peroxide and cytokines stimulate iNOS and further lead to the formation of 13HODE; 13HODE activates PPAR gamma, which is active in endothelial cells including vascular cells, has roles in immune function including macrophage and microglial activation and is involved in the upregulation of iNOS. PPAR gamma activates oxidised LDL receptors including CD36 and OLR1, thus synergistically increasing the supply of peroxisomal lipid substrate and LA oxylipin-activating factors, and at the same time upgrading macrophage and iNOS activity. Rats supplemented with 20 % LA '*demonstrated elevated inducible nitric oxide synthase expression*' [229].

The catalase gene it appears only has one main form in animals [230]; activators include peroxide [231], oxidised lipids and PPAR alpha and gamma (in normal tissue, but not glioma) [232]; however, the activity of haem-based catalase [233, 234] is blocked by excess NO. '*The iNOS protein associates with peroxisome*(s)'. '*iNOS might localise to peroxisomes in long-lived cells*' [235]. During phagocytosis, peroxisomes may fuse and/or interact closely with macrophages [236]. During immune function and tissue repair stress, NO is acutely produced in response to PPAR gamma, peroxide and cytokine activation [237, 238] of iNOS [239–241] in relevant tissues such as '*endothelial cells, neurons and platelets, or after induction of hepatocytes, myocytes, smooth muscle cells,* etc'. NO blocks the catalase enzyme recycling function [242], and following activation over several hours [243] results in a net excess of peroxide over catalase and related tissue damage. At lower maintenance-related activation levels, NO may assist in oxidative stress limitation [244, 245] by binding with peroxide [246].

Macrophages may use PPAR gamma-related peroxisomally produced peroxide, combined with iNOS related NO suppression of catalase rather than myelo-peroxidase produced peroxide [247, 248], to help fuel oxidative phagocytic bursts. Consistent with this concept, PPAR gamma deletion in macrophages '*gravely impairs their ability to induce oxidative metabolism, as evidenced by reduced rates of β-oxidation of fatty acids and the blunted mitochondrial biogenic response*' [13].

Peroxisomes associate with macrophages and, during phagocytosis, may assist by beta-oxidising LA and other substrates including prostaglandins, and as well as supplying peroxide, provide short fats to fuel macrophage mitochondria, '*oxidative metabolism is accompanied by an influx of fatty acids, nutrients that serve as the substrates for β-oxidation*', and furnishing ACoA as substrate for macrophage expansion repair and maintenance.

The differential presence in the different tissues of PPAR alpha and delta or PPAR gamma related peroxisomes, as well as differing levels of iNOS expression, and so NO catalase blocking capacity, might help explain differing tissue variations in the levels of production of catalase by PPAR alpha-, delta- and gamma-related peroxisomes.

Oxidative Stress and DNA Damage

Excess oxidative stress, excess LA, deficit of ALA, imbalances and excesses of LA oxylipins, and related products, and antioxidant pathway required nutrients will ultimately cause DNA and protein damage, inflammation and overactivation of the immune inflammatory processes including macrophage and microglial systems. Damage by radicals and oxylipin products to the more vulnerable mitochondrial DNA; blood and tissue proteins; and wider cellular membrane components, including cardiolipin, leads to the disruption of substrate transport, enzyme and thus cellular function, and energy pathways, ultimately resulting in apoptosis.

Cancer

There is mounting evidence that excess dietary LA, its long-chain derivatives and oxidised products increase the risk of incidence, progression and metastasis of cancer, and that dietary ALA and its derivatives reduce that risk.

Role of Epithelial Tissues in Cancer

Ninety per cent of carcinomas originate in epithelial cells [249]. Organs rich in epithelial cells, such as thyroid, pancreas, breast, prostate, are particularly susceptible to carcinomas. LA-related oxidised stress factors—net surplus peroxisomal peroxide production; related hydroxyl radical cascades; wider oxylipins; reduced internal antioxidant function including catalase; high iNOS and NO activity [250]; cardiolipin species related mitochondrial dysfunction and so energy dysregulation and apoptosis; wider inflammatory and oxidative stress related dysbiosis [251] with the consequent increased risk of mitochondrial DNA and wider protein damage—are responsible for initiation, recurrence and severity of cancer [252].

LA oxylipin-related pathways have the ability to trigger angiogenesis, peroxisomal activation, multiple growth factors and promote new tissue formation, which are common factors between reproductive pathways, immune function and cellular proliferation.

In terms of preventing initial damage to mitochondrial DNA [253] and onset of mitochondrial dysfunction including likely impaired cardiolipin species capacity to assist cellular apoptosis, it is important to control oxidative stress levels and the presence of products capable of damaging DNA and diminishing mitochondrial functionality, including LA oxylipin products, HODEs and aldehydes such as MDA and 4HNE.

Relationship of Oxidised Products of LA with Cancer

At levels outside the physiological norms, the exclusive omega-6 metabolites 13HODE and/or 13Oxo-HODE, 4HNE and non-exclusive LA metabolite MDA have recognised roles in cancer:

- **4HNE**: 4HNE is an exclusive product of omega-6. At lower concentrations, 4HNE is a signalling agent; in excess, 4HNE can modify proteins, react with double bonds and DNA. It is one of the most abundant and reactive aldehydes in mammalian cells, has been linked cancer and with a number of other diseases. It is mutagenic and genotoxic in bacteria, viruses and human cells. 4HNE forms adducts with the p53 gene, '*a mutational hotspot*' in a bacterial model [254]. 4HNE has been reported to induce proliferation and migration [255]. As discussed, oxidised cardiolipin is a source of 4HNE formation. 4HNE may reduce mitochondrial function. Reduced mitochondrial function is a factor in many cancers [256].
- **13HODE**: 13HODE is an exclusive omega-6 product, produced by a number of mechanisms, including the action of LOX12/15. LOX12 is overexpressed in breast cancer patients with poor survival prospects. 13HODE was significantly more expressed in malignant cancer, more so in higher grades, and associated with poorer prognosis in all types of breast cancer; this effect may also involve 13HODE derivative 13Oxo-HODE. HODEs through the excess activation of PPAR gamma pathways upregulate angiogenesis, tissue growth-related pathways, macrophage expression and OLR1, which has been associated with cancer pathways. 13HODE has also been shown to increase EGF [257] and VGEF. Direct 13HODE administration increased a marker of tumour growth substantially. Interestingly, melatonin, a powerful diurnal antioxidant and factor in sleep, inhibits cancer by reducing 13HODE [258, 259]. Blocking of 13HODE, but not unoxidised LA, arrested the growth [260].
- **MDA**: MDA is produced from other polyunsaturated fats as well as LA, including ALA and in greater quantities, but given the overwhelming predominance of LA amongst polyunsaturated fats in plasma, a significant amount of MDA logically will originate from LA. MDA factors may be both a marker of oxidative stress and carcinogenic due to its ability to damage DNA [261]. MDA in breast and lung cancer patients was significantly higher than in controls [262], two to three times higher in pre-cancer and oral cancer patients [263]; oropharyngeal cancer patients with lower MDA were more likely to remain in remission [264]; MDA increased the incidence of pancreatic and thyroid cancer in rats [265]; in dogs, MDA was significantly higher in 80 tumour-bearing dogs than in controls independent of cancer type [266]; ovarian cancer patients had significantly higher levels of MDA and lower catalase levels than controls [267]; prostate cancer cases had higher level of MDA and lower related antioxidant levels [268].

Oxidised Lipoprotein Receptor OLR1

HODEs are the strongest endogenous activators of PPAR gamma, which significantly uprates OLR1 activity. '*OLR1 may act as an oncogene*'. Many of the genes activated by OLR1 were '*NF-kB target genes responsible for proliferation, migration and inhibition of apoptosis*'; OLR1 is '*a possible link between obesity, dyslipidaemia and cancer*' [269]. OLR1 is key to post-traumatic angiogenesis in vivo, activating multiple pro-angiogenic signalling pathways. Angiogenesis is central to cancer growth and proliferation. OLR1 may have a role in prostate cancer [270].

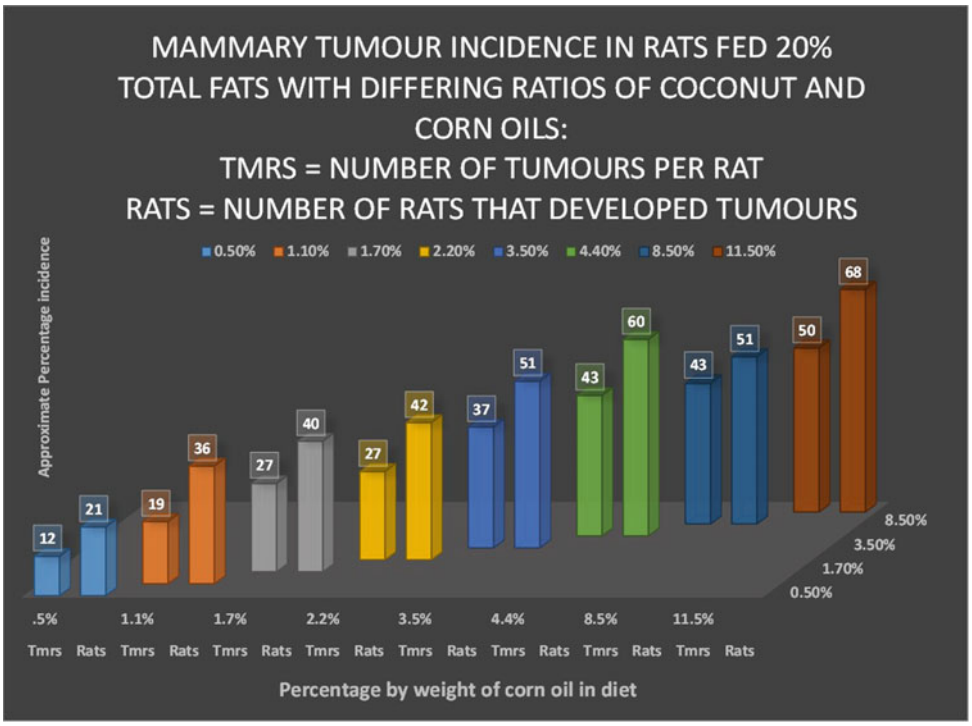


Fig. 28.7 There was a striking difference between the groups, both in the number of rats that developed tumours, and the subsequent increase in tumour rates. Interestingly, there seems to be a plateau at about 4 %, which is consistent with work of Lands on highly unsaturated fatty acid tissue membrane uptake, which suggested a ceiling at around 4 % (Lands—Fish Omega-3 and Human Health. p23). There are possibly two major sets of lipid pathway effects involved in the tumour development: those of the AA-based prostaglandins and those of the LA oxylipins, which will also interact as LA is a preferred LOX12/15 substrate to AA as well as a lesser competitor for COX2. The 4 % LA intake plateau may be where the effect of the AA prostaglandin cascade has reached a maximum because the membrane is AA saturated, and so has reached maximum PLA2 release potential. The effect of AA released from the membrane is then diminished as LA intake continues to rise and so increasingly competes for the attention of the COX2 enzyme and LOX12/15. Then as the effects and amounts of oxidised LA products rise further, they too increase oxidative damage and so tumour incidence, which would account for the resumption of tumour increase at higher LA intakes. The data were abstracted from graphs 3 and 4 of '*Requirement of essential fatty acid for mammary tumorigenesis in the rat*' with very grateful thanks to the authors: Ip et al. [271]

Cancer Is Complex, but LA May Increase and ALA May Reduce the Risk of Cancer Initiation

Long-term excess oxidative products of LA set in the context of a Western diet may prove to be a significant initiating factor in cancers, by promoting mtDNA mutation, protein damage, mitochondrial dysfunction and angiogenesis.

Fascinating studies in the 1980 s looked at the impact of dietary polyunsaturated lipid intakes on cancer development and progression. Authors of one such study, looking at the effects of linoleic acid on the progression of DMBA-induced breast cancer tumours in Sprague Dawley rats, said '*It is thought that linoleate may be the essential fatty acid primarily responsible for the tumour-promoting effect of unsaturated fatty acid'*. Interestingly, one arm of the study used coconut fat as the non-polyunsaturate, which has a very low omega-6 LA content (1 %); data have been abstracted from the graphed results and re-graphed to highlight the impact on both tumour numbers and the numbers of rats affected [271] (Fig. 28.7).

It is generally suggested that omega-3 s are protective and excess omega-6 s increase risk factors. Cancer trials looking at ALA intake are limited; however, studies suggest that low ALA in breast tissue is associated with an increased risk of breast cancer and spread [272, 273]. Dietary ALA [274] and flaxseed [275] may reduce prostate cancer risk factors. Inconsistent results of observational and epidemiological prostate cancer ALA studies looking at wider tissue levels might be influenced by hormone-related changes in desaturase function.

There is a large amount of research looking through a variety of mechanisms at the wider effect of imbalances in

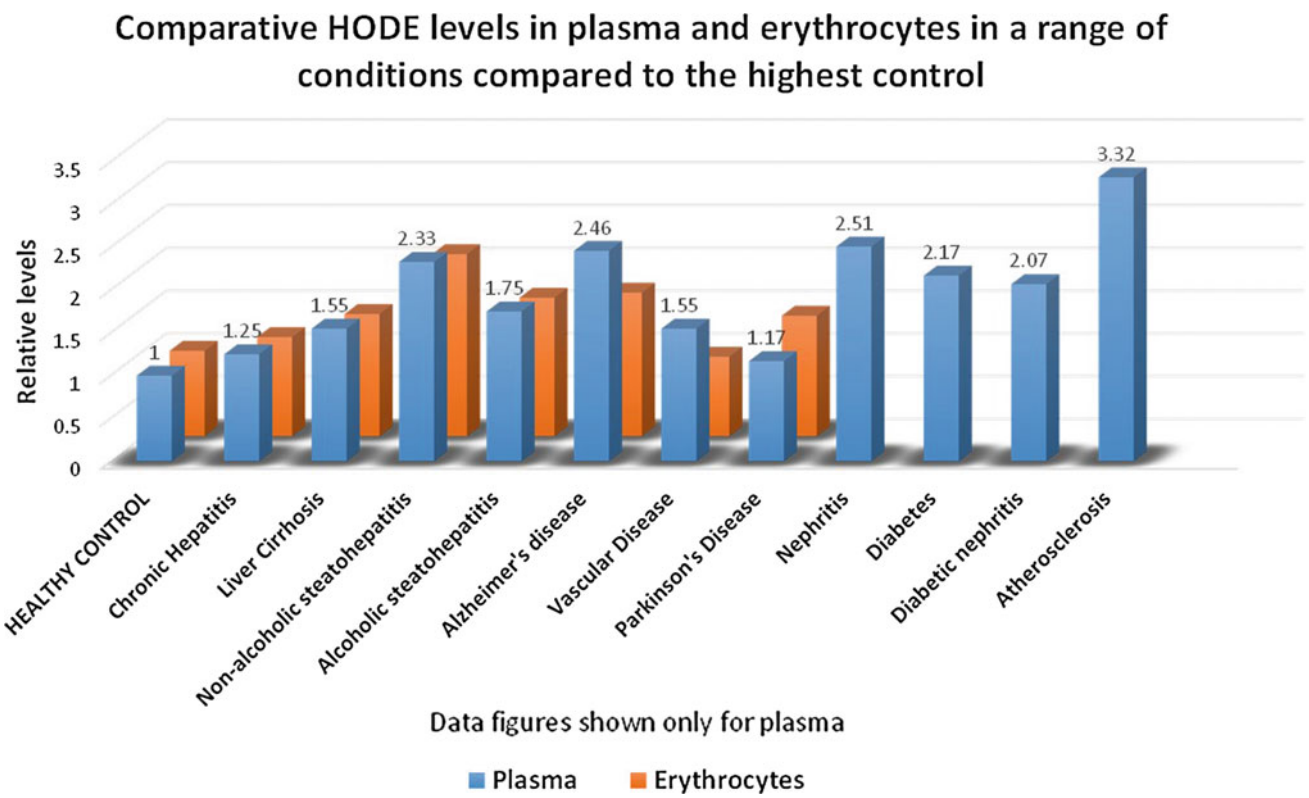


Fig. 28.8 '*It has been reported in numerous studies that the extent of lipid oxidation is elevated in diseased patients compared to normal subjects*'; '*linoleates are the most abundant PUFAs in vivo*', '*these products exert cytotoxic and genotoxic effects*'; these observations of the authors are supported by figures showing relative levels of hydroperoxyoctadienoic acid (HODEs) in plasma and erythrocytes of patients suffering from a range of common 'Western diseases' compared to healthy controls, graphed from selective data abstracted from Fig. 4 of '*Lipid peroxidation biomarkers for evaluating oxidative stress and assessing antioxidant capacity in vivo*'. with very grateful thanks to the authors: Yoshida et al. [139]

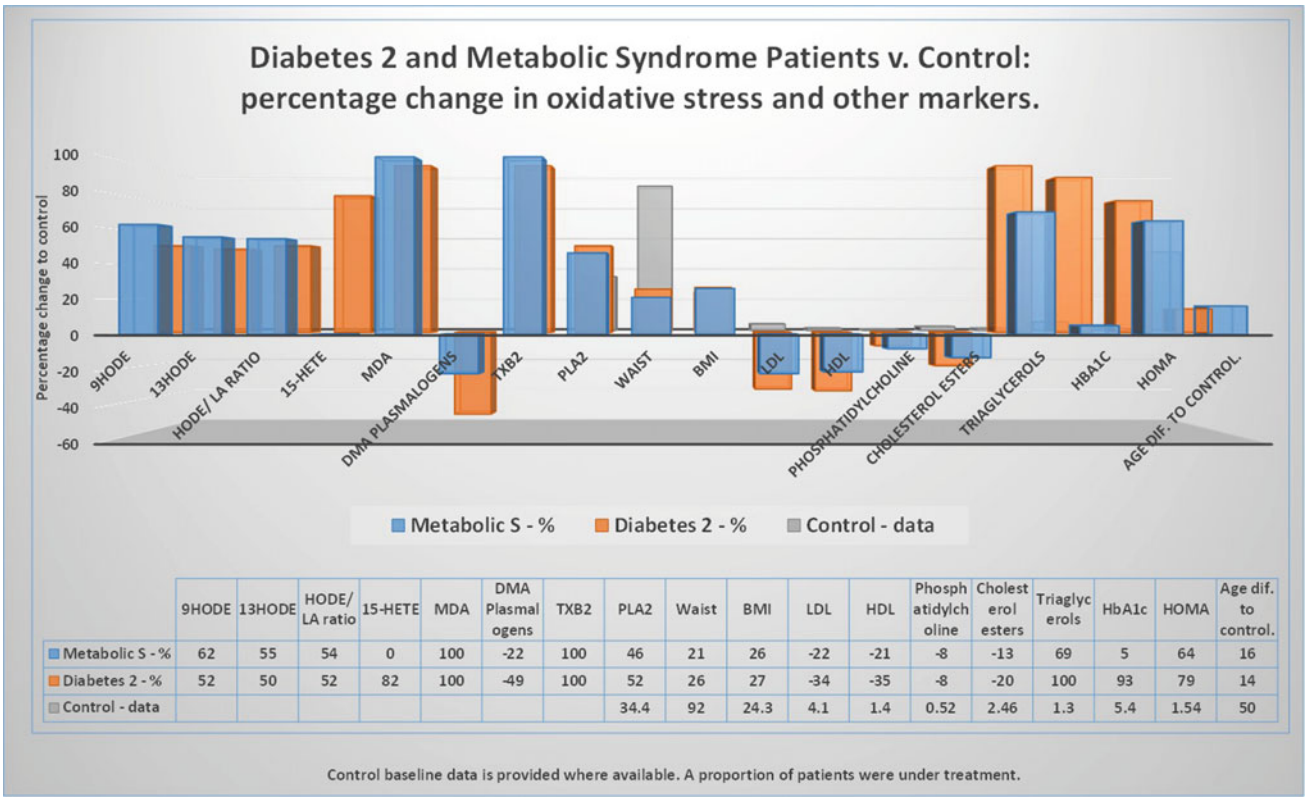
the omega-3 and 6 lipid pathways, including LA, EPA as well as DHA, and the incidence of cancer, and as a very general trend omega-3s tend to diminish and omega-6s increase risk of negative outcomes; other risk factors include wider oxidative stress, nutrient deficiencies generally and particularly those of iodine and vitamin D.

Diet and Western Disease—Conclusion

A large number of people globally are insufficient in iodine [276, 277], vitamin D [40], one or more minerals [278] and other nutrients that have on impact antioxidant pathways, and have excessive intakes of LA and lack omega-3 s. Multiple pathways highlight that the excess of omega-6 oxylipins are significant factors in, and markers for, a range of increasingly common Western oxidative stress-related non-communicable 'diseases' including obesity, diabetes, fertility-related conditions, atherosclerosis, NAFLD and Alzheimer's disease (Fig. 28.8).

Western 'diseases' of civilisation in pre-industrialised populations with no access to refined food, irrespective of their dietary profile, were very low [279–281]. LA, ALA and their oxidised products have simple structures, but when imbalanced oxidised or present in excess, in the long term and in the context of a Western diet, have extensive significant undesirable physiological effects on human health.

Reversal of the recent explosion in rates of early-onset Western non-communicable 'diseases' requires widespread recognition, that adequate nutrients and regulation of redox are both absolute conditions of our existence; consequently, reversal of this trend requires holistic optimisation of the nutrient content of food from farm and factory to fork, and minimisation of oxidative stress (see Fig. 28.9) [195, 282, 283] including crosslinking during production and processing; these criteria should be the core values to be applied when growing, processing and marketing the huge quantities of food needed to feed planet Earth's growing human population.



	9HODE	13HODE	HODE/ LA ratio	15-HETE	MDA	DMA Plasmalogens	TXB2	PLA2	Waist	BMI	LDL	HDL	Phosphatidylcholine	Cholesterol esters	Triaglycerols	HbA1c	HOMA	Age dif. to control.
Metabolic S - %	62	55	54	0	100	-22	100	46	21	26	-22	-21	-8	-13	69	5	64	16
Diabetes 2 - %	52	50	52	82	100	-49	100	52	26	27	-34	-35	-8	-20	100	93	79	14
Control - data								34.4	92	24.3	4.1	1.4	0.52	2.46	1.3	5.4	1.54	50

Control baseline data is provided where available. A proportion of patients were under treatment.

Fig. 28.9 Oxidative stress marker comparison between healthy controls versus obese patients with metabolic syndrome and diabetes type 2. (No, 10 in each group). 9HODE, 13HODE, HODE/LA ratio, MDA and TXB2 are all, in part or whole, omega-6 derivatives. Other markers including CRP (mg/l) were also significantly raised in the metabolic (3.61) and diabetes groups (4.82) compared to control (0.54), and platelet aggregation increased by 197 and 251 % in metabolic and type 2 diabetes patients respectively, but were not graphed for reasons of scale. CRP is a wider marker of oxidative stress and '*robustly associates with increased risk of all-cause mortality*' [282]. HbA1c, also a marker of oxidative stress, is associated '*with increased mortality at high and low HbA1c levels*' [195]. In contrast, HOMA, and so insulin sensitivity, is not correlated with all-cause mortality [283]. Oxidative stress is arguably a common factor and marker for all Western diseases. Selected data were abstracted from the text and Tables 2 and 3 of '*LDL from obese patients with the metabolic syndrome shows increased lipid peroxidation and active platelets*' with very grateful thanks to the authors: Colas et al. [145]

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29 In a Western Dietary Context Excess Oxidised Linoleic Acid of Dietary and Endogenous Origin by Over-Activation of PPAR Gamma so Immune and Inflammatory Pathways, and through Cardiolipin Damage, Increases Cardiovascular Risk

Robert Andrew Brown

Terms	
AA	Arachidonic acid (Omega 6; 20 carbon derivative of LA)
ACOX1	Acyl-CoA oxidase (First step in peroxisomal beta-oxidation)
ACoA	Acetyl coenzyme A (Raw material for the energy/cholesterol pathways)
AGE	Advanced glycation end product (Non-enzymatic covalently bound sugar to protein or lipid)
ALA	Alpha-linolenic acid (Omega 3; 18 carbon plant-based polyunsaturated fat)
APOE	Apolipoprotein E (Lipid transport signature protein)
ATP	Adenosine triphosphate (Enzyme used as an energy carrier)
CD36	Cluster of differentiation 36 (Fatty acid translocase receptor)
COX	Cyclooxygenase (Enzyme-catalysing oxidation of fatty acids)
CoQ10	Ubiquinol (Fat-soluble component of mitochondrial electron transport)
CPT1	Carnitine palmitoyl-transferase (Acts as shuttle mainly for long-chain fats C:16–18 into mitochondria)
DHA	Docosahexaenoic acid (Omega 3; 22 carbon derivative of ALA)
EPA	Eicosapentaenoic acid (Omega 3 fatty acid C20:5)
iNOS	Inducible nitric oxide synthase (Inducible isoform involved in stress response in macrophages microglia and other tissues)
LA	Linoleic acid (Omega 6; 18 carbon plant-based polyunsaturated fat)
LOX5	Lipoxygenase (Enzyme-catalysing oxidation including AA and EPA)
LOX12/15	Lipoxygenases (Enzyme-catalysing oxidation of multiple lipid-based substrates)
LDLR	Low-Density Lipoprotein (LDL) Receptor (LDL receptor for minimally oxidised LDL)
LPL	Lipoprotein lipase (Mobilises lipids from chylomicrons, VLDL, LDL both at the vascular face and intercellularly)
MDA	Malonaldehyde (Non-exclusive oxidation product of omega 6)
MCT	Medium chain triglyceride (Triglyceride containing fats between C6 and C12)
MCF	Medium chain fatty acids (Fatty acids between C6 and C12)
MUFA	Monosaturated Fatty Acid (Monosaturated fatty acid)
NAFLD	Non-alcoholic fatty liver (Fat deposition in the liver not due to alcohol)
NO	Nitric oxide (An important signalling messenger and oxidant)
OA	Oleic acid (Omega 9 monosaturated fat C18:1)

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OLR1	Oxidised LDL receptor 1 (Receptor for oxidised LDL sometimes called LOX1)
Oxo-HODE	Oxooctadecadienoic acid (Oxidation products of HODEs also called KODEs)
PA	Palmitic Acid (Saturated fat C:16)
PPAR	Peroxisome proliferator-activated receptor (3 forms alpha, gamma and delta)
P450	Cytochromes P450 (Family of often oxidative enzymes)
PUFA	Polyunsaturated fatty acid (Polyunsaturated Fatty Acid)
SA	Stearic Acid (Saturated fat C:18)
SFA	Saturated Fatty Acid (Saturated Fatty Acid)
SCD1	Stearoyl-CoA desaturase (Delta-9-desaturase so key to formation of OA)
SN2	SN2 position (Location of fat in triglycerides or phospholipids)
VEGF	Vascular endothelial growth factor (A protein signalling angiogenesis)
Wy14643	PPAR alpha activator (Activates PPAR alpha-related peroxisomes)
4-HNE	4-Hydroxynonenal (Exclusive omega 6 fats peroxidation aldehyde)
9-HODE	9-hydroxy-10E, 12Z-octadecadienoic acid (Major LA oxidation product of LOX12/15, COX, photo-oxidation and auto-oxidation)
13-HODE	13-hydroxy-9Z, 11E-octadecadienoic acid (Major LA oxidation product of LOX12/15, COX photo-oxidation and auto-oxidation)
13-HOTE	13-OH-9Z, 11E, 15Z-octadecatrienoic acid (Major ALA oxidation equivalent of LA product 13-HODE)
15-HETE	15-hydroxy-eicosatetraenoic acid (Major AA LOX15 oxidation product)
15d-PGJ2	(15-deoxy-Δ12, 14-Prostaglandin J2 (Downstream AA COX2 oxidation product and PPAR gamma activator)

Importance of Lipids to Cardiac Function and Cardiovascular Health

Lipids are exceptionally important to cardiac function acting as; cellular structural membrane components including in mitochondrial cardiolipin; oxidised messengers for immune function and repair; raw materials for maintenance substrate, as well as providing as much as 70 % of cardiac fuel energy requirement. The prime cardiac lipid delivery mechanisms are as follows:

- In the fasted state, LDL and
- In the fed state, chylomicrons.

Healthy cardiac and related vascular tissue function requires efficient vascular delivery and uptake of fat of endogenous and dietary origin, including the diet-derived essential nutrients LA and ALA, and that they should not be excessively pre-oxidised due to processing or other factors.

Is excess Oxidised LA within a 'Western' Dietary Context a Key to Cardiovascular Disease?

LA, ALA and/or their derivatives are essential nutrients that must be derived from diet; they have extensive families of oxidised products, 'oxylipins', which are the most common oxylipin products in plasma, and crucial messengers in multiple pathways and organs. Oxidised lipids are both created internally and absorbed in pre-oxidised form across the gut [1]. LA is also crucially the major species in cardiac cardiolipin, which is very susceptible to oxidation.

Long-chain Omega-3 and 6 fatty acids also form large families of highly bioactive oxidised messengers, but have much lower plasma presence, and are not under consideration in this review.

A mix of exogenous and endogenous LA-oxidised products are essential to signalling, but in 'excess' and long term in the context of a low-nutrient, pre-oxidised antioxidant-diminished 'Western diet' are core activators of PPAR gamma inflammatory-related processes including; diversion of peroxisomal beta-oxidation output substrate to repair and lipid storage rather than energy, macrophage and immune function activation, angiogenesis, tissue destruction, repair, and creation, cardiolipin damage thereby reduced mitochondrial energetics, ultimately in concert contributing to serious cellular malfunctions.

Under activation of PPAR alpha, due to lack of energy deficit during exercise or fasting, or to a lesser extent due to lack of omega 3s ALA and/or DHA, so diminished energy provision as well as lack of oxidative LOX COX and P450 enzyme competition by ALA, are also important factors in cardiovascular disease as well as in other non-communicable Western diseases.

Delivery of LA and ALA to Tissue: Phospholipids Chylomicrons and LDL

The Digestion, Delivery, Absorption and Uptake Pathways of LA and ALA

The digestion, delivery, absorption and uptake pathways of LA and ALA impact cellular metabolism, organ function and are ultimately important factors in cardiovascular health and generally, because they influence the LA and ALA content of LDL and chylomicrons, which are the major delivery mechanisms to cardiac tissue.

Delivery of dietary unoxidised and oxidised LA and ALA to cells for incorporation by a combination of esters triglycerides and phospholipids, directly by endocytosis including of LDL, and indirectly including by action of LPL on chylomicrons, influences; cell membrane content hence flexibility and wider function; cardiolipin content so mitochondrial function; peroxisomal function, beta-oxidation, metabolic rate including thermogenesis; desaturase function so conversion to long-chain derivatives; LOX, COX and P450 enzyme function; nitrous products; immune system regulation including macrophage activation as well as respiratory burst capacity; tissue repair including angiogenesis; pain pathway activation; downstream oxylipin signalling, prostaglandin formation and the wider oxidative stress tone of blood and tissues; as well as more widely regulating reproductive ability including through; steroid hormone production, metabolic rate, fat deposition, thereby influencing the risk of cardiovascular disease as well as other Western conditions. As the lipid content of LDL and chylomicrons changes, vascular function, cardiac energetics and related pathways including oxidative stress levels will also change.

'Western' LDL generally contains significant amounts of OA LA and cholesterol, some saturated fats, limited ALA, and very limited long-chain EPA and DHA. Phospholipids form a significant part of LDL maybe 30–40 %, underlining the importance of phospholipids as a significant component part of the delivery mechanisms of polyunsaturated fats to vascular epithelial membranes.

Absorption, Chylomicrons and Portal Vein

Delivery of LA and ALA to wider tissue are made by a mix of phospholipids, triglycerides, possibly galactolipids, via chylomicrons, LDL IDL and VLDL, by reverse transport though HDL, as well as by albumin and free fatty acids.

At low dietary intakes, possibly up to 50 % of the polyunsaturated fat intake travels from the gut via the portal vein [2], maybe to ensure adequate LA and ALA reach the liver in times of famine [3], including for elongation to essential AA and DHA. At higher intakes, most dietary polyunsaturated fat is transported from the gut in chylomicrons initially in lymph [4], and subsequently in the circulatory lipoproteins.

Oxidised dietary fats including aldehydes, as well as protein sugar lipid cross-linked products actively cross the gut membrane to be incorporated for onward transport [5], both direct through the portal vein to the liver and/or via chylomicrons.

Chylomicrons contain triglycerides (95–90 %) at their core, with an outer layer of phospholipids (6–9 %) and identifier apolipoproteins A, B, C and E (see Fig. 29.1).

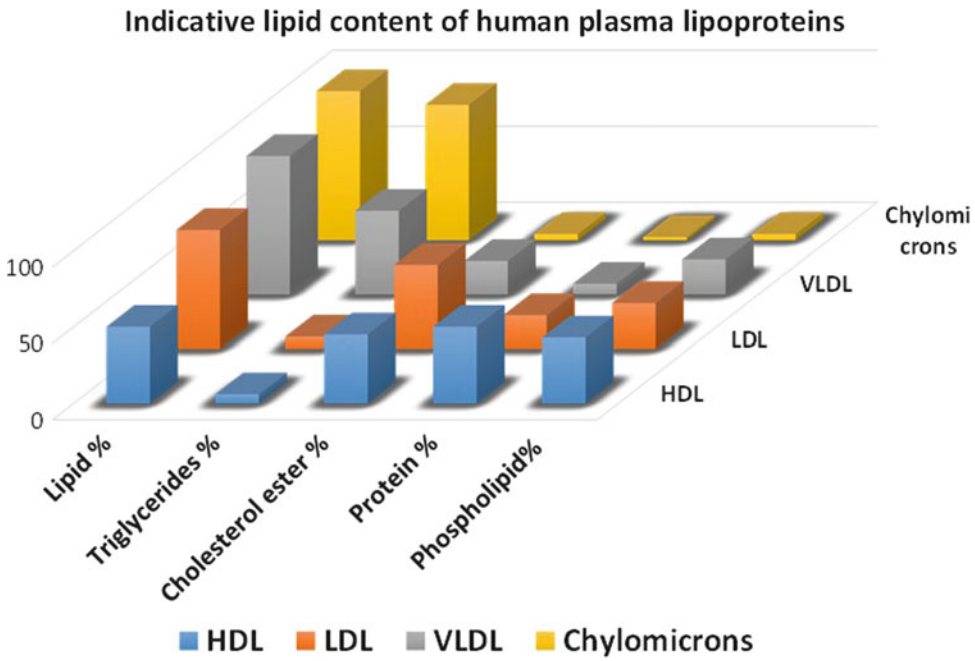


Fig. 29.1 An indication of the lipid content of lipoproteins based on human plasma; data abstracted from the text on pp. 1 and 2, of '*Functions And Interrelationships Of Different Classes Of Plasma Lipoproteins*' with very grateful thanks to the author: Nichols A., N.A.S. Symposium On Lipoproteins Vol. 4 1969

Phosphatidylcholine is a major constituent of the chylomicron shells (75 %) [6]; it is also the primary phospholipid in LDL.

Phospholipids in the chylomicron shell of plant dietary origin will likely be rich in ALA at the SN2 position. Whilst leaf lipids are primarily in galactolipid and phospholipid form, in contrast seed lipids are mainly comprised of triglycerides. The composition of triglycerides in the chylomicron interior is very dependent on diet, including changes in ALA intake.

Rats fed 300 mg of flaxseed oil, increased ALA in chylomicron triglycerides from below 1 % to over 30 %, of which 17–28 % was in the SN2 position [7]. ALA in sheep chyle was increased by flaxseed, and also when ALA was directly introduced into the digestive tract in such a way as to bypass the rumen [8]. Similarly dietary GLA increased GLA in rat chylomicron triglyceride [9].

Absorption of LA and ALA in ruminants is different, because a significant proportion of the LA and ALA in their diet is broken down or converted to other fats by bacteria in the rumen; consequently, their tissue levels of LA and ALA are much lower than they would otherwise be.

As omnivores, we and our prey, now our livestock, would have eaten much more green material than we do today; our diet contained much greater amounts of ALA from plant based galactolipids, phosphatidylcholine and to a lesser extent triglycerides and consequently greater ALA in our chylomicrons, so LDL, and ultimately in our cell membranes.

In LDL, after ALA supplementation the increase of ALA in relative terms is high, but ALA remains limited in quantity compared to LA [10]. LDL nonetheless provides a mechanism for delivery to cell interiors of ALA in triglycerides or phosphatidylcholine originating from the liver, or alternatively acquired whilst in transit in plasma through phosphatidylcholine transfer from chylomicrons as part of active lipid restructuring including re-acetylation of lysophosphatidylcholine.

Whilst a limited number of plant seeds contain significant ALA, LA is the predominant polyunsaturated fat in triglyceride-rich plant reproductive tissue, and grain-fed animal adipose tissue. By reducing and altering the amount of plant-based phospholipid and galactolipid content of our diet, and hugely increasing vegetable oil intake, we have increased the LA-rich lipid triglyceride content, and altered the dietary chylomicron, triglyceride and phospholipid lipid compositional balance.

Phosphatidylcholine Composition Is Affected by Diet thereby Altering Lipoprotein Shell Composition and Delivery from Lipids to Tissue

Phosphatidylcholine is the second most common membrane component in green plant material after galactolipids, and the most common phospholipid in the human body an important component of human cellular membranes; it mainly carries LA and/or ALA at the SN2 position. Lipid uptake differs depending on whether lipids are delivered by triglycerides or phospholipids [11].

Subject to the sources of dietary fat intake, a significant proportion of phosphatidylcholine usually contains a polyunsaturated acid at the SN2 position [12], and so potentially ALA, as well as LA and longer polyunsaturates. A plant-rich diet will logically result in a greater proportion of ALA in the phospholipid-rich outer shell (6–9 %) of chylomicrons, a significant portion of which will be directly transferred into VLDL LDL and HDL, which in turn will be taken up by cells.

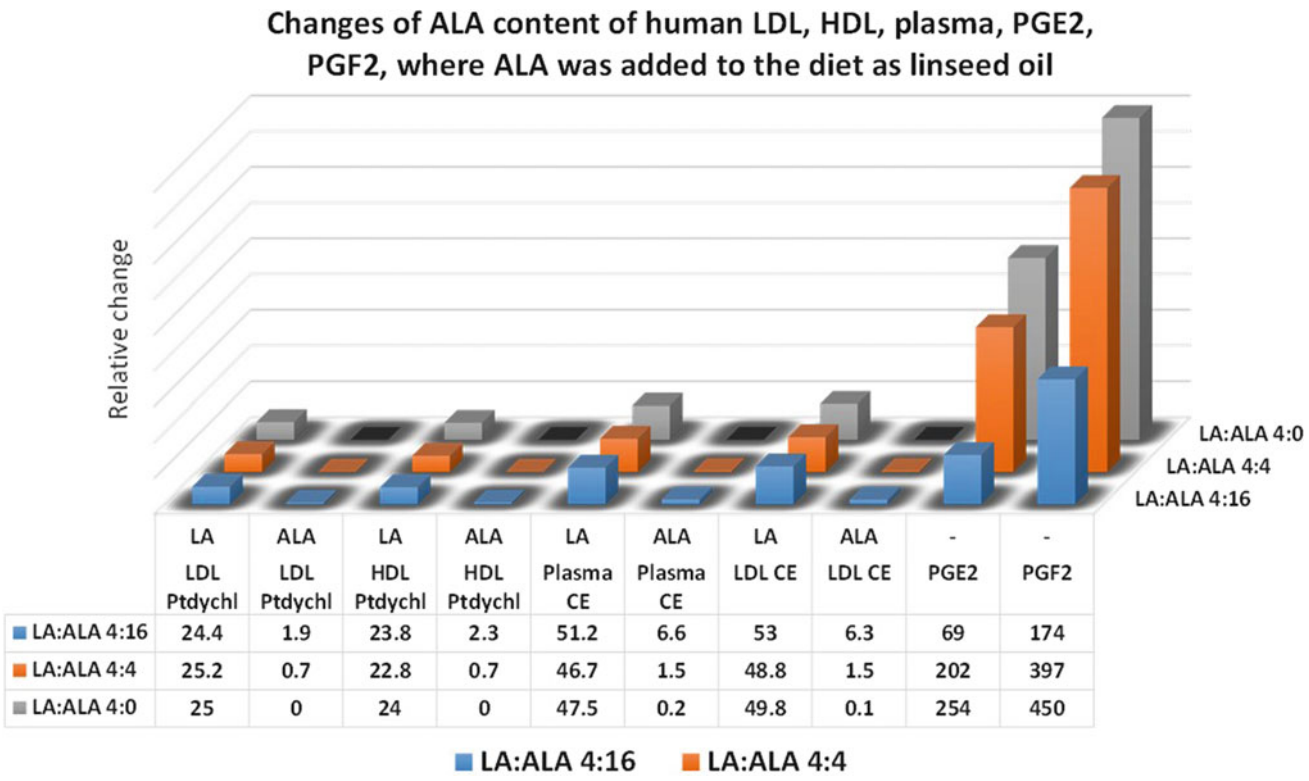
Phosphatidylcholine an Important Mechanism for Delivery of Lipids and Choline

Phosphatidylcholine is the primary outer shell component of lipid delivery vesicles including LDL, HDL and chylomicrons [13] hence a delivery mechanism of lipids and dietary choline. Uptake via chylomicrons of ingested phospholipids potentially provides an important mechanism for delivery of omega 3 fatty acids, including ALA. Dietary phosphatidylcholine can be directly and rapidly passed from chylomicrons to LDL and HDL [14], creating a very direct liver independent link between dietary phospholipid intake of ALA and LA including oxidised content, and wider cell membrane composition.

Cattle fed additional linolenic acid in the form of flax oil had increased ALA in muscle and adipose tissue in both phospholipids and triglycerides, which given the preferential oxidation of ALA by the liver, and low level of ALA in LDL suggests the ALA is likely in the main to have originated from direct lipoprotein lipase action on chylomicrons and/or from direct delivery in phospholipids. Interestingly, and consistent with this, the fraction of ALA in muscle and adipose tissue was principally in the phospholipid rather than the triglycerides fraction [15].

In humans, both total parenteral nutrition and dietary variation in LA, resulted in significant changes in chylomicron [16] and plasma phospholipids LA content. As detailed in the abstracted data below (see Fig. 29.2), humans on an isocaloric dietfed ALA in the form of linseed oil for 2 weeks at 4–16 % of energy intake saw significant changes in the ALA content of LDL, HDL and plasma, in both the cholesterol ester and phosphatidylcholine moieties [17].

The rise in ALA was compensated for by a corresponding drop in OA content; LA, AA and EPA remained comparatively stable. Further ALA reduced COX prostaglandin AA products, presumably through inhibition and/or competition.



	LA LDL Ptdychl	ALA LDL Ptdychl	LA HDL Ptdychl	ALA HDL Ptdychl	LA Plasma CE	ALA Plasma CE	LA LDL CE	ALA LDL CE	- PGE2	- PGF2
LA:ALA 4:16	24.4	1.9	23.8	2.3	51.2	6.6	53	6.3	69	174
LA:ALA 4:4	25.2	0.7	22.8	0.7	46.7	1.5	48.8	1.5	202	397
LA:ALA 4:0	25	0	24	0	47.5	0.2	49.8	0.1	254	450

Fig. 29.2 Changes in the ALA content of LDL, HDL and plasma, in humans on an isocaloric diet, fed ALA in the form of linseed oil for 2 weeks each at 4–16 % of energy intake. The competitive effects of rising ALA, presumably primarily for the COX enzymes, on AA so the production of prostaglandins PGE2 and PGF2 is thought provoking. The data are abstracted from Tables 2, 3 and 6 of: '*Effect of alpha-linolenic acid in the human diet on linoleic acid metabolism and prostaglandin biosynthesis'* with very grateful thanks to the authors: Adam et al. [17]

The changes in our phosphatidylcholine ALA intake, consequent on reduction in plant material intake has been amplified by grain feeding of livestock, due to distortion of the ALA:LA balances in livestock phospholipids, as well as in their triglycerides leading to altered lipoprotein function and changes in delivery of lipids to cell membranes.

Importance of Phosphatidylcholine to Lipoprotein Membranes

In the LDL shell, phosphatidylcholine cannot be substituted for by other phospholipids; inhibition of liver phosphatidylcholine production reduces liver VLDL output [18]. Dietary choline deficiency, causes liver dysfunction including lipid accumulation, in the laboratory is used to generate a NAFLD model, and leads to 'remarkable' increases in oxidative stress in mice [19]. Choline-related compounds improve glutathione production [20, 21]. Choline has many other roles [22].

Phosphatidylcholine appears to have a particular structural/polar relationship with cholesterol and fatty acids possibly explaining its selection for use in lipoprotein shells. It may increase lipid solubility as seen in its use for localised fat lump management [23]. Phosphatidylcholine is also bioactive when oxidised, increasing inflammation [24], immune and related activity. Interestingly when injected in a study for cosmetic fat reduction as well as leading to lipolysis, it caused; fibrosis, inflammatory infiltration, fat necrosis, micro-calcification, cyst and micro-abscess formation [25].

Galactolipids

Galactolipids are the most common fats in green plant material, but whilst human triglyceride metabolism is fairly well understood, knowledge on the human metabolism of galactolipids is limited.

ALA is the predominant fatty acid in galactolipids, more commonly attached at the SN1 position than LA, and is often found attached at both the SN1 and SN2 positions [26, 27]. (ACOS Lipid library) Trials suggest, given sufficient time, both the SN1 and SN2 fats can be enzymatically cleaved in the gut, but are some galactolipids also absorbed across the gut membrane in a similar way to phospholipids with one fat remaining attached. Are they transported in chylomicrons? This may be significant, particularly if the

brain, heart or lungs preferentially absorb galactolipids, as happens for phospholipids [28].

Plant-type galactolipids have not been identified as having wide-ranging roles in humans, but interestingly are found in relatively small amounts in the brain [29], and may have wider functional effects. Small amounts of galactolipids 0.1–0.6 % with similar structures to those in plants (ACOS Lipid Library) form part of astrocyte and neuronal membranes [30]. Galactolipids are essential to myelin formation and stability, as well as oligodendrocyte formation. Galactolipid-deficient animals exhibit defective nervous system function [31].

Albumin as a Transport Mechanism

Whilst albumin has many roles, it is known to transport DHA [32, 33], and low albumin may be associated with cardiovascular disease risk [34], but albumin does not appear, including in analbuminemia patients, to be a core lipid delivery mechanism to cardiac tissue [35], but may provide alternative pathway capacity or due to subtleties of delivery have greater relevance to membrane phospholipid composition. Exposure of endothelial cells to LA and ALA attached to albumin resulted in significant LA ALA changes in the phospholipid fraction [36] so membrane composition, but not the triglyceride fraction possibly suggesting a specific important but limited lipid delivery role for albumin. Interestingly, LA alone increased intact cross-membrane transfer of albumin [37].

The abnormal lipid deposition profiles in analbuminemia suggest important roles for albumin in export of stored adipose lipids. Albumin contains a number of fat binding sites, varying as to strength by site, and competed for by other substrates. Intriguingly albumin preferentially binds with 13-HODE in preference to unsaturated fats [38]. Albumin also transports glycated proteins, reducing albumin fatty acid-binding sites including for LA [39]. Reduced reverse albumin transport capacity from adipose tissue, due to blockage of sites by oxidised and glycated products, could have a range of consequent effects, including on adipose tissue accumulation and distribution.

Export of LA from the Liver and Implications for Non-alcoholic Fatty Liver Disease

LA arriving in the liver from the portal vein, via chylomicron remnants of which LA forms a significant proportion, or HDL, must be beta-oxidised, stored or re-exported in VLDL. Logically, the best option to prevent liver lipid build-up, including NAFLD, is export of accumulated LA through increased VLDL output. Excess LA and raised LA oxylipins in the liver are associated with NAFLD.

Those with hypobetalipoproteinaemia so inability to normally form LDL develop enlarged fatty livers, as well as having low tissue levels of fat-soluble antioxidants and neurological impairment [40].

Given the importance of regulating lipid cholesterol and LA levels in the liver, onward wider tissue requirements for LA and cholesterol, evolutionary pressures to store them in adipose tissue, and need to prevent NAFLD, as might be expected LA-related synergistic mechanisms exist to commensurately increase LDL output proportionately to the rate of LA arrival in the liver, thereby normally preventing liver lipid build-up [41].

Synergistically, oxidised LA promotes VLDL component production including OA, POA and cholesterol. The oxidised products of LA, including 13-HODE and Oxo-HODE, are primary endogenous activators of PPAR gamma, which synergistically activates SCD1, a key enzyme in the conversion of the respective parent saturated fats to OA and POA. PPAR gamma also activates HMGCoA reductase, which increases cholesterol and CoQ10 production from ACoA. Activation of HMGCoA reductase also diverts resources from PPAR alpha-related energy pathways and ketone production, thereby supporting increased LDL production.

Higher levels of liver LA will result in higher HODEs which will stimulate activity of PPAR gamma-related tissue maintenance peroxisomes and peroxide production, hence oxidative stress, lipid and cholesterol production, as well as additonal production of LA oxylipins including 13-HODE, thereby further raising PPAR gamma activity and creating a feed forward loop. Consistent with this PPAR gamma [42], and 13-HODEs [43] were significantly elevated in obese NAFLD patients.

In a hepatic cell line PPAR gamma activation enhances lipogenesis uprating '*genes including adipose differentiation-related protein* (*ADRP*), *adipocyte fatty acid-binding protein 4, sterol regulatory element-binding protein-1* (*SREBP-1*), *fatty acid synthase* (*FAS*), *and acetyl-CoA carboxylase*' [44]. A PPAR gamma null leptin-deficient mouse had '*dramatically decreased triglyceride* (*TG*) *content*' [45].

Consistent with PPAR gamma activation being a part of the mechanism by which cholesterol production may by controlled, peroxisomes are necessary contributing process components of the cholesterol production pathways [46, 47]. Peroxisomes also play important roles in the bile acid pathways, and in the formation and breakdown of cholesterol, as well as having roles in wider tissue; consistent with this '*Studies from other tissue macrophages have shown that PPAR gamma regulates cholesterol influx, efflux, and metabolism*' [48].

PPAR gamma, by increasing peroxisomal beta-oxidation so ACoA production and related gene expression, promotes creation of substrate including fats and cholesterol for tissue including liver creation and repair, and/or for export along with LA in VLDL, and/or for accumulation in the liver.

Conversely, energy stress-related PPAR alpha activation may ameliorate NAFLD [49], consistent with this mice that are PPAR alpha and ACOX1 null (the first oxidative enzyme in the peroxisomal beta-oxidation chain), exhibited dramatic steatosis [50, 51].

LA Promotes Increased Liver LDL Output yet LA Reduces Plasma LDL: A Paradox?

The LA, OA and cholesterol, cleared from the liver via LA oxylipin promoted enhanced VLDL production, would lead to increased plasma LDL in response to increased LA intake, but paradoxically it is accepted that LA induces falls in plasma LDL.

A likely explanation is that LA and HODEs by activation of LDL receptors such as LDLR, CD36 and OLR1 [52, 53] in epithelial and adipose-related vascular membranes, also synergistically signal for increased abstraction of non-oxidised and oxidised LDL from the blood, logically by greater amounts than the corresponding increase of VLDL/LDL output by the liver. It makes evolutionary design sense that the body would have a compensatory mechanism to prevent undue lipid build-up in the blood, and in the 'natural' world to secure greater delivery of essential lipids to cells to support reproductive-related function when the environment was fecund. Consistent with this, oestrogen promotes LDL uptake [54, 55].

Reduction in LDL consequent on increased LA intake would therefore represent increased vascular receptor up take of oxidised and unoxidised lipids across the vascular membranes, hence reduced plasma LDL, but when heavily oxidised, endocyted LDL is likely to increase rather than reduce risk of vascular damage.

LDL a Major Transporter of LA to Vascular Epithelial Cells Hence Cardiac Tissue

LDL is composed in significant part of cholesterol esters and phospholipids, and LDL are a major and obligate bulk transporter of LA OA and POA including to cardiac tissue; in the fasting state 70 % or more of cardiac energy is supplied by LDL. In a group comprising mainly Caucasian women cholesterol esters represent approximately 1/3 of total plasma lipids; phospholipids represented over 1/3, triglycerides under 1/3, and free fatty acids between 3 and 4 % (Table 3) [56]. Ratios will vary, diet being one variable.

MUFA PUFA and Saturated Fat Content of LDL

In common 'Western' human dietary scenarios, the PUFA and MUFA content of LDL vary in relation to dietary intake and inversely with dietary intake of each other, they are also impacted by endogenous OA and POA production. In contrast, saturated fat content of LDL is comparatively stable.

'*Despite wide variations in SFA intake, the SFA content of LDL was not statistically different between the four diets (25.8–28.5 % of LDL fatty acids). By contrast, the PUFA (43.5–60.5 % of LDL fatty acids) and MUFA content of LDL (13.7–29.1 % of LDL fatty acids) showed a wider variability dependent on diet*'.

Plasma saturated fats may rise in those on very-low-fat diets. In fasted rats on a 4 % fat laboratory diet, of which 1.1 % was saturated (Harlan Teklad) the PA and SA lipid percentage contents of plasma were 25.9 and 9.7 %, respectively [57], plasma and LDL lipid data on humans on equivalent low-fat diets could not be found.

LDL: An Evolutionary Lipid Supply Mechanism, to Support Reproductive Tissue Needs, Adipose Tissue Lipid Supply, and Sequester Blood Borne Oxidised Material and Pathogens into Tissue for Reprocessing?

Given the crucial importance of LA and cholesterol to both tissue repair and reproduction, combined with the need for storage of them, it is suggested that LDL, as well as providing a mechanism to transport environmental food chain dependent signalling messengers that regulate reproductive capacity, and support cellular reproductive nutrient needs, is an evolutionary mechanism to supply LA, OA, cholesterol, and other nutrient and lipid-soluble antioxidant products found in or attached to LDL, for direct delivery by endocytosis to the core of adipose and epithelial-related cells, thereby securing their storage, and further ensuring secure controlled delivery and safe sequestration into tissue of oxidised LDL content, pathogens and detritus for reprocessing.

Only during famine, essential fatty acid deficiency scenarios, and very high omega 3 intake as seen in Inuit, will LA levels in the liver and LDL likely be very low. In famine, reduced LDL LA content, and consequent diminished availability of raw materials for products such as prostaglandins, will reduce immune capacity and inhibit reproductive capacity.

Even at relatively low dietary LA intakes, LDL contains 40 % plus of LA, which suggests LA and accompanying cholesterol have particular functional and physiological relevance to their destination tissues. The liver also appears to accumulate LA even at very low intakes [58].

In a study, linoleic acid (LA) content of plasma cholesterol esters varied between 47 and 77 %, and oleic acid

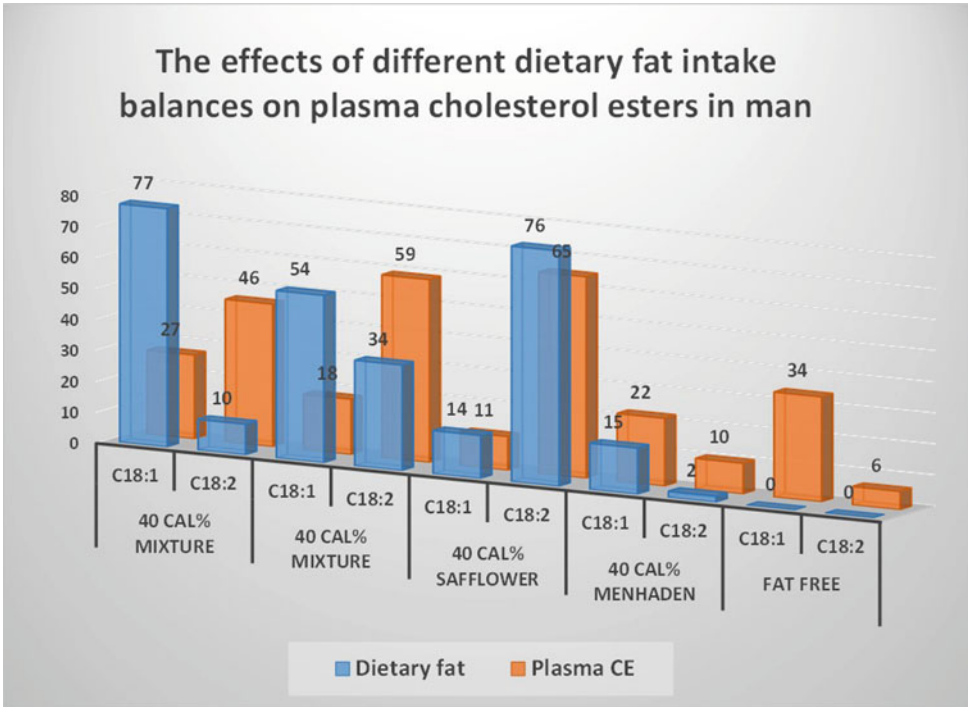
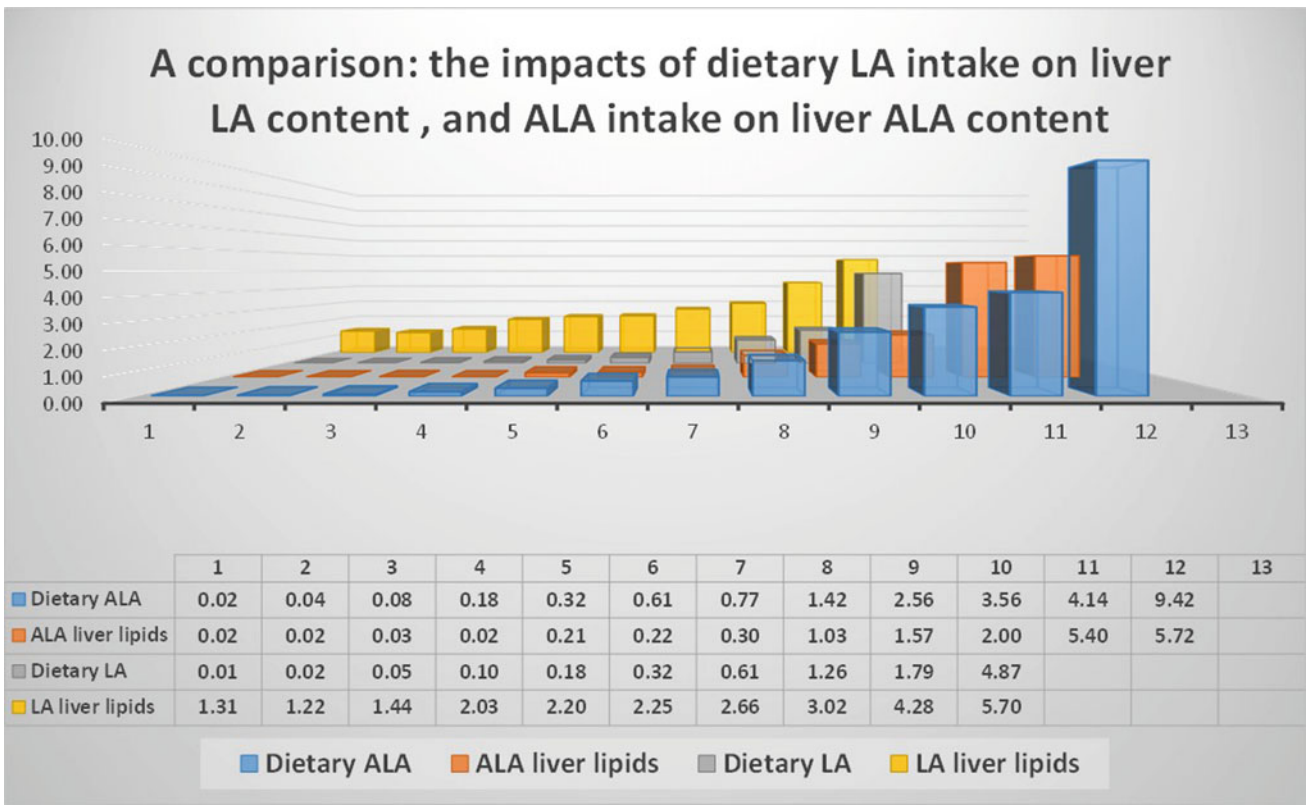


Fig. 29.3 The percentage lipid composition of plasma cholesterol esters can be changed significantly by the percentage in calories of OA and LA in dietary fats. The uptake of LA in plasma cholesterol esters shows a tendency to flatten off at higher intakes. The data are abstracted from Table 4, p. 71 of '*Atherogenic Effect of Different Cholesteryl Esters*' with very grateful thanks to the authors: Gottenbos et al. [59]



	1	2	3	4	5	6	7	8	9	10	11	12	13
Dietary ALA	0.02	0.04	0.08	0.18	0.32	0.61	0.77	1.42	2.56	3.56	4.14	9.42	
ALA liver lipids	0.02	0.02	0.03	0.02	0.21	0.22	0.30	1.03	1.57	2.00	5.40	5.72	
Dietary LA	0.01	0.02	0.05	0.10	0.18	0.32	0.61	1.26	1.79	4.87			
LA liver lipids	1.31	1.22	1.44	2.03	2.20	2.25	2.66	3.02	4.28	5.70			

Fig. 29.4 Omega 6 levels in the liver in rats remain relatively high even on very low omega 6 intakes as a percentage of calories, the saturation effect referred to by Professor Lands [64]. LA liver lipid content rises much faster than ALA content. The data are abstracted from Table 2 of '*The effect of dose level of essential fatty acids upon fatty acid composition of the rat liver*' with very grateful thanks to the authors: Mohrhauer et al. [58]

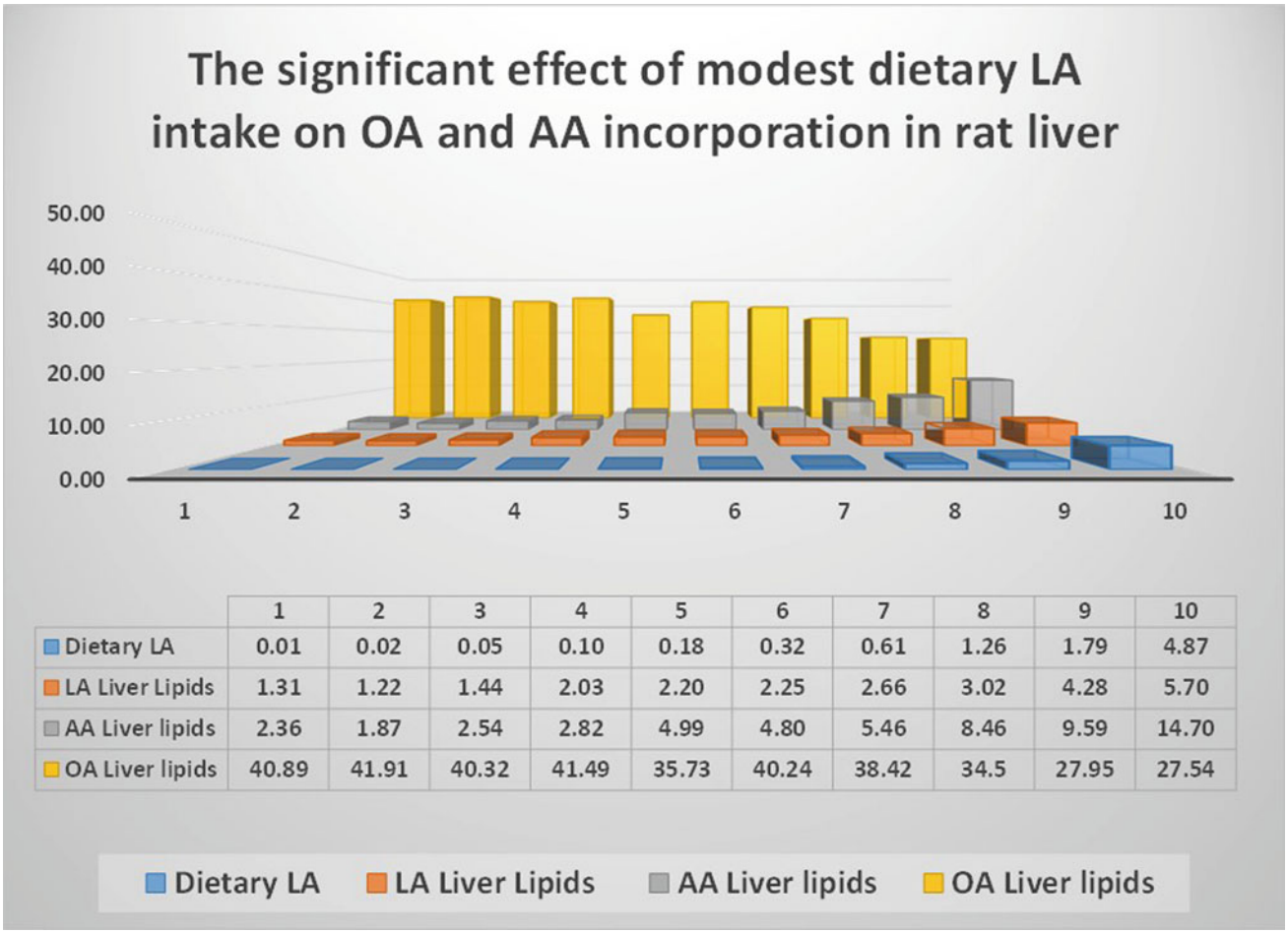
between 7 and 32 % [59] (see also The Human Serum Metabolome table LA-1506, OA-704, PA-405 and POA 119 [60]). LDL LA levels are moderated by diet [61] but remain significant even at intakes around 1 % of dietary calories (Kitavans 42.6 %) [62].

The exceptions shown to high LA in LDL esters were as follows: menhaden oil feeding reduced OA and LA cholesterol esters to 22 and 10 %, respectively; and a no fat diet for a week resulted in OA of 34 % and LA of 6 % (subjects in 1970s may not have had significant amounts of LA in their adipose tissue) [59] (Fig. 29.3). Low LA in cholesterol esters is also seen in Inuit living on a traditional diet, around 20 % (1970), but not in Westernised Inuit (55 %) [63]. Indications of the dietary effects of a variety of LA and/or ALA intakes on LA [64], AA, ALA and OA levels in liver lipids and cholesterol esters can be shown in Figs. 29.3, 29.4 and 29.5.

LA content of LDL rises significantly where LA intake is high. On a weight maintaining diet in humans, LA in LDL cholesterol esters rose by 9.3 % for each 10 % of energy intake as LA; for OA, the figure was 6.5 %; whilst the changes for saturated fats were only 1–2 % [65].

In 'Western' diets, compared with LA saturated fat content of LDL appears relatively smaller and stable, adding to evidence saturated fat intake per se may not be a major factor in cardiovascular disease. Interestingly, and consistent with low-fat diets potentially increasing the saturated fat content of LDL, in subjects with metabolic syndrome on either a high- or low-fat diet, there was a slight tendency to increased saturated fat in blood lipids on the high carbohydrate diet [66].

LA falls are likely in famine and during EFA deficiencies [67]. LA is then primarily replaced by endogenously produced OA and POA. The changing balance of LA:OA:POA



	1	2	3	4	5	6	7	8	9	10
Dietary LA	0.01	0.02	0.05	0.10	0.18	0.32	0.61	1.26	1.79	4.87
LA Liver Lipids	1.31	1.22	1.44	2.03	2.20	2.25	2.66	3.02	4.28	5.70
AA Liver lipids	2.36	1.87	2.54	2.82	4.99	4.80	5.46	8.46	9.59	14.70
OA Liver lipids	40.89	41.91	40.32	41.49	35.73	40.24	38.42	34.5	27.95	27.54

Fig. 29.5 There is a reciprocal relationship between omega 9 OA, omega 6 LA, and AA in plasma and liver tissue. Increased dietary omega 6 as a percentage of calories will displace omega 9. The lipid profile of the liver will logically be reflected in the lipid content of LDL, and those lipids will be delivered to tissues. Further given AA is the substrate of the prostaglandin pathways these changes will have wider and significant physiological effect. The AA content of the rat liver content rose significantly with increased LA intake as illustrated by the attached data abstracted from Table 2 '*The effect of dose level of essential fatty acids upon fatty acid composition of the rat liver*' with very grateful thanks to the authors: Mohrhauer et al. [58]

and their oxidised products in LDL provides a mechanism to alter wider cell membrane and cardiolipin composition, and circulating oxidised messengers, so to change metabolics, physiology and behaviour in response to food availability, which in turn is ultimately dependent on the impact of environmental growing conditions on plant fecundity.

Importance of Peroxisomes

Peroxisomes work in synergy with mitochondria and are key to efficient cardiac energy production, and repair. PPAR alpha, delta and gamma are all present in cardiac tissue, PPAR alpha energy production-related peroxisomes predominate in normal hearts. Long chain fats are beta-oxidised by peroxisomes to short fats, ACoA, and byproduct peroxide. Short fats are used by mitochondria for fuel. ACoA may be used as a fuel substrate, or directed to production of lipids and/or cholesterol dependent if PPAR alpha or gamma pathways are activated.

In cardiac as in other tissues, PPAR alpha, delta and gamma pathway-related peroxisomes have different roles and are activated by different mechanisms and substrates. The decision whether the ACoA product of peroxisomal beta-oxidation of the LA, as well as the OA PA and POA (lesser substrates of peroxisomes) delivered by LDL, is directed to tissue repair, or mitochondrial fuel, is significantly determined by whether PPAR alpha and delta, or alternatively PPAR gamma, and their respective target gene sets are activated.

Peroxisomal beta-oxidation product ACoA may also be used to make malonyl-CoA, which inhibits carnitine assisted takeup of long chain fats by the mitochondria. The creation of malonyl-CoA from ACoA produced by peroxisomes may be a mechanism to inhibit carnitine pathways so drive fats to beta-oxidation in peroxisomes, rather than direct beta-oxidation in the mitochondria.

The products of peroxsiomal PPAR alpha-, delta- and beta-oxidation activated by energy demand following exercise or in fasting and/or to a lesser extent by omega 3s, and related gene expression pathways, tend to be directed to mitochondrial energy production and also synergistically promote reduced oxidative stress.

The products of excess peroxisomal PPAR gamma activation and related gene pathways are directed to tissue maintenance, immune function lipid storage and increased oxidative stress. PPAR gamma is activated primarily by dietary and/or endogenous enzymatic and free radical oxidised products of LA, including HODEs, which are the most common oxidised lipid in plasma.

Imbalances in PPAR alpha-, delta- and beta-oxidation in relation to PPAR gamma beta-oxidation could lead to a lack of substrate being directed to energy production, and an excess being directed to substrate creation, resulting in both mitochondrial energy deficits and intracellular lipid accumulation, which effects are present in many cardiovascular conditions.

Potential Recycling of Oxygen

Activation of peroxisomal pathways by increased concomitant production of catalase potentially recycles peroxide to oxygen and may moderate net overall combined peroxisome mitochondrial pathway oxygen demand per unit of mitochondrial ATP production.

Short Saturated MCTs and Cardiovascular Function

Peroxisomes produce MCFs as a by-product of beta-oxidation. They are fed back to the mitochondria: peroxisomal MCFs are likely a significant cardiac fuel. C:8 is the shortest fat that mammalian peroxisomes can produce. Short fats are poorly metabolised by peroxisomes (Masters P.101), but well oxidised by mitochondria, and bypass the need for carnitine in some but not all tissues.

In a human study in subjects with a variety of long-chain fatty acid mitochondrial transport-related oxidation disorders including of CPT1 and CPT2, MCTs were found to lower cardiac exercise workload, improve oxidative capacity, and decrease glucose usage [68].

Dietary MCTs, by bypassing blocked and damaged pathways will often improve conditions of metabolic dysfunction including metabolic syndrome [69], suggesting such conditions at least in part involve lack of PPAR alpha and delta function, due to; polymorphisms; tissue damage; lack of periods of energy deficit and/or other activators such as fasting exercise and to a lesser extent omega 3s.

Peroxisomal Dysfunction-Related Cardiac Impairment

Cardiac function impairment, particularly under load, is observed in models of and those with peroxisomal disorders [70], such as Refsum's and Zellwegger's. In arrhythmogenic right ventricular dysplasia (ARVD) '*decreased PPAR-α, and increased PPAR-γ expression in the right ventricle*' was '*characterised by a progressive fibrofatty infiltration*' [71].

Cardiac effects of PPAR gamma agonists are mixed, however, high doses of some PPAR gamma agonists have been observed '*to induce cardiac dysfunction*'. '*PPAR-γ leads to elevations in lipogenic enzymes, which subsequently increase triglyceride production*'. '*PPAR-γ ... lipogenic effects, may contribute to intracellular triglyceride*

accumulation and cardiac lipotoxicity' 'Despite many beneficial features of glitazones, they also exhibit adverse effects, such as oedema, heart failure, weight gain, bone fractures, and increased risk of myocardial infarctions'.

PPAR alpha knockout mice exhibit impaired antioxidant function, age-associated functional decline, impaired antioxidant capacity, and failure to adapt to increased cardiac workload '*PPAR-α KO mice exhibit progressive cardiac fibrosis with abnormal mitochondria and myofibrils*' … '*significant cardiomyocyte hypertrophy*' … '*muscle exhibits reduced shortening velocity and isometric tension*'.

In contrast in humans use of PPAR alpha activators in '*several large studies have shown that the use of PPARα-activating drugs is favourable in the reduction of CVD risk factors, as summarised by the FIELD* (*Fenofibrate Intervention and Event Lowering in Diabetes*)' reduced arterial occlusion, infarction, and cardiovascular events [72].

Impact of Failure to Activate PPAR Alpha Through Energy Restriction, Exercise and/or ALA Availability, and Over Activation of PPAR Gamma

In a modern sedentary world, in which periods between food intake generally are not sufficient to result in a 'fasted' state, exacerbated by 24/7/365 access to food, rich in oxidised LA oxylipins, low in ALA- and lipid-related antioxidants, and lacking in physical activity, PPAR gamma pathways in cardiac tissue are likely significantly overactivated, and PPAR alpha underactivated, leading to mitochondrial energy disruption, and long term to cardiac lipid build-up [73].

In the postprandial state in a pre-agricultural scenario, LA intake was lower seasonal and more in balance with ALA, intervals between meals were greater, and food required activity to acquire, consequent activation of PPAR alpha by short-term fasting and/or exercise would divert excess LA delivered by LDL to cardiac tissue energy, and reduce available LA quantities competing for LOX12/15 and COX enzymes, so production of oxidised LA PPAR gamma activators; this would mitigate inflammatory and oxidative cascade effects of oxidised LA, cardiolipin oxidation, overactivation of the immune system, and both localised cellular and wider manufacture and deposition of cholesterol lipids and related products.

In the fed state when ingested lipids are supplied to cardiac tissues by newly arrived chylomicrons, the body is arguably expecting the chylomicrons to contain some ALA, and in diets rich in marine foods significant DHA, which Omega 3s would help activate PPAR alpha pathways. However, the modern diet is high in LA and low in ALA and long-chain omega 3s.

Benefits of Short-Term Fasting

Consistent with fasting activating PPAR alpha, increasing energy production, and reducing inflammatory and adipose-related PPAR gamma activity, anecdotal reports by specialist doctors and a review suggest that part-day fasting is a useful strategy for managing diabetes and cardiovascular risk.

In a randomised study of young overweight women '*intermittent energy restriction is as effective as continuous energy restriction on weight loss and metabolic disease risk markers*' [74] and '*Alternate day calorie restriction reduces markers of oxidative stress and inflammation in overweight adults with moderate asthma*' [75]. Further intermittent energy restriction '*was as effective as continuous dieting over 8 weeks and for weight loss maintenance at 12 months*' [76].

Delivery of Lipids and Oxidised Substrate to the Heart

Lipid supply mechanisms to cardiac tissue comprise, a mix of transport by; albumin; LPL facilitated uptake from chylomicrons and VLDL; by endocytosis of LDL [77], and potentially endocytosis of some VLDL [78]. It would make sense that the lipid circulatory delivery mechanisms would adapt and change, as the predominant lipid source moved from postprandial dietary intake to fasted lipolysis of adipose fat stores [79].

The ability of endothelial tissue to take up lipids will depend on its functional status; foam cell formation, placque and inflamed tissue are likely to impair normal cardiac uptake pathways including LPL action and endocytosis of LDL. Further heavily oxidised LDL will be directed to macrophages for disassembly and reprocessing, so will not be available as a potential mechanism to supply raw materials to cardiac cells.

Receptors for Oxidised LDL Including CD36 and ORL1

LDL is taken up by vascular membranes by a variety of receptors. LDL receptor CD36 (also known as FAT1) [80, 81] is a receptor for oxidised and 'non-oxidised' or 'native', meaning not significantly oxidised, LDL [82]. Observations of humans with LDL receptor CD36 polymorphisms suggest that CD36 [83] is necessary for normal cardiac uptake of fats [84]; significantly lower fat uptake 57–73 % was seen in humans homozygous for the mutation [85]. Impaired CD36 function may be a factor in heart disease [86] and is associated with cardiac fibrosis.

CD36 is likely primarily activated by PPAR gamma, which in turn is primarily activated by LA oxylipins HODEs, and to a much lesser extent by AA oxylipins, but logically might also be activated to some extent by PPAR alpha in some tissues, given the importance of CD36 lipid uptake to cardiovascular metabolism.

CD36 is widely expressed in adipose tissue, skeletal muscle, cardiac tissue, digestive tract, insulin beta cells and the brain. CD36 is found on the surface of macrophages and is a '*principal receptor*' for binding oxidised LDL [87]. Indicative of its importance CD36 deficiency also reduces obesity.

LDL receptor OLR1 (also known as LOX1) [88] in contrast to CD36 appears to be activated by oxidised LDL but not 'native' minimally oxidised LDL. It sits in a cancer-related gene sequence; is 'oncogenic', detected in vascular plaque close to macrophage-rich regions; and is demonstrated to have a role in cardiovascular disease, '*The evidence for a pathogenic role of LOX-1 in cardiovascular diseases was demonstrated by a wealth of experimental animal studies, in which genetic LOX-1 abrogation significantly diminished disease progression*' [89]; and factors in oxidative stress induced mitochondrial damage to DNA [90]. OLR1 is also regulated by PPAR gamma.

Receptors CD36 and OLR1 coactivate immune response [91, 92], tissue repair and catabolism, including involvement in leucocyte chemotaxis and rolling. HODEs will activate PPAR gamma, which further activates both OLR1 and CD36, accelerating processes [93, 94]. PPAR gamma activates macrophages, has roles in wider immune function and tissue repair including angiogenesis and promotes adipose tissue deposition including via ORL1- and CD36-driven LDL uptake.

Excess PPAR gamma activation is itself likely to increase oxidative stress through increased peroxisomal activity, additional net peroxide production which will stimulate iNOS, so NO production, and blocking of catalase enzyme activity.

Fed State

In the fed postprandial state, in contrast to the fasting state, chylomicrons with some assistance from VLDL, are likely a major source of lipids for cardiac tissue, possibly as much as 60–80 %. They appear to be a significantly more efficient delivery mechanisms to cardiac and adipose tissue than albumin [95].

Lipoprotein lipid content and oxidation status will reflect, oxidative blood status, adipose lipid content, and in the fed state particularly, dietary intake, including of oxidised fats and lipid-soluble antioxidants.

In the fed state, ALA and DHA intake, and/or exercise v oxidised LA blood stream products, will determine if PPAR alpha or gamma cardiac peroxisomal pathways are activated, so direction of lipids between energy provision and maintenance including lipid accretion. As discussed, in the fed state chylomicrons may subject to dietary intakes, be a significant source of ALA to cardiac tissue.

ALA is preferentially released compared to LA by LPL. LPL releases lipids from chylomicrons at the vascular membrane; LPL also smooths lipid fuel supplies, by release of fats from temporarily stored intramuscular cardiac lipids [96].

The heart and lungs, then brain, via the actions of LPL, would have the first opportunity to abstract LA and ALA from the 'new' chylomicron surface phospholipids and triglyceride cores, as well as to a lesser extent via LDL receptors including LDLR and CD36, from LDL containing fresh phospholipids recently acquired from chylomicrons.

Intermediate State Between Fed and Fasted

There will be an intermediate state where cardiac lipid fuel supply is supplied by a mix of recent intake, stored local intracellular fat and adipose tissue. The activation of PPAR alpha- and/or gamma-related pathways will depend on a mix of factors, including ingested LA, oxidised products, oxidative bloodstream status, LA lipid storage status and activity levels.

Non-fed Fasted State

In the non-fed fasted state, when chylomicron supplies would be limited, LDL appears to be the major delivery mechanism for lipids to the heart. The fasted lipid content of LDL will be determined by stored tissue fat.

Low or high oxidative status of LDL and its components significantly determines which vascular LDL receptors are activated, and, respectively, if the content will be used as substrate by cells or diverted for reprocessing by macrophages.

High levels of LA and likely oxidised product are found in Western adipose tissue, which aggressively accretes available oxidised and unoxidised LA into storage. LA-oxidised product of adipose origin, or oxidised in a blood stream, and immune activity will activate PPAR gamma.

Relevance of LDL LA-Related Oxidative Stress to Cardiovascular Disease

Given seventy percent of cardiac energy is derived from fats, and in the fed state up to 70 % are delivered by LDL, the cardiac-related vascular tissue endocytosis of LDL

containing excess LA oxylipins, damaged proteins, AGEs [97], and other oxidised LDL-related contents including oxidised cholesterol, absent sufficient antioxidant factors, may in significant part be responsible for vascular epithelial damage and related cardiovascular malfunction.

Mechanisms by which excessive supply of oxidised LA influences cardiac function include; alteration of gene pathway expression; cardiolipin oxidation so reduced mitochondrial energetics; cardiolipin release of oxidised LA species products including 4-HNE and MDA so damage to mitochondrial enzymes and DNA; wider oxidative damage to vasculature including overwhelming of macrophage capacity to recycle oxidised LDL, so macrophage 'foam cell' accumulation leading to arterial lipid build-up, inflammation and impairment of lipid supply; overactivation by LA HODEs of the PPAR gamma-related repair pathways; linked overactivation of the immune inflammatory and intracellular lipid deposition pathways; and inhibition of the PPAR alpha peroxisomal energy production-related pathways.

Oxidised Cholesterol Phosphatidylcholine and LA Significantly Influence of Gene Expression

Oxidised products of cholesterol phospholipids and LA have significant biological effects including through alteration of gene expression. Exposure of human coronary endothelial cells (HAEC) to oxidised LDL for 12 h resulted in up- or downregulation changes greater than 1.5 in almost 1500 genes and twofold changes in 596 genes [98].

Oxidised phospholipids also significantly altered the expression of over 1000 genes in HAECs [99]. Interestingly, '*The hydroperoxy derivatives of 18:2 and 20:4 (13(S) HODE, 15(S) HETE) were shown to be 100 times more potent than H_2O_2 in oxidising*' some phospholipids [100].

Relevance of Oxidative 'Western' Dietary Factors to Cardiovascular Disease

For avoidance of doubt, the negative effects of excess LA seen in those on Western diets is not simply due to excess omega 6 intake, but likely due to a combination of factors including excess calories particularly in refined form, lack of periods of energy deficit stress, 'food' damage due to refining and processing generally, nutrient deficiencies, degradation of lipid-related antioxidant food products, and pre-oxidation of foods including the LA they contain.

A diet low in antioxidant factors and high in easily oxidised and/or pre-oxidised foods will lead to increased levels of oxidation of; LDL, polyunsaturated fats, sugars, blood proteins and cholesterol. Oxidised products found in or attached to LDL include a range of oxylipins, oxidised phosphatidylcholine and lysophosphatidylcholine, damaged carrier proteins, AGEs, pathogens, and damaged blood components.

Higher levels of oxidised products are seen in the systems of those with or at greater risk of cardiac disease. Sixty patients with cardiac disease had higher oxidised LDL than matched controls [101]. Glycation may increase LDL oxidisability. Glucose will auto-oxidise under diabetic conditions. Oxidised LDL is higher in diabetics [102].

Ultimately damaged LDL will be taken up to some extent by cardiovascular-related tissues and impact cardiovascular health and function.

Oxidised Lipids and Other Products Including AGE Actively Cross the Gut Membrane

Oxylipins generally, including those generated in frying, such as 13-HODE [103], AGEs, as well as cross-linked fats and proteins, will pass through the human gut membrane as demonstrated with oxidised products in walnut oil [104], ultimately to be incorporated in VLDL, LDL, and/or wider bloodstream products such as albumin and haemoglobin [105]. Dietary-oxidised cholesterol is also incorporated in serum including likely chylomicrons, LDL and HDL [5].

The Relative Importance of LOX12/15 and COX to Oxidative Stress Induction

In an animal model in the absence of dietary ALA, LA is the next preferred LOX12/15 substrate. Overstimulation of LOX12/15 is associated with vascular disease. LOX 12/15 is uprated in heart disease and may be involved in heart failure [106]; LOX12/15 inhibition in mice reduces damage during heart failure. LA product 13-HODE is produced likely by LOX12/15 action by epithelial cells on a minimally stimulated basis [107, 108], which again emphasises the importance of excess activation of the LOX12/15 pathways and potential importance of the competitive presence of ALA. Conversely LOX12/15 plays protective roles during 'normal' physiological activation [109].

LA Oxylipin Including HODE-Related Oxidised Stress and Damage to Vascular Membranes

LA oxylipins such as the HODEs as well as being the primary endogenous activators of PPAR gamma pathways, which '*may play a role in atherogenesis*' [110], induce damage to vascular membranes.

'*Endothelial cells of the aorta of a rabbit injected with LA hydroperoxide was deformed by an enormous expansion of*

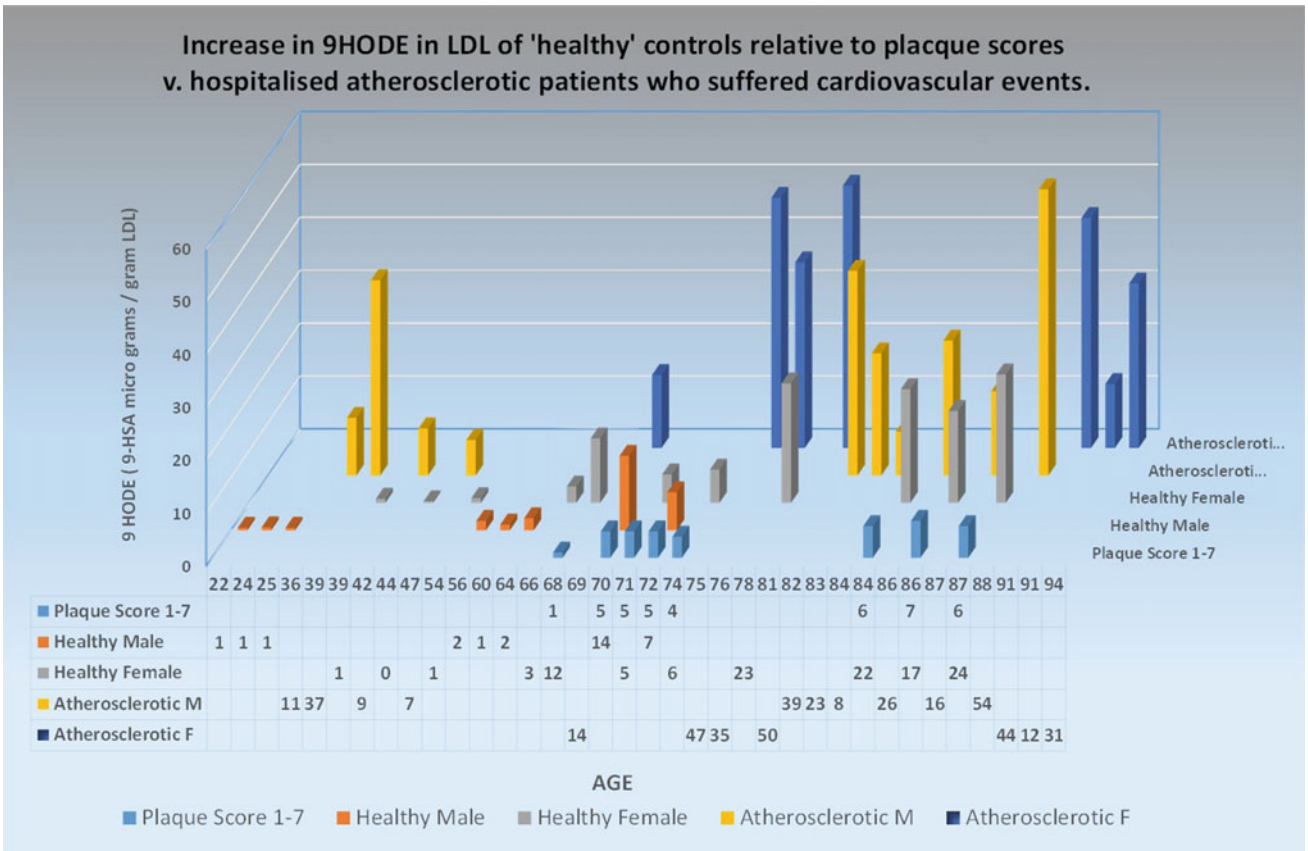


Fig. 29.6 Striking changes in relative levels of 9-HODE between men and women with age, in relationship to vascular placque levels and cardiovascular disease based on data abstracted from Tables 1, 2, and 4 of '*Strong increase in hydroxy fatty acids derived from linoleic acid in human low density lipoproteins of atherosclerotic patients*' with very grateful thanks to the authors: Jira et al. [112]

the endoplasmic reticulum … the basement membrane had disintegrated, and the subendothelial space was filled with plasma fluid' [111].

In atherosclerotic patients aged 36–47 years, 9-HODE increased 20-fold compared to healthy volunteers from the same age group and increased 30–100-fold in atherosclerotic patients ages 69–94 years, compared to young healthy individuals, and 2–3 times compared to 'healthy' individuals of the same age group [112] (see Fig. 29.6).

13-HODE increases angiogenic factor VEGF, which is '*remarkably expressed in activated macrophages, endothelial cells, and smooth muscle cells within human coronary atherosclerotic lesions*' [113], and induces endothelial proliferation.

In the epithelial membranes excess 13-HODE and PPAR gamma activation [114] helps recruit and differentiate macrophages, which are associated with foam cell formation and so plaque, as well as increasing macrophage infiltration into and damage to vascular and cardiac tissue.

LA oxidation products 13-HODE, 4-HNE and MDA are linked to activation of the oxidised LDL receptors; cardiac cardiolipin oxidation; mitochondrial damage and energy dysfunction. 13-HODE in cardiac vascular tissue may be a better marker of oxidative stress than AA isoprostanes [115].

Dietary Oxidised LA: A Significant Factor in Cardiac Disease

In excess, in the context of a Western diet, oxidised dietary LA oxylipins are likely much bigger factors in a range of Western disease than realised. The LA oxylipin enantiomers found in plaque suggest non-enzymatic oxidative origins, at least in part, so potentially pointing to dietary sources of oxidised products as possible factors in cardiac disease.

Importantly, as well as being made in situ in the body, LA oxylipins 13-HODE, 9-HODE, downstream 4-HNE and MDA, with a whole basket of other oxidised and cross-linked products, including AGEs, are present, sometimes in significant amounts, in frying oils [116] and related foods containing polyunsaturated vegetable oils.

Oxidised products pass across the gut membrane [117], may be incorporated into LDL or indirectly increase LDL oxidation, and are a contributory factor to oxidative stress

and so cardiovascular disease, including more than doubling vascular plaque formation increases in rabbits and mice [118, 119].

If oxidised LA products get across the gut membrane and into the circulation, there are limited effective antioxidant systems, including HDL, to reduce them. Dietary oxylipins can, by exchange between chylomicrons and LDL, avoid the liver and end up in active vascular tissue or being stored in adipose tissue, with all the consequent effects.

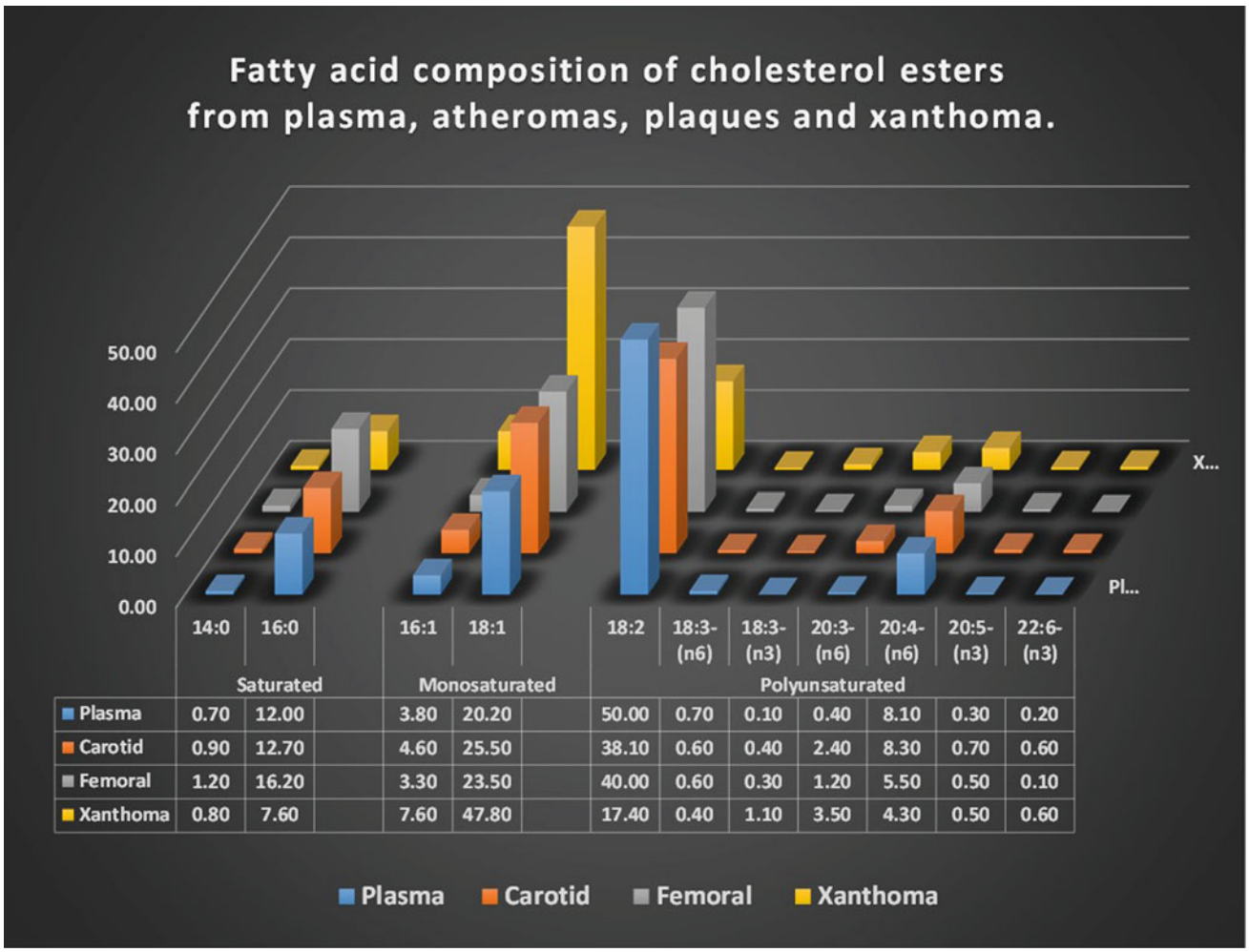
In evolutionary terms, the ability of oxidised dietary LA to reach tissue may have been a signalling system linking internal cellular function to external environmental status. The overamplification of those signalling pathways on a year-round basis by pre-oxidised dietary content, particularly LA, combined with a lack of ALA, in the context of the Western diet, compounded by endogenous oxidation, is likely a major factor in a wide range of Western including vascular diseases.

LA-Not-Saturated Fat Is the Primary Constituent of Vascular Plaque

The common perception that Western arteries are primarily blocked by dietary saturated fats is misplaced. The amount of saturated fat in LDL is limited, and relatively stable, [120] Saturated fat only forms 10–20 % of plaque [121, 122].

In those on Western diets, LA (40–60 % of LDL lipid content) and cholesterol, rather than saturated fats, must obligatorily be the largest components of plaque, because in Western diets they always form the largest proportion of lipids in endocyted LDL, which when heavily oxidised via foam cell formation are the predominant source of lipids in plaque (see Fig. 29.7).

LA plus AA can form 30–45 % and more of polyunsaturated fat in Western atheromas of which 30 % are oxidised. Further '*The cholesteryl linoleate content of aortae has been reported to increase with progression of atherosclerotic disease*' [123].



	14:0	16:0	16:1	18:1	18:2	18:3-(n6)	18:3-(n3)	20:3-(n6)	20:4-(n6)	20:5-(n3)	22:6-(n3)
	Saturated		Monosaturated		Polyunsaturated						
Plasma	0.70	12.00	3.80	20.20	50.00	0.70	0.10	0.40	8.10	0.30	0.20
Carotid	0.90	12.70	4.60	25.50	38.10	0.60	0.40	2.40	8.30	0.70	0.60
Femoral	1.20	16.20	3.30	23.50	40.00	0.60	0.30	1.20	5.50	0.50	0.10
Xanthoma	0.80	7.60	7.60	47.80	17.40	0.40	1.10	3.50	4.30	0.50	0.60

Fig. 29.7 Lipids in placque based on data abstracted from Table 1 '*Lipids of human astherosclerotic placques and xanthomas; clues to the mechanism of plaque progression*' with very grateful thanks to the authors; Rapp et al. J. Lipid Res. 1983. 24:1329–1335 [121]

Given LA-oxidised products HODEs are the predominant oxidised lipids in plasma, and oxidised cholesterol is a significant plasma component, it is unsurprising 9- and 13-HODEs and 7-ketocholesterol are major oxidised components of vascular plaque [124, 125].

HODEs are abundant in vascular lesions, and 20 times higher in patients with atherosclerosis than controls [126]. The amount of 9- and 13-HODEs present also links closely with severity of plaque [127].

Oxidised cholesterol also comprises a significant component of plaque. Fibrous plaque contained 89 mg/g, and ulcerated plaque 335 mg/g, of sterol-ester oxylipins. The ratio of hydroxy LA to non-oxidised LA is a useful marker of oxidative stress in vascular lesions and has been found to correlate with their cholesterol content [128].

LDL via Receptors Sweep and Regulate Levels of Oxidised Product in the Blood

Phosphatidylcholine and the ALA and LA, it may be attached to, are very easily oxidised, allowing it as a significant component of chylomicron, VLDL and LDL shells, to act as a broom to sweep the blood of 'detritus' including staphylococcal and mycobacterial organisms; parasites; viruses; apoptotic cells; and AGEs [129].

The order of oxidisability of polyunsaturated fat in phosphatidylcholine interestingly was LA AA > DHA, again emphasising the physiological importance of LA. The location of phosphatidylcholine in the outer shell of LDL makes it easily accessible by oxidising factors as well as vascular receptors. Oxidised phosphatidylcholine, and its derivative lysophosphatidylcholine, as well as damaged carrier proteins, will deform the LDL membrane making it recognisable to oxidised LDL vascular uptake receptors, giving oxidised phosphatidylcholine and its derivatives particular functional relevance.

The consequent oxidised products of LA, ALA and phospholipids are highly bioactive and play essential roles including activation of LDL receptors, creating a feed forward mechanism stimulating vascular receptor-related oxidised LDL endocytosis, so uptake of oxidised and pathogenic material safely into cells for reprocessing, as well as induction of necessary related immune and repair pathways, which includes stimulation of denovo in situ lipid and cholesterol production.

LDL are often suggested to be well protected in the circulation by the extensive presence of antioxidants in plasma, but that would not fit with LDL being a sweeping mechanism, because the process of sweeping of the blood occurs in part through oxidation of LA, phosphatidylcholine, LDL-related protein and/or electrophilic attachment to LDL of oxidised 'detritus'. Prevention of oxidation of LDL would inhibit that sweeping process [130].

Consistent with its role as a sweeper, LDL unlike HDL does not contain mechanisms to break down or reduce oxidised products.

'*LDL is not a homogeneous entity but a group of particles that differs in density, size, electric charge and composition*' [131]. The change in electropositivity and negativity of LDL may factor in regulation of blood pH, as well as assisting sweeping of the blood of a mix of electropositive and negative bodies.

Omega 3 Fats and Vascular Plaque

In Inuit, despite high fat intake, levels of vascular disease were historically low, although recent papers report the existence of significant cardiovascular disease [132, 133], which may be due to Westernisation of even traditional diets [134, 135]. Inuit dietary fats were marine based, and so rich in omega 3s which formed 13 % of their fat intake, compared to 0.8 % in Danish food; monounsaturated fat intake was also much higher, 57 % compared to 35 % [136]. Northern Intuit mostly ate food raw [137], so it was subject to limited oxidation, and thus provided the benefits of the antioxidants in prey tissues.

Cardiolipin Damage

In normal circumstances, LA is the major component 'species' of mature cardiac cardiolipin. Cardiolipin is crucial to mitochondrial bioenergetic capacity and function, including having roles in Complexes I, III, IV, V of the electron chain, and apoptosis. Cardiolipin can contain four LA, so is very susceptible to oxidative stress. Oxidative damage to cardiolipin LA species is a contributory factor in cardiovascular disease.

DHA is also a significant but lesser constituent of cardiolipin, the quantity present reflects dietary DHA intake. Like LA, it will be oxidised in situ. DHA is a competitive substrate for LOX12/15. Little appears to be known about the LOX12/15 products of DHA, which may have roles in controlling apoptosis [138].

ALA is not found in cardiolipin species to any significant extent, so not available in cardiolipin as a substrate for oxidation to downstream messengers, consequently ALA will not have as much influence on mitochondrial function as LA and DHA. ALA may be present in other mitochondrial phospholipids.

LA is the predominant cardiolipin species, and 13-HODE is a predominant oxylipin in cardiac tissue [139]. 9- and 13-HODE, 4-HNE and MDA are factors in apoptosis, and

mitochondrial oxidative cardiolipin-related energy production inhibition. They are generated by oxidation of mitochondrial cardiolipin, through LOX12/15 activity, hydroxyl radicals generated from peroxide, and the action of the cytochrome C enzyme. These oxylipins are soluble, can travel, and on mitochondrial death will be released into the aqueous compartment, leading to oxidative cascades.

A streptozotocin diabetic induced rat, on a high-LA diet (but not on normal chow), so largely exclusively reliant on the beta-oxidation of LA for energy, in an oxidative environment including of high NO via iNOS activation, and lipid peroxides (an effect of streptozotocin [140, 141]), so at risk of high 13-HODE and PPAR gamma activation, and oxidation of LA species in cardiolipin, suffered substantial loss of cardiolipin and very significant damage and shrinkage of mitochondria [142] (see Fig. 29.8).

Reduced mitochondrial energy output factors in many conditions include Parkinson's disease; metabolic disorders including diabetes and obesity; recurrent pregnancy loss and male sterility; heart failure; and diabetic cardiomyopathy [143, 144].

HDL: Antioxidant Transport for Oxidised Phospholipids and Cholesterol

HDL is a transport system from the vascular membranes and potentially both to and from adipose tissue, of excess damaged or oxidised; cholesterol, cholesterol esters, lipids and phospholipids, including phosphatidylcholine and the choline it contains; for transport to the liver for recycling or incorporation into bile and excretion [145]. HDL

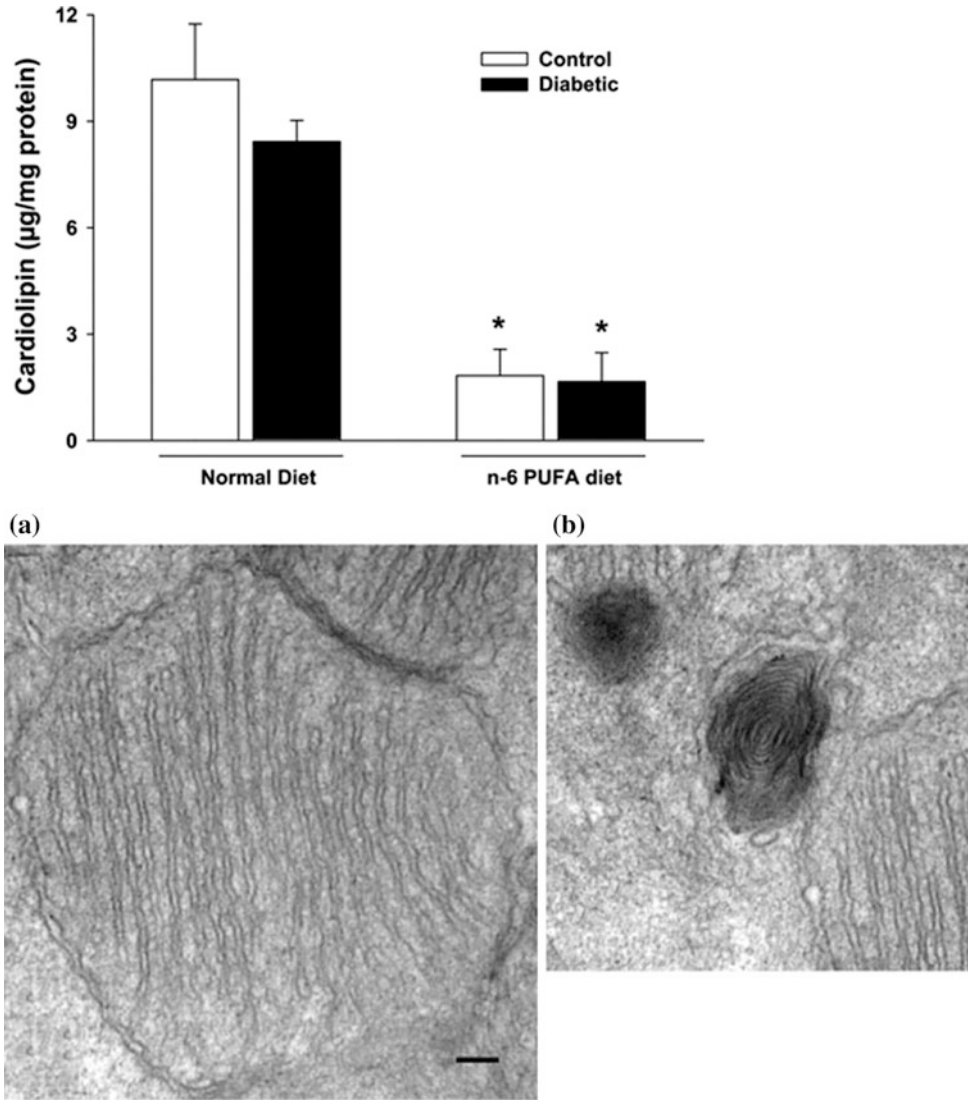


Fig. 29.8 '*Biochemical and ultrastructural changes in cardiac mitochondria after PUFA feeding and diabetes*'. Mitochondrial damage in Wistar rats fed a high omega 6 linoleic acid diet, with induced STZ diabetes and likely significantly raised oxidative stress, compared to controls fed the same diet but with no induction of diabetes. Substantial disruption to glutathione function is also observed, as well as loss of cardiolipin and lower ATP generation. From '*Brief episode of STZ-induced hyperglycaemia produces cardiac abnormalities in rats fed a diet rich in n-6 PUFA*' (Fig. 29.5) with very grateful thanks for permission to use the illustration to the authors: Ghosh et al. Am J Physiol Heart Circ Physiol. 2004 Dec;287(6): H2518–27 [142]

accumulates oxidised products in the blood stream including by transfer from LDL and promotes efflux of 7-keto-cholesterol from inflamed vascular sites [146].

Consistent with this role, HDL contains oxidised cholesterol esters, phospholipids, and apolipoproteins; some of which were abstracted from the vascular membrane and some of which were oxidised enroute. Interestingly, increased levels of lysophosphatidylcholine in HDL appear to increase its ability to abstract cholesterol from the intima of the vascular walls. The oxidised contents of HDL are increased by plasma oxidants in vitro and vivo.

HDL Unlike LDL Contains Antioxidant Enzymes Including Glutathione and Paraoxonase

HDL being oxidised in preference to LDL acts as a sacrificial antioxidant system. HDL in contrast to LDL contains the antioxidant enzyme glutathione, as well as paraoxonase PON1 and PON3 enzymes produced by the liver and secreted into HDL, which degrade oxidised cholesterol esters and phospholipids [147], reducing endothelial cell oxidative stress and related inflammatory and immune activation [148]. Impaired paraoxonase status has been associated with a number of conditions including diabetes.

HDL in Extreme Oxidative Scenarios May Lose Its Antioxidant Capacity and Become Proatherogenic

However during vascular stress, such as seen postsurgery, or in advanced vascular conditions, HDL may lose its antioxidant capacity becoming proatherogenic. Riwanto and Landmesser based on data from a study by Morgantini et al. [149] (Fig. 29.9) postulated that '*oxidised fatty acids (5-HETE, 9-HETE, 12-HETE, 15-HETE, 9-HODE, and 13-HODE) in HDL isolated from the type 2 diabetic patients may account for the impaired antioxidant properties of the lipoprotein* (HDL)'.

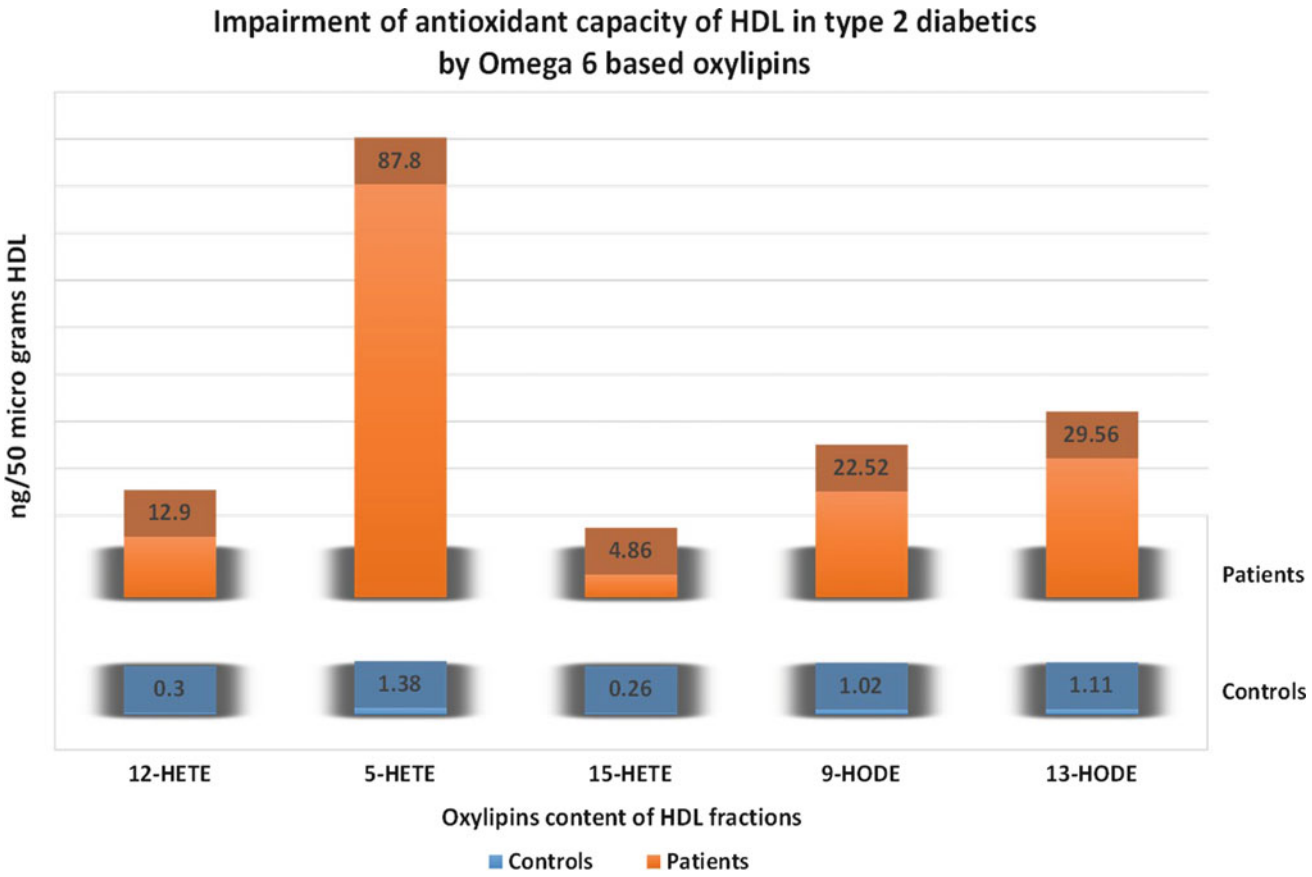


Fig. 29.9 The relative content of omega 6-based oxylipins in HDL in controls (No. 8) compared to type 2 diabetics (No. 11), subsets of larger groups. In the wider the patient group, the total cholesterol, LDL, HDL, and triglycerides are 182, 109, 46, and 171 (mg/dl), respectively, compared to 201, 128, 50 and 109 (mg/dl) in controls. CRP increased from 1 (mg/L) in controls to 9 (mg/L) in patients. The high level of AA-based product 5-HETE is interesting. The graphed data are abstracted from Tables 2 and 3 of '*Anti-inflammatory and antioxidant properties of HDLs are impaired in type 2 diabetes*'. with very grateful thanks to the authors: Morgantini et al. Diabetes. 2011 Oct;60 (10):2617–23 [149]

They also observed that the inflammatory HDL index was much higher in diabetic patients (0.65 v 1.45 approx.), that cholesterol efflux was impaired and that inflammatory HDL profiles were linked to intima media thickening and inflammatory plaque size. Interestingly total, LDL and HDL cholesterol were lower in patients than controls.

Morgantini observed that oxidised HDL loses their capacity to assist cholesterol efflux and that HDL may change from becoming anti-inflammatory to being pro-inflammatory. This would seem to suggest that high HDL in patients with cardiovascular disease cannot be automatically assumed to be a desirable marker, and indeed HDL when heavily oxidised may become pro- rather than anti-inflammatory.

Populations with High-Saturated Fat Intake

Animal fats, often commonly referred to as 'saturated fats', for a long time have been held responsible for cardiovascular disease. However within normal parameters, and as discussed below, 'pure' saturated fats in LDL, of either dietary or endogenous origin, are unlikely to be significant factors in cardiovascular disease. However, oxidised LA, cholesterol and lipid antioxidants in animal fat products may in excess have contributed to cardiovascular disease.

Saturated fats, whilst activators of genes, important fuel substrates, and structural membrane components, are relatively inert. In contrast, polyunsaturated fats including LA and ALA, and their respective elongation products are relatively easily oxidised, forming extensive arrays of far-reaching bioactive oxylipins with wide-ranging impacts, including on peroxisomal pathways.

In those on diets with no 'Western' influence, including those high in saturated fats, plaque may have been present [150], but as in Masai and other peoples free of 'Western dietary' influence, did not progress to vascular disease [151–154].

The absence of cardiovascular disease in groups with traditional diets high in saturated fats and low in polyunsaturated fats, and within normal parameters, absence of obvious pathways by which saturated fats could cause extensive cardiovascular dysfunction, throws into question the perceived role of saturated fats, as against highly oxidised LA-rich animal fats, in cardiovascular disease.

Coconut Fat—A Dietary Staple, Highly Saturated and Significant Source of MCTs

In 1978, the UN Demographic Yearbook records Sri Lanka as having the lowest mortality from heart disease; at that time, their predominant dietary fat was saturated coconut fat [155]. Kitavans Tokelauans and Pukapuka also had high coconut fat intake up to 64 % of calories, but vascular disease was pre-Westernisation uncommon [156].

Coconut fat has a unique lipid composition. In addition to ~10 % PA, coconut fat contains significant amounts of medium chain saturated fats (MCTs) including C:12 lauric acid ~50 %, as well as C:8 and C:10 ~ 20 %, which fats do not need carnitine to enter the mitochondria in some but not all tissues, and are better mitochondrial fuel than longer saturated fats, including in the heart and brain [157].

Saturated Fat and Cardiovascular Disease?

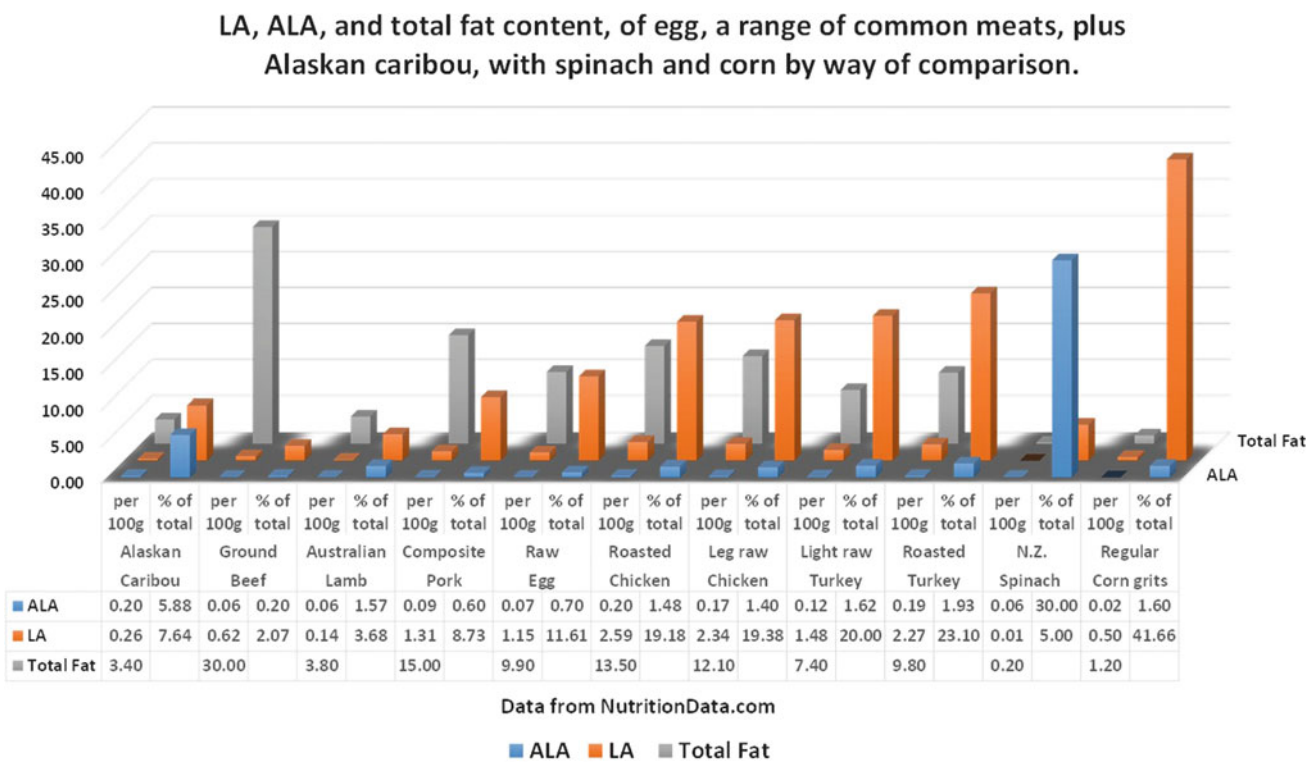
The assertion that oxidised LA-rich 'saturated' animal fats derived from industrially produced grain-fed pigs and chickens raised indoors, or hydrogenation or transesterification of vegetable oils, once processed so further oxidised, and consumed in the context of a Western diet, may increase cardiovascular disease more than relatively lightly oxidised processed vegetable oils is probably tenable.

In diet trials examining the effects of saturated fats, the words 'saturated fat' have come to be commonly used as a descriptor for animal fats or 'solidified' hydrogenated or interesterified vegetable oils; the reality is that industrially raised non-ruminant animal fats and solidified vegetable oils are not pure saturated fats, but contain significant amounts of polyunsaturated fats, as well as cholesterols and/or plant sterols, which along with their oxidisable lipid-soluble antioxidant coproducts are likely at least partially oxidised in processing.

High-fat (60 % cals.) rodent diet D12492 first produced in 1999 uses a mix of mainly lard with a small amount of soy oil, and over 10,000 kg is sold per month [158]. Lard may contain 10 % and more LA, potentially oxylipins and other oxidised product. D12492 contains 2205 kcal% lard, 225 kcal% soybean oil, 275 kcal% sucrose and 500 kcal% maltodextrin as a proportion of total 4057 kcal%.

Fat of grain-fed swine, poultry and non-ruminants can contain 10 and 25 %, and more, respectively, of LA (see Fig. 29.10). These amounts are well above the LA levels found in wild livestock, which vary seasonally between approximately 1–4 %. Additionally, the net amount of fat tissue compared to muscle in modern livestock is hugely increased over that seen in their wild equivalents; so multiplying the effect of an excess of LA in total quantity terms.

Ruminant monogastrics and non-ruminants have different lipid ratios, which are further altered by grass or grain feeding [159] (see Fig. 29.11). There were also significant differences in the fat-soluble antioxidant tissue contents between grain- and grass-fed animals. Beta-carotene levels in 3 studies for grass-fed steers were 0.74, 0.45, 0.16, and for the grain-fed animals 0.17, 0.06 and 0.01 μg/g of tissue,



	per 100g	% of total	per 100g	% of total	per 100g	% of total	per 100g	% of total	per 100g	% of total	per 100g	% of total	per 100g	% of total	per 100g	% of total	per 100g	% of total	per 100g	% of total	per 100g	% of total
	Alaskan Caribou		Ground Beef		Australian Lamb		Composite Pork		Raw Egg		Roasted Chicken		Leg raw Chicken		Light raw Turkey		Roasted Turkey		N.Z. Spinach		Regular Corn grits	
ALA	0.20	5.88	0.06	0.20	0.06	1.57	0.09	0.60	0.07	0.70	0.20	1.48	0.17	1.40	0.12	1.62	0.19	1.93	0.06	30.00	0.02	1.60
LA	0.26	7.64	0.62	2.07	0.14	3.68	1.31	8.73	1.15	11.61	2.59	19.18	2.34	19.38	1.48	20.00	2.27	23.10	0.01	5.00	0.50	41.66
Total Fat	3.40		30.00		3.80		15.00		9.90		13.50		12.10		7.40		9.80		0.20		1.20	

Fig. 29.10 Weight and ratio of total fat, LA and ALA, in eggs and meats (grms. per 100 g). The amounts of LA and ALA are also expressed as a percentage of total fat to highlight the relative as well as absolute indicative amounts of LA and ALA in common foods. Spinach and corn grits are included to raise the issue of the comparative total and relative quantities of LA and ALA in plant-based foods. The data are abstracted from and with very grateful thanks to Nutrition Data.com

respectively. Alpha-tocopherol in grain-fed beef was 0.75–2.92, and in pasture fed 2.1–7.73 μg/g, respectively. Grass-fed animals also were higher in glutathione SOD and catalase (Daley Tables 3, 4).

The LA, cholesterol, and antioxidants in 'saturated' animal fat-rich meat are very prone to oxidation during industrial processing including fat extraction and clarification; mechanical meat recovery; mincing; grinding; mixing with marrow bone and air; mixing with other easily oxidised proteins and sugars, biocide usage including bleach; frying; and roasting etc.

The risk of oxidation and wider cellular stress due to processing will be increased in meat from industrially produced livestock, due to greater biocide usage in processing, because they get; little exercise so are subject to increased oxidative stress, little or no sunshine so have limited or no opportunity to make vitamin D, and limited dietary green plant-based antioxidants such as polyphenols.

The production of 'saturated' trans and or interesterified fat products including from LA-rich vegetable oils has significantly increased. Increased use of such fats, in addition to industrially grown LA-rich poultry intake, together with an increasingly processed pre-oxidised diet, lack of nutrients including vitamin D, may help explain the very high and growing incidence of obesity diabetes and related conditions including cardiovascular disease [160], in a number of Middle Eastern countries.

Monosaturated Fats and Cardiovascular Disease

Antioxidant factors in unrefined oils high in monosaturated lipids, such as olive oil, may factor in their reported cardioprotective effects. Interestingly, a two-week crossover study in a closed environment, with prepared food, looking at the effect of different lipids in the diet on oxidisability of LDL, found that LDL oxidisability was lowest in the olive oil group, intermediate in the rapeseed group, and highest in the sunflower oil group [161].

Clearly if the test olive oil was relatively unrefined compared to the rapeseed and sunflower oils, the polyphenol and phospholipid content of the olive oil, as well as the displacement of LA content by OA, could have contributed to the reduction in LDL oxidation; in contrast, the higher level of omega 6 and level of processing in rapeseed oil may

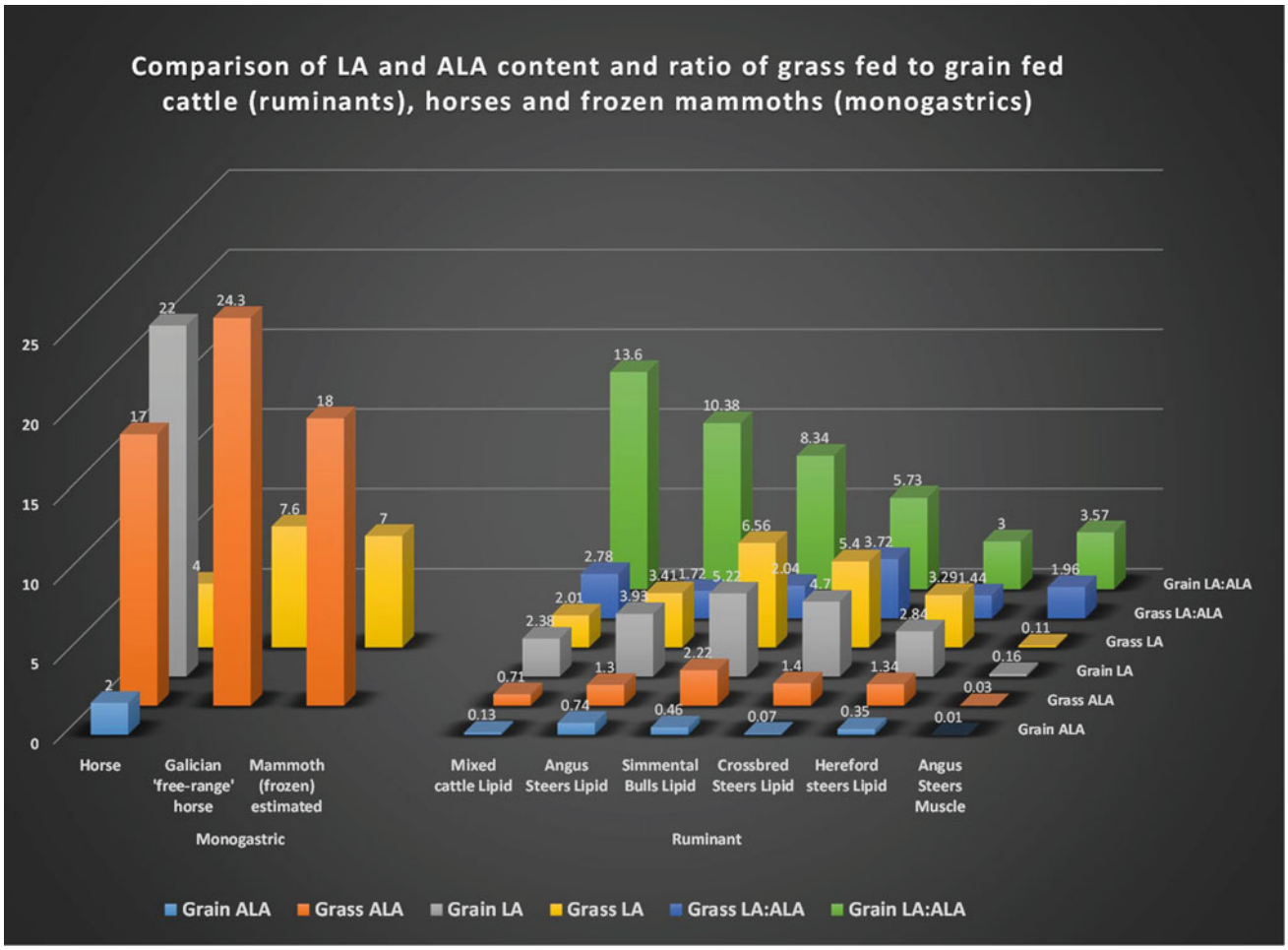


Fig. 29.11 A graph indicating the consequential changes in LA:ALA lipid profile of livestock due to grain- v grass-based feeds, domestication, and the lipid profile differences between ruminants and monogastrics using data abstracted from Table 2 of '*A review of fatty acid profiles and antioxidant content in grass-fed and grain-fed beef*' with very grateful thanks to the authors: Daley et al. Nutr J. 2010 Mar 10;9:10. doi:10.1186/1475-2891-9-10 [159]

have negated some of the likely expected omega 3 benefits. Olive oil only contains limited ALA.

Cardiac Protective Effects of Unrefined Cold Pressed Oils

As discussed, unrefined oils with moderate LA content and high in antioxidant factors may be protective against vascular disease [162, 163]. Antioxidant factors in red palm oil were protective in atherogenic rabbits [164], but refined palm oil was significantly more atherogenic [165]. Reheated palm oil increased vascular damage in rats [166]. This is consistent with palm oil containing approximately 10 % LA, which will be oxidised in refining and subsequent reheating.

The results emphasise the importance of wider dietary antioxidants and food oxidative status including of vegetable oils in setting the oxidative stress tone of the blood.

The Importance of Plant-Based Fat-Soluble Antioxidants

The effect of plant antioxidants logically starts in the gut, continues during absorption and transport by chylomicrons, and as components of LDL. When dietary and/or endogenously produced antioxidant factors capable of reducing oxylipins and the hydroperoxides are missing, such as CoQ [10, 167] glutathione, and lipid-soluble plant-based phyto-antioxidants, plasma and LDL will be more susceptible to oxidation.

Plant fat-soluble antioxidants such as vitamin E, beta-carotene, lutein and zeaxanthin, when present, reduce LDL oxidation, by competing for the LOX12/15 enzyme reduce LA oxylipins, emphasising their dietary importance [168]. Plant antioxidants will be substantially lacking in highly processed foods and may be reduced in crops grown in low UV glass houses.

Low-Fat Diets, Low in Antioxidants, Containing Oils with Pre-oxidised LA Content

Those on low-fat 'healthy' diets, eating for example, industrial grain-fed LA-rich, antioxidant and nutrient-diminished chicken, and hydroponically grown salads, potentially low in nutrients including antioxidants, dressed with LA-rich vegetable oils, and possibly a few crisps as treats, due to nutrient insufficiencies and peroxidation of food including LA may actually be at greater risk of cardiovascular disease and wider Western diseases than somebody who is not dieting, and at face value eating a less 'healthy' but more diverse diet containing 'saturated' fats.

ALA and Vascular Disease

As discussed it is clear that PPAR alpha pathways are important to energy production in the heart [169] and strongly expressed in cardiac tissue [170]. ALA intake is generally associated with reduced cardiovascular risk, including with lower carotid vascular plaque [171]. '*Nine major studies have reported that ALA levels are inversely correlated with primary cardiovascular events*' [172].

Peroxisomes prefer ALA as substrate. Energy deficit and to a lesser extent ALA is associated with PPAR alpha activation [173] and so production of energy substrates for mitochondria and improved antioxidant status.

A greater ALA to LA ratio will; increase the ALA content of membrane phospholipids, through desaturation will increase EPA and may increase DHA availability, will alter membrane fluidity so function, and influence downstream COX and LOX12/15 oxylipin products, so alter inflammatory profile; and will increase PPAR alpha activity so diversion of lipid resources to energy pathways and away from PPAR gamma related maintenance pathways. ALA is the preferred substrate of LOX 12/15 enzymes and ALA oxylipins are likely less 'inflammatory' than LA LOX12/15 oxylipins, and further in a murine model, LA and ALA are preferred COX substrates compared to EPA [174], which also raises interesting questions as to the effects of excess LA and related hydroperoxides (Lands p. 140), and lack of ALA and EPA on the production of COX oxylipin products.

Population Studies: LA Intake, LDL Plasma Levels, Oxidative Stress and Vascular Disease

Recent re-analysis of the Minnesota Coronary Experiment [175] and Sydney Diet Heart Studies [176] suggests that the 'diet-heart hypothesis', which held that saturated fat promotes cardiovascular disease and LA reduces it, was not in retrospect borne out by the then-observational evidence.

The diet-heart hypothesis in part arose out of promulgation of observations of those who worked in Africa including D P Burkitt FRS (Birkitts' Lymphoma), and H C Trowell OBE MD FRCP authors of '*Western Diseases; their emergence and prevention'* [177] that groups that had not adopted 'Western' refined foods were generally free of cardiovascular disease and generally ate diets with moderate to low-fat content and high plant fibre content.

A perception that those in the West who ate higher amounts of animal fat had higher coronary disease, together with a view that high cholesterol was associated with high fat intake, and equated to cardiovascular disease and consequent mortality risk, lead to dominance of the diet-heart health hypothesis proposed by Keys. Animal fat consumption became equated to saturated fat consumption.

The short 1957 book '*Fat Consumption and Coronary Disease, an Evolutionary Answer To This Problem*' by Cleave [178] who also in 1974 wrote the 'Saccharine Disease' [179] usefully illustrates the then-thinking, debates, state of knowledge and general consensus that higher 'Western' fat intake (perceived as mainly animal fat intake, although vegetable oil intake and related solidified product intake was even then long standing and growing) was primarily responsible for cardiovascular disease.

Knowledge of the biochemistry of polyunsaturated fats in the 1950s was truly in its infancy. In 1956, Dr. Hugh Sinclair had proposed that 'Western' degenerative diseases were caused by lack of 'essential' fats; he had observed consumption of EFAs by Western society was less than that by those on non-Westernised diets [180]. The nuances of omega 3s and 6s and Sinclair's prescient concerns as to the potential negative health impact of highly processed vegetable oils got lost in translation.

It was from these historical perspectives that the advice eat less animal 'saturated' fat and more polyunsaturated fat emerged. The change in the make-up of non-ruminant animal fats with industrialisation, and vast increases in processed food consumption was not foreseen.

A great deal of evidence and underlying biology has emerged since the hypothesis was made that now points to excess oxidised LDL and omega 6-related products and pathways, including LOX12/15, HODEs, MDA [181], 4-HNE [182], CD36, OLR1, and PPAR gamma, being powerful agents in inducing vascular and cardiac damage. The contention that pure saturated fat causes, and increased dietary LA lowers, the risk of cardiovascular disease is now arguably weak, and strongly contested [183].

'High-Fat' Preindustrialised Diets Are not Comparable to Modern 'High-Fat' Diets

Groups such as Inuit (marine fats largely uncooked), Masai (Dairy fat), Nunamuit (Caribou) who ate higher fat diets are limited in number. Wild animals have lower total fat, higher antioxidant content, better omega 3; 6 balance and very much lower omega 6 LA intake, so their 'wild' 'high-fat' 'low-carb' 'paleo' diets are not in any way comparable in terms of total fat intake, fat lipid polyunsaturated fat content and ratios, antioxidant capacity, or overall nutrient content, to a modern high-fat diet.

Hypertension and Cardiovascular Disease Were Unknown to Those on Traditional Pre-industrialised Diets

The biology of vascular and cardiac disease in hugely complex, but simplistically and of fundamental importance, hypertension and cardiovascular disease were virtually unknown to those on traditional pre-industrialised diets, and much less common around 1900 in the UK. In the 1900s–1930s, non-Westernised populations eating traditional foods with no refined Western components were observed and reported by trained doctors to be virtually free of cardiovascular disease; in the words of Sinclair '*it was strikingly rare*' (p. 14); and Donnison of atheromas based on autopsies said '*Medial degeneration of marked deg LA oxylipin promoted ree I LA oxylipin promoted have never seen*' (p. 18) [145, 177, 178].

Primary Causes of Cardiovascular Disease—Summary

The primary cause of cardiovascular disease is arguably not pure saturated fats per se, but raised plasma oxidative stress including in LDL and damage to cardiolipin species, due to excess LA oxylipins of dietary and endogenous origin, consequent overactivation of the AA COX pathways as well as the oxidised LDL receptors CD 36 and OLR1 and PPAR gamma pathways, so excess inflammation and immune activation including through iNOS and peroxisomal peroxide-related oxidative stress; as well as LA oxylipin promoted PPAR gamma activation and diversion by PPAR gamma activation of lipid resources to maintenance and substrate rather than energy, hence related lipid and cholesterol accumulation, combined with lack of ALA as a competitive oxylipin substrate and PPAR alpha activator, as well as lack of PPAR alpha activation through exercise or fasting resulting in mitochondrial energy deficit, and consequent damage to tissue as well as mitochondria, cardiac cell damage, vascular damage, and resultant malfunction; all facilitated by pre-oxidised nutrient and antioxidant depleted, AGE and cross-linked protein product-rich, LA ALA-imbalanced, LA oxylipin rich, 'Western diets'.

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30 Linoleic Acid and Alpha-Linolenic Acid Have Central Roles in Brain Energy Substrate Provision, Endogenous Lipid Production, Immune and Repair Function, via Peroxisomal Beta-Oxidation-Related Pathways?

Robert Andrew Brown

Terms	
AA	Arachidonic acid (Omega-6; 20 carbon derivative of LA.)
ACoA	Acetyl coenzyme A (Raw material for the energy/cholesterol pathways.)
ALA	Alpha-linolenic acid (Omega-3–18 carbon plant-based polyunsaturated fat.)
BBB	Blood–brain barrier (Barrier between blood stream and brain.)
CD36	Cluster of differentiation 36 (Fatty acid translocase receptor.)
COX	Cyclooxygenase (Enzyme-catalysing oxidation of fatty acids.)
CPT1	Carnitine palmitoyltransferase (Acts as shuttle mainly for long-chain fats C:16–18 into mitochondria.)
DHA	Docosahexaenoic acid (Omega-3–22 carbon derivative of ALA.)
EPA	Eicosapentaenoic Acid (Omega-3 fatty acid C20:5.)
HMGCoA	3-hydroxy-3-methyl-glutaryl-CoA (Found in two forms, reductase and synthase. Reductase regulates cholesterol production. Synthase regulates HMGCoA production. HMGCoA is substrate for ketones or cholesterol.)
iNOS	Inducible nitric oxide synthase (Inducible isoform involved in stress response in macrophages, microglia and other tissues.)
LA	Linoleic acid (Omega-6–18 carbon plant-based polyunsaturated fat.)
LOX12/15	Lipoxygenases (Enzymes-catalysing oxidation of multiple lipid-based substrates.)
MCAD	Medium-chain acyl-coenzyme A (Dehydrogenation of fats C:6–12 in mitochondria and present in inner mitochondrial membrane.)
MDA	Malonaldehyde (Non-exclusive oxidation product of Omega-6.)
MCT	Medium-chain triglyceride (Triglyceride containing fats between C:6 and C:12.)
MCF	Medium-chain fat (A fat between C:6 and C:12)
NO	Nitric oxide (An important signalling messenger and oxidant.)
OLR1	Oxidised LDL receptor 1 (Receptor for oxidised LDL sometimes called LOX1.)
PA	Palmitic acid (Saturated fat C:16.)
PPAR	Peroxisome proliferator-activated receptor (3 forms alpha, gamma and delta.)
SA	Stearic acid (Saturated fat C:18.)
SCD1	Stearoyl-CoA desaturase (Delta-9-desaturase key to the formation of OA.)
SOD	Superoxide dismutase (Reduces superoxide to oxygen or peroxide.)
Wy14643	PPAR alpha activator (Activates PPAR alpha-related peroxisomes.)
4HNE	4-hydroxynonenal (Exclusive Omega-6 fats peroxidation aldehyde.)
4HHE	4-hydroxy hexenal (Exclusive Omega-3 fats peroxidation aldehyde.)
4HPNE	4-hydroperoxy 2-nonenal (Oxidation product of Omega-6 LA and likely AA.)

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13HODE 13-hydroxy-9Z, 11E-octadecadienoic acid (Major LA oxidation product of LOX12/15, COX photo-oxidation and autoxidation.)
13HOTE 13-hydroxy-9Z, 11E, 15Z-octadecatrienoic acid (Major ALA oxidation equivalent of LA product 13HODE.)

Linoleic Acid (LA) and Alpha-Linolenic Acid (ALA) Multiple Roles in the Brain Including as Potential Energy Substrates

Brain lipid research primarily focuses on AA and DHA, but ALA and LA, as preferred peroxisomal beta-oxidation substrates, and to a lesser extent PA and OA, likely have underappreciated but fundamental roles in the brain as the primary substrates for peroxisomal beta-oxidation, so indirect sources of:

- mitochondrial "fuels" (MCFs, ACoA and derivatives) from which to make ATP;
- substrate (ACoA) for "endogenous" lipid manufacture within the BBB;
- peroxide as a signalling agent and basis of oxidative stress;
- promotion of catalase production through peroxisomal induction;

and LA and ALA also have more specialist roles in the brain including the following:

- diet-dependent LA presence in cardiolipin modulates brain energetics;
- as the preferred substrates for LOX12/15 when released into damaged brain tissue;
- LA-/ALA-oxidised products in injured brain tissue moderate immune function;
- LA HODEs activate PPAR gamma which impacts on microglial function;
- LA oxylipin promoted iNOS-based NO production inhibits catalase assisting microglial oxidative including phagocytic function and/or promoting wider oxidative stress and related signalling.

Current Metabolic View as to Brain Energy Sources

Current metabolic considerations of "fuel" sources for brain energy, and substrate creation, generally focus on glucose and externally derived ketones. However, the healthy existence of Inuit, with a common Inuit CPT1A carnitine polymorphism that substantially inhibits mitochondrial uptake of long fats, who apparently were not in ketosis, and had little access to glucose, suggests that humans may have the capacity to fuel their brain very largely from the only other clear potential option, peroxisomally produced MCFs, ACoA and derivatives.

The health, and ability to build and run a brain, of neonates nourished with breast milk, which is high in lipids and low in carbohydrates, whom are very rarely in significant measured ketosis, adds to evidence that the brain is able to metabolise fats as a major energy source.

ALA and LA Presence in Structural Brain Lipids

In the brain, during normal function, LA and ALA overall are only present in structural brain lipids in the limited amounts; based on a porcine model, possibly 1 % LA and 0.2 % ALA of lipids overall; the phospholipids fraction is likely to be diet and development stage sensitive [1] and, in mature brains, may contain more LA and ALA, approximately double for phosphatidylcholine, further there is significantly more LA (but not ALA) in cardiolipin possibly 10–15 %. Cerebrospinal fluid interestingly also contained higher levels of LA 7 % and ALA 2.5 % [2].

However, during brain damage or ischaemia, LA and ALA levels in tissues may rise, possibly due to less efficient peroxisomal beta-oxidation of them, and/or increased BBB uptake, giving them wider roles as oxylipin-signalling systems, including for peroxisomal related oxidative forward-feeding oxidative cascades, in both the areas of immediate damage, and through their ability to travel and signal in more distant tissue.

LA, ALA, PA, OA and POA Uptake and Usage by the Brain

Radioactive tracer experiments confirm ALA [3], LA, OA [4], EPA [5] and palmitate [6–8] are imported intact by the brain, but limited amounts are known about their uptake. The uptake of ALA, LA, PA and OA by the brain is likely impacted by the following:

- the proportions of LA and ALA in the diet so plasma content,
- the lipid carriers to which they are attached and the brains' preference for them,
- the metabolic energy status of the brain and wider body.

Factors regulating the metabolic energy status and substrate requirements of the brain include; genetic

polymorphisms, physical and brain activity levels, wider body energy status including glucose availability fasting and exercise, and impairments in glucose metabolism as seen in metabolic syndrome for example, brain uptake of labelled palmitate was 86 % higher in metabolic syndrome subjects compared to controls [9].

ALA followed by LA, and to significantly lesser extents PA and OA, is the preferred peroxisomal beta-oxidation substrate. Brain uptake amounts and ratios of, ALA, LA, PA, POA and OA will ultimately depend on plasma availability, including lipoprotein content. Synergistically, and if available, ALA will be preferentially taken up through the BBB by the brain compared to LA and in preference to PA and POA [10]. However, total lipid take and mix across the BBB will also depend in part on the plasma lipid status, which will reflect the dietary status.

Perfusion experiments [11] and use of labelled fats [12] suggest uptake mechanisms include a mix of; passive transport; absorption following lipid lipase release from chylomicrons [13] and albumin; activation of transporters, and through endocyted LDL via receptors such as CD36 [14]. Oxidised LDL is also taken up by the brain through oxidised LDL receptors CD36 [15, 16] and OLR1 [17]. Much is still unknown.

The preference of the blood–brain barrier, including the astrocyte "feet" that synergistically contains peroxisomes and forms an integral part of the BBB, for different lipid delivery systems including LDL, will help determine which lipids are taken up, because different delivery systems contain different lipid mixes, and uptake selection takes different forms, including lipolysis and endocytosis.

Interestingly those with hypobetalipoproteinemia, so inability to normally form LDL are at risk of significant neurological impairment [18]. LDL in normal circumstances carries mainly LA, OA, PA and POA, interestingly the main preferred peroxisome beta-oxidation "fuels", as well as important lipid-related antioxidants. LDL CD36 receptors have been identified in the brain for example in the cortex post traumatic brain injury. However it is currently generally thought that structural brain cholesterol is made endogenously in the brain. If LDL were taken up by receptors intact by astrocyte 'feet' or disassembled there, the question of what happens to the cholesterol delivered in LDL would remain; and if LDL is not taken up through astrocyte feet what is/are the plasma sources of ALA, LA, and other lipids likely taken up by the brain. The fact LDL is a primary transporter of, substrates that are important to peroxisomes at least LA, OA and PA, and of crucial brain relevant fat soluble antioxidants, combined with the neurological impact of hypobetalipoprotemia, the conundrum how CPT1 altered Inuit and babies fuel their brain, the origin of cerebral arterial atheroma and role of oxidised LDL in their formation, and puzzle why the brain takes the trouble to make a resource it potentially has pentiful access to, add to the unanswered questions. Determination that the brain does endocyte LDL and cholesterol would help explain communalities between neurological and wider health conditions such as vascular disease diabetes and Alzheimer's. Further "fresh" chylomicrons may be an important transport mechanism for delivery of fresh dietary ALA to the brain.[1,2]

In animal studies, a '*substantial proportion*' of labelled dietary ALA ends up in the brain as monosaturated fats, saturated fats and cholesterol; it was not clear whether this was consequent on direct uptake and/or via beta-oxidation products being recycled in the liver and subsequent product uptake by the brain, but was likely due to endogenous conversion. High levels of LA beta-oxidation (likely peroxisomal) products were seen in male rats; 86 % of the LA brain uptake was beta-oxidised within 5 min, but under 1 % of LA, it was converted to AA [19].

Most ALA and LA that cross the BBB logically are **likely** peroxisomally beta-oxidised in astrocytes consistent with this they are not utilsed or found in the structure in significant amounts, and likely cannot be taken up by mitochondria due to CPT1 or malonyl-CoA blocking. Dietary PA that has crossed the BBB, consistent with it being less actively metabolised by peroxisomes, is partially peroxisomally metabolised and partially directed to structural incorporation. Significantly more palmitate than ALA and LA ends up as a structural lipid brain component, likely due to less efficient peroxisomal beta-oxidation of palmitate.

There is evidence that OA may cross the BBB. Some suggest that SA is taken up [20–22] and others do not. Further lipid uptake may be different in maturing and developed brains, and depend on plasma lipid availability and brain energy glucose requirement.

Selective brain tissues "outside" the BBB needing to sense signalling messengers in the blood stream, such as the pituitary and hypothalamus, fenestrated sections of brain tissue and the choroid plexus, may be better able to take up ALA and LA [23].

Astrocytes Are Numerous, Populate the Blood–Brain Barrier and Contain Peroxisomes

Once across the BBB, during normal non-trauma scenarios, ALA and LA are likely mainly directed into astrocytes, which have an overwhelming presence in the blood–brain barrier and contain peroxisomes, where logically, ALA and

[1] http://scholarcommons.usf.edu/cgi/viewcontent.cgi?article=6897&context=etd

[2] https://www.researchgate.net/publication/13999309_Brain_and_skeletal_muscle_bioenergetic_failure_in_familial_hypobetalipoproteinemia

LA as the primary- and secondary-preferred peroxisomal beta-oxidation substrates, are very largely peroxisomally beta-oxidised, including to short fats, and ACoA that can be used to form mitochondrial fuel substrates including acetate malate and ketones, and/or be directed to lipid and cholesterol creation, to support structural and maintenance requirements, depending whether PPAR alpha and delta, or PPAR gamma peroxisomal pathways are activated.

Peroxisomes (and in wider brain tissues microperoxisomes) are widespread in some brain cells, including in the oligodendrocytes [24] and astrocytes [25]. Mammalian astrocytes outnumber neurons by possibly 10:1, forming 25–50 % of total volume in some brain areas [26], and their structure results in a sheath over approximately 90 % or more of the BBB [27], which has a surface area of 15–25 m^2 [28].

Given the widespread nature of astrocytes, astrocytic peroxisomes are likely to have significant functional relevance. Glioma cells have been shown to have '*active lipogenic and cholesterologenic activities*' [29]. Malignant glioma significantly relied on beta-oxidation, expressed beta-oxidation related enzymes, and produced ACoA. All three PPAR peroxisomal-related pathways are expressed in astrocytes.[3]

ALA and LA, as the preferred substrates for peroxisomal beta-oxidation, are likely in large part peroxisomally beta-oxidised, and PA and OA partially beta-oxidised and partially directed to structural brain components:

- via PPAR alpha and delta peroxisomal beta-oxidation, by astrocytic peroxisomes to short fats and ACoA for use as energy to support astrocyte mitochondria directly, and neuronal mitochondria directly and/or indirectly, through the production of MCFs and ACoA, which opens up a wide range of alternate substrate possibilities for fuelling neurons, including ACoA and derivatives [30] such as, acetate [31] and malate [32], which pathways may also be of benefit during ischaemia, and genetic- or disease-based brain mitochondrial energy pathway malfunction.
- via PPAR gamma peroxisomal beta-oxidation, likely by astrocytic peroxisomes to short fats for; energy and ACoA as substrates; for lipids including cholesterol for brain tissue building and repair; and immune function; further and synergistically, peroxisomal pathways are essential to cholesterol synthesis containing the enzymes thiolase and mevalonate kinase, further PPAR gamma-related peroxisomal pathways activate genes related to lipid and cholesterol synthesis, including SCD1 desaturase activation, fatty acid synthase (FAS), sterol regulatory element-binding protein (SREBP-1) and HMGCoA reductase.
- Via peroxisomal beta-oxidation to by-product peroxide;
- Peroxisomal beta-oxidation will be accompanied by the activity of the catalase enzyme, which will be raised during PPAR alpha activation, and diminished during 'supra-homeostasis' PPAR gamma activation, but potentially increased during homeostasis.

The absolute amount of ALA, LA, OA, PA and other fats beta-oxidised in the brain will depend on a mix of BBB uptake preferences, lipid availability and mix of plasma lipid carriers.

Importance and Presence of Peroxisomes in the Brain

Peroxisomes are recognised as having key roles in brain function. The extent and importance of peroxisomes to brain and wider function are evidenced by the variety of significant issues in those with peroxisomal disorders such as Refsum's and Zellweger's.

Brain peroxisome malfunction issues include build-up of very long-chain fatty acids, loss of plasmalogens, inability to make DHA, excess oxidative stress, low catalase and oxylipin imbalances. Wider peroxisomal pathway lipid imbalances are also associated with a number of neurological and neurodegenerative conditions including Alzheimer's [33], which has been likened by some to a peroxisomal disorder.

Research into peroxisomal activity in brain energetics appears limited and, to date, only occasionally peripherally recognises, but does not focus on, the potential implications of peroxisomal dysfunction on brain energy pathways and as a provider of lipid and cholesterol substrate.

Brain Mitochondria Have Limited/Very Limited Ability to Beta-Oxidise Long-Chain Fats

Long-chain fats are largely blocked from entering neuronal mitochondria, and also likely astrocytic mitochondria, by a combination of low function of CPT1 enzymes [34, 35], and/or glucose-related ACoA Randle cycle malonyl-CoA blocking, on which basis it is often proposed the brain is primarily fuelled by glucose or ketones.

Three CPT1 isoforms are present in the brain, but CPT1 (c) is only found in neurons. Specialised metabolic-sensing neurons do appear to respond to some extent to long-chain fats, which ability is used as part of the appetite-control process. Neurons also intriguingly respond to lipoprotein receptor CD36 activity.

[3] http://neuro-oncology.oxfordjournals.org/content/early/2016/06/29/neuonc.now128.full.pdf+html

Astrocytes But Not Neurons Can Metabolise Medium-Chain Fats MCFs

Astrocytes have been observed to significantly beta-oxidise fatty acids [36]. Interestingly, it appears astrocytic mitochondria [37], but not the neuronal mitochondria [38], can metabolise MCFs including those produced by astrocytic peroxisomes.

It is not clear whether astrocytic beta-oxidation of long-chain fats takes place primarily in astrocytic mitochondria, or peroxisomes or both, or whether the ACoA from which ketones are produced was of astrocytic or mitochondrial origin, albeit it is likely the ketones were primarily made from ACoA produced by peroxisomes.

There is also debate as to whether neurons use glucose direct, or products of glucose, including lactate supplied by astrocytes: Peroxisomal ACoA and derivatives might also be capable of direct utilisation by neuronal mitochondria.

Protection of Long-Chain Fats in the Brain Structural Membrane from Beta-Oxidation

To protect the structural fats of the brain from use as beta-oxidation fuel substrates, it would make sense if astrocytic mitochondria, whilst able to metabolise MCFs, had limited CPT1A-related ability to metabolise long-chain structural brain fats. Eighteen carbon-saturated fats are poor peroxisomal beta-oxidation substrates, so will not be readily beta-oxidised by peroxisomes, hence if not metabolised by mitochondria due to malonyl-CoA and/or CPT1 restriction or by the peroxisomes in signifcant amount, the structural 18 carbon fats would be protected from significant beta-oxidation. Indeed the mix of; peroxisomal beta-oxidation preferences, lipid uptake rates across the BBB, and low CPT1 activity combined with Randle cycle related malonyl-CoA blocking of carnitine function may be signficant factors in the determination of the balance of structural lipids in the brain.

Due to lipid peroxisomal beta-oxidation preferences, ALA would be present in the structural brain lipids in lesser amounts than LA; palmitic and oleic acid and their desaturase derivatives would be present in greater but variable quantities; stearic acid would be present in greater amounts still; and very long-chain fatty acids that cannot be metabolised at all by mitochondria would build up in those with peroxisomal disorders, as seen in brain lipid analyses.

Are Ketones or MCFs and ACoA the Primary Alternative Brain Fuel to Glucose?

Ketones are generally suggested to be the primary brain fuel alternative to glucose; however, astrocytes from developing rat brain used fats even more efficiently than ketones [39]. Irrespective of debates as to whether the ketones suggested to fuel the brain originated in the liver or the brain itself, evidence suggests that at least during short term glucose fuel shortages MCFs and ACoA products including ketones, likely of peroxisomal brain endogenous origin, are a major fuel source for the brain:

- Neonates on breast milk, which is low in carbohydrates, only have mild ketone elevation, and only a fraction of a per cent were observed to be in significant ketosis.
- Inuit with a widespread Inuit CPT1 variant, traditional diets, so low-dietary glucose, not observed to be in ketosis, generally live healthy "ordinary" lives.
- Ketosis is unable to prevent neurological damage during hypoglycaemia in those with MCAD deficiency disorder.
- The ketogenic-fasting response is almost entirely blocked in PPAR alpha null mice, suggesting peroxisomes are the prime source of ketone substrate ACoA [40].

Further, oral administration of MCT oil, and/or coconut oil rich in shorter fats, improves memory and cognitive function in Alzheimer's patients and, in other conditions, hinting at the importance of short fats as brain fuels [41].

Endogenous in situ cellular ketones produced and utilised within brain astrocytes and wider body cells, might not show up on a traditional blood or urine-based assay of ketosis, but would likely effect acetone in the breath. There is no suggestion that neonates or CPT1A variant Inuit exhibit noticeable levels of acetone in breath, suggesting the ketone production even if taking place in situ is likely a minor component, of the fuel mix in CPT1A variant Inuit and breast-fed neonates. Further ketosis also takes signifcant time hours to days to fully develop, so as seen in MCAD deficiency cannot replace a dietary induced glucose deficit.

Interestingly, astrocytes do produce ketones from labelled PA, but not by "conventional" pathways [42], raising the possibility that they were, as suggested above, produced through the use of peroxisomal beta-oxidation product ACoA and related pathways, rather than through mitochondrial ACoA production pathways. Ketones derived from the liver may become a more important energy source during long term food deprivation such as famine, as against shorter term deficits.

Do Peroxisomal Pathway Substrates Fuel the Brain of Inuit with a CPT1A Variant?

In northern Inuit with a widespread (in excess of 80 % of 422 consecutive births) "broken" CPT1A polymorphism [43–45] resulting in very low 2 % CPT1A function, in combination with low-dietary availability of glucose, a lack of observed regular ketosis suggests that alternate fuel

sources to glucose and ketones must be available to fuel the brain, likely based on the peroxisomal beta-oxidation of polyunsaturates and a portion of the palmitic acid (and oleic acid to the extent that it crosses the BBB which is debated) that enters the brain, to MCFs, ACoA and related downstream products.

This common Inuit mutation only came to light by chance in 2001 in a patient with muscle cramps, vomiting and occasional loss of consciousness, as it generally has no significant health effects from the perspective of those with the variant. '*The adults homozygous for the P479L variant in this study mostly denied any ill health that could be attributed to CPT-I deficiency*'.

Their traditional diet '*consisted of 80–85 % fat, 15–20 % protein and, apart from a little muscle glycogen, almost no carbohydrate*'. These Inuit have '*a mutation that decreases beta-oxidation and ketogenesis*' at least via mitochondrial pathways.

CPT1 is active in the brain along with CPT1B and C [46]. CPT1A mutations are a recognised disorder; symptoms include energy-related musculature issues, but there is no common suggestion of related neurological consequences, pointing to a non-critical energy role for CPT1A, and so non-critical role for mitochondrial beta-oxidation of long-chain fats as a fuel for the brain.

Neural CPT1A in any event may have low functional transport capacity in the wider population [47], possibly due to malonyl-CoA inhibition consequent on ACoA derived from glucose and/or peroxisomal beta-oxidation, giving peroxisomal neural likely astrocytic MCF and ACoA substrate production pathways that fuel mitochondrial beta-oxidation bypassing CPT1A blockages great relevance.

Mitochondrial long-chain beta-oxidation is likely very limited (2 %) in CPT1A defective Inuit [44]. In the absence of; significant glucose; liver-based ketogenesis; or short fats in plasma, the only other option is, **in situ energy deficit or high-Omega-3 PPAR alpha activation-related peroxisomal beta-oxidation** of long fats, providing short fats, ACoA so malate and acetate, that is primarily directed by PPAR alpha-related gene subset activation to mitochondrial energy pathways, including through the related use of ACOA as raw material including for gluconeogenesis and ketones.

The traditional high-fat high-Omega-3 marine-related diet in these Inuit, where lean meat was traditionally given to their dogs, and the constant energy demands of a cold harsh active lifestyle, would preferentially activate PPAR alpha-related peroxisomal beta-oxidation pathways and synergistic energy production-related gene sets, rather than PPAR gamma-related repair and maintenance-related pathways.

The authors of the Inuit related paper remarked '*for this variant to attain such high prevalence in this population it must confer a significant evolutionary advantage*'. PPAR alpha activation would provide, thermogenesis, potentially greater ATP/oxygen consumption through catalase-based peroxide recycling ($2H_2O_2$ to $2H_2O + O_2$), and higher antioxidant catalase function. Increased thermogenesis would be of significant benefit to survival in a cold climate, and increased antioxidant production and/or reduced oxygen consumption could be of use in a number of ways. Increased peroxisomal activity would also provide a mechanism to down-regulate membrane and adipose tissue stores of DHA and related long-chain marine food based polyunsaturated fats, which are easily oxidised; storage of which would demand high antioxidant resources and in excess place cells at signficant risk of oxidative stress. Further DHA is too long to be metabolised by mitochondria even if CPT1 was fully functional.

Short fats in the brain can originate from peroxisomal oxidation within the brain, or externally from plasma, but whilst albumin does transport octenoate, it appears it is quickly taken up, and plasma levels in humans except in Rey's syndrome are normally low [48], further pointing to peroxisomal beta-oxidation of long-chain fats to shorter fats within the brain itself as their primary source. Further and interestingly high levels of short-chain fats in Rey's can lead to seizures and other symptoms [49].

In conclusion, the healthy existence of non-functional CPT1A Inuit, who are likely not generally in ketosis [50], strongly supports the idea, brain astrocytes, the predominant site of brain beta-oxidation [51], can through peroxisomes beta-oxidise long-chain fats to substrates that do not require CPT1A for mitochondrial access. These include short fats and ACoA; so acetate, malate and potentially ketones and even glucose, substrates that likely fuel the glial [52] and/or neuronal [53] mitochondria, as well as providing brain endogenous substrate for the production of structural c-holesterol, saturated and Omega-9 fats, through a mix of PPAR alpha gamma and delta activation of peroxisomes.

Potential Use of Peroxisomal Pathways to Fuel Neonate Brains

As in CPT1A variant Inuit, intriguingly it is possible that peroxisomal beta-oxidation is important in providing mitochondrial substrate to fuel brain growth and function in neonates. Human breast milk contains 6–7 % carbohydrates [54]. Available glucose-derived carbohydrate is likely mainly used to support glutathione reduction, lipid synthesis and other substrate via the pentose phosphate pathway.

Consistent with low carbohydrates in breast milk, and primary usage of available glucose for maintenance rather than energy, it is believed the neonate brain relies primarily on non-glucose fuels, and focus to date has generally been on ketones [55, 56]. However, it appears most neonates have only a small increase in ketones, and in a screening programme, a fraction 0.01 % had significant ketosis [57, 58]. Brain albumin levels interestingly are also higher in neonates [59].

The developing brain consumes 60 % or more of the basal metabolism [28], PPAR alpha pathway derived mitochondrial substrate produced from proxisomal beta-oxidation of fats, compared to direct mitochondrial beta-oxidation of the same fats, may result in higher ATP per unit of oxygen and/or oxygen recycling, as well as increased PPAR alpha catalase production and so reduced oxidative stress, and increased thermogenesis, which all would all be of use to developing infants. High basal metabolism might also drive neonate metabolism towards PPAR alpha activation. Concurrent but tissue related, or possibly diurnal activation of PPAR gamma related beta-oxidation, would provide substrate for new tissue creation and related growth.

The absence of significant ketosis in human and swine [60] neonates, and likely low available glucose but high metabolism, is suggestive of a role for peroxisomes in helping provide substrate for mitochondrial APT production including in the brain, and substrate for tissue building.

Polyunsaturated Percentage Content of Breast Milk Is Significant and Has a Wide Range

Polyunsaturates are an important component of breast milk which, as a percentage of total lipid, ranged widely from 14 % (Sweden), 26 % (China), to 62 % (in mother who ate a lot of fish including higher DHA and EPA) [61]. Palmitic acid that is less well beta-oxidised by peroxisomes forms 20–25 % of human milk [62].

PPAR Alpha Null Rodent Pups Exhibit Increased Mortality

Whilst rodent models are not fully applicable to ketosis in human neonates, in the rat, PPAR alpha null pups' mortality is increased, and further mortality is increased in those born to diabetic dams [63]. PPAR-knockout neonatal mice also exhibited impaired metabolism and significant lipid build-up in the liver [64]. Their ability to survive as against thrive is likely due to the direct oxidation of fats by the mitochondria and/or dietary glucose sources.

MCAD Deficiency Disorder

In contrast to CPT1A variants that primarily block long-chain fat uptake, MCAD deficiency disorder prevents mitochondrial metabolism of shorter fats C:4–C:12 and **does** result in significant neurological issues; the condition can be fatal [65, 66].

Consequently on an energy stress event following '*failure to frequently feed*' '*twenty-five percent of untreated individuals with MCAD deficiency will die during their first episode and, of the remainder, half will have neurological impairment*'. '*The USA survivors showed cerebral palsy (9 %), seizure disorder (14 %), muscle weakness (16 %), speech disability (22 %) and/or abnormal results on formal psychodevelopmental assessment (32 %)*' [67, 68].

The above neurological impacts of MCAD deficiency, consequent on low glucose, confirms the importance of short fats and ACoA, likely of brain endogenous peroxisomal origin, as a rapidly available fuel source to facilitate normal brain metabolism during the periodic energy deficit, including during inter-meal fasting in the wider population.

The effects of '*failure to feed frequently*' in MCAD patients likely took place in a short time frame, so providing little opportunity to develop ketosis, and diets used by MCAD patients, are unlikely to produce ketosis; further, in all MCAD patients ketones were absent or lower than expected [65].

Those with MCAD deficiency likely have full CPT1 function; it follows that in the absence of adequate glucose, CPT1 related brain mitochondrial fat-beta-oxidation capacity and/or functionality, as a generality, appears inadequate to provide sufficent energy to prevent brain dysfunction, yet will support wider body function, suggesting metabolic differences between CPT1 activity in the brain and wider body. The serious consequences of the inability of MCAD deficiency patients to metabolise MCFs, and inability of CPT1 pathways to adequately compensate, and absence of similar effects in the wider population, suggest that in the wider population ACoA and derivatives, and particularly MCFs, of peroxisomal beta-oxidation origin rather than ketones alone, are central pillars to brain metabolism as alternatives to glucose.

Role of Palmitate and Other Fats as Peroxisomal Substrates

Most of the LA, ALA and EPA, and much of the PA (60 %) that enters the brain, are likely largely peroxisomally beta-oxidised, consistent with this scenario they do not form a significant part of the brain structure, and likely are poorly beta-oxidised by the mitochondria due to CPT1 'blocking'. During famine or low polyunsaturated fat intake, and in the absence of glucose, PA uptake across the BBB and astrocytic peroxisomal beta-oxidation of a proportion of that PA, would become a primary fuel provider for the brain.

In monkeys injected intravenously with palmitate, and where beta-oxidation (logically mainly peroxisomal) was chemically blocked with methyl-2-tetradecylglycidate, which importantly also may block peroxisomal function [69], oxidation of palmitate fell from 60 to 15 % and significant amounts of radioactivity accumulated in brain tissues, mainly in the phospholipids [70].

This confirms palmitate crosses the BBB, and in the absence of other fuel substrates may be significantly peroxisomally beta-oxidised, and further, that rates of peroxisomal oxidation of incoming lipids will influence brain structural lipid composition.

Alternate Potential Fuel Products of ACoA

As discussed, additive to peroxisomal short-chain fat output, peroxisomal ACoA can be converted locally into acetate, malate and ketones. Malate is a component of the mitochondrial Krebs ATP cycle, so a potential fuel substrate. Glutamate in combination with malate may be a more efficient fuel than malate and pyruvate, which might be of importance in the brain's selection of fuel substrates [71]. Malate can also be converted to glucose. It would also make synergistic sense if ACoA was capable of being fed directly into mitochondria.

Wider Importance of Brain Peroxisomes

Roles of PPAR Gamma

As in the wider body, PPAR gamma-related peroxisomes produce ACoA, as well as medium-chain fats and peroxide, and further, PPAR gamma activates genes related to lipid metabolism, including the production of cholesterol, which pathways could be used to supply lipid structural substrate to the brain, and for repair and immune-related activity.

Long-term overactivation of PPAR gamma by excess LA oxylipin HODEs, over-direction of available peroxisomal beta-oxidation substrate, to ACoA-based denovo lipid and cholesterol production, combined with uprating-related gene pathways, including SCDI and HMGCoA reductase, would lead to structural brain lipid compositional change; displacement of LA by inclusion of greater amounts of SA OA as well as POA and related Omega-7 and Omega-9 polyunsaturates, including mead acid, in both cardiolipin and wider membranes, which as well as altering membrane function, would likely reduce mitochondrial energy availability [72]. It would also lead to intracellular lipid build-up. OA lipid build-up and SCD1 activation have been implicated in Alzheimer's; and in an animal Alzheimer's model inhibition of SCD1 '*rescued proliferative defects*' [73].

Impact of PPAR Gamma Peroxisomal Activation on Oxidative Stress and Its Relationship with iNOS

Peroxisomal beta-oxidation produces peroxide, which is '*profoundly implicated in the transcription of genes supporting the endothelial response to injury*' [74], and through the oxidation of LA produces HODEs, the primary endogenous activators of PPAR gamma.

Macrophages and microglia are impacted by PPAR gamma activation; insofar as PPAR gamma activation results in excess activation of the related peroxisomal pathways, diversion of substrate to lipid and cholesterol production, and increased peroxide-related oxidative stress, outcomes are likely to be negative; however, in more physiologically normal scenarios, PPAR gamma activation outcomes may be positive.

Microglia '*the professional phagocytes of the CNS*' [75] can constitute possibly between 2 and 10 % of all cells found in the brain. LA oxylipins HODEs are the primary endogenous activators of PPAR gamma. '*PPARγ is the major form expressed in microglia, suggesting a role for PPARγ in microglia-mediated neuroinflammation*', including in Alzheimer's [76], '*microglia sense CNS damage and can act as versatile effector cells during neuropathological conditions*' [77].

Excess long-term PPAR gamma activity, concurrent cytokine activation and peroxide production can induce iNOS, and so leads to NO production; iNOS is primarily associated with tissues connected with repair and immune function such as epithelial tissues. High iNOS levels may induce neuronal death [78]. '*Because of its inducible nature, upregulation of iNOS in glia leads to huge production of NO, which is mostly cytotoxic*' [79], NO in 'excess' blocks catalase enzyme function, allowing a build-up of peroxisomal peroxide, which may contribute to phagocytic oxidative bursts. Induction of iNOS takes many hours and may last 24 and up to 72 h or more [80].

'*Mice lacking Mfp2 a peroxisomal b-oxidation enzyme develop robust neuroinflammation*' but '*do not upregulate pathways associated with phagocytic clearance, lysosomal activity and reactive oxygen/nitrogen species production*' [77], adding to evidence that peroxisomes have a central role in assisting the peroxide- and/or iNOS-related oxidative stress necessary to phagocytosis.

PPAR Gamma Induced Glial Death: PPAR Alpha Activation in Contrast Did Not

PPAR gamma activator, '*15d-PG J2 was able to induce cell death in a dose-dependent manner in all glial cell types tested, being more toxic for primary astrocytes than for C6 glioma cells*'.

In contrast PPAR alpha activation did not cause astroglial cell death; '*cell viability was unaffected by WY14643 at all doses and cell types studied (up to 100 μm).*' '*PPARγ ligands other than glitazones, but not PPARα ligands, can cause astroglial cell death in our experimental system*' [81].

PPAR Alpha-Related Peroxisomes: Activation by Energy Deficit Stress Including Through Fasting and Exercise

PPAR alpha peroxisomal pathways are activated primarily by energy deficit stress including due to fasting, exercise, and to a lesser extent Omega-3 s. Fasting increases peroxisomal activity in the brain, along with energy-related genes, including MCAD, mHMG-CoA synthase and acylCoA oxidase [40, 82].

PPAR Alpha Activation Increases Antioxidant Production Potentially Protecting the Brain from Inflammation

'*Chronic Intermittent Fasting Improves Cognitive Functions and Brain Structures in Mice*' [83] and has '*anti-inflammatory effect on the neuroimmune system which a high-fat diet prevents*' [84] (Rodent diet D12492—Lard 2205, Soybean Oil 225, of total 4057 kcal). Lard may contain 10+ % of LA and likely includes preoxidised product.

PPAR alpha-activated peroxisome pathways appear, in contrast to PPAR gamma pathways, to increase net antioxidant including catalase capacity. This makes energy deficit stress following exercise or fasting, as well as to a lesser extent Omega-3 intake, which also activates PPAR alpha, potentially important to neurological health and the maintenance of brain function into old age.

Role of PPAR Delta in Brain Antioxidant Protection

PPAR delta, also called PPAR beta, is active in the brain, and consistent with its possible role in uprating antioxidant function, also appears to have a protective role against oxidative stress and may be anti-inflammatory '*PPARβ-null mice exhibited a significant increase in the infarct size*' [85]. However, redox is multi-faceted, and 13HODE may inhibit PPAR delta, which in the case of cancer cells may assist apoptosis [86].

Dietary Balance of ALA and LA: Relevance to Brain Function

The amounts and balance of LA and ALA entering the brain, energy sufficiency/deficiency status of the wider body and brain, and oxidised stress status, are important in determining the nature and balance of peroxisomal astrocyte metabolism, namely the balance of PPAR alpha, delta and gamma activity, and consequent direction of substrate to energy, or repair and immune-related pathways, and the resultant increase or reduction in brain oxidative stress and lipid substrate availability.

Sensitivity of the Brain to Oxidative Stress

The brain locally extracts and uses 20 % of body energy, is highly dependent on oxidised substrate for signalling and requires efficient antioxidant systems, because approximately 25 % of its structure depending on cell type is composed of easily oxidisable long-chain polyunsaturated fats, of which a significant portion is DHA.

Oxidised stress is known to cause brain damage and functional decline. Control of oxidative stress is crucial to daily brain function and maintenance into old age. Retention of healthy neurons is important because in most brain tissues, except those related to memory, neurons are designed to last a lifetime and not replaceable.

Peroxide production in the brain is high, and neurons are especially sensitive to it [87]. Transgenic targeting of catalase production to mitochondria extends life in mice. Trials repeatedly connect oxidative stress generally, including high levels of 9 and 13 HODE, 4HNE and MDA, to neurological diseases including Alzheimer's and Parkinson's, as well as less "serious", but no less debilitating conditions such as self-harm and depression [88, 89].

Peroxisomal dysfunction has been associated with Alzheimer's [90]. Perspectives on the presence of peroxisomes and/or microperoxisomes in neurons differ; catalase staining is seen in some neurons, as is peroxisomal membrane protein PMP70, but is PMP70 also found in microperoxisomes; microperoxisomes like peroxisomes produce catalase but it is less clear whether microperoxisomes produce peroxide; from a design standpoint, it would no make sense to have significant quantities of active peroxide producing bodies, in a cell that in many instances has to last a lifetime.

Brain antioxidant enzyme function is lower than the liver despite its high-energy consumption. Neuron antioxidant production capacity is particularly limited. External including diet-derived antioxidant support options are limited by the BBB. Neurons rely on additional antioxidant substrate support from astrocytes, which have higher antioxidant production potential, and act as donors including of glutathione and catalase [91].

Cardiolipin: Mitochondrial Function Including Energy Production and Apoptosis

LA forms a lower proportion of brain cardiolipin than in the wider body, but as in the wider body, oxidation of cardiolipin LA would have significant effects. In Wistar Rats,

increased LA intake from 2 to 10 % w/w, within a week or so, results in brain LA cardiolipin increase from approximately 10 % to possibly 15 % [92].

LA increases in the lipid constitution of cardiolipin will change mitochondrial energetics and, in the absence of sufficient lipid protective antioxidant capacity, make them more susceptible to damage, which would reduce cytochrome C-related function so downstream ATP production and result ultimately in mitochondrial damage and release of LA-based oxylipins including HODEs and 4HNE [93].

Cardiolipin oxidation is associated with apoptosis, low cytochrome C function and so reduced energy production, and postulated to be a significant factor in brain trauma [94], including release of cell '*death factors*': following brain impact trauma, the number of oxidised cardiolipin species increased considerably. LA was the most prevalent species in the basket of major brain cardiolipin species that underwent oxidation (Supplementary Table 1 of reference) [95].

Potential Role of LOX12/15 and LA Oxylipin HODEs During Brain Damage or Injury

LA oxylipins are seen in quantity particularly during brain damage in the form of oxylipins, such as 4HNE [96] and 13HODE, adding to evidence LA crosses the BBB. During trauma, if LA was not metabolised by the peroxisomes, it would logically be available for oxidation to oxylipins including by the LOX12/15 enzyme, of which ALA followed by LA is the preferred substrate. Further astrocytes in ischaemia may take up plasma-oxidised LDL [97].

LOX12/15 is active in the brain, but its role is not yet fully defined. In a canine model of ischaemia '*The predominant oxidized lipids were identified by gas chromatography/mass spectrometry as 13- and 9-hydroxy-octadecadienoic acids (13- and 9-HODE)*' [98].

In contrast, ALA oxylipins, including those of the LOX12/15 pathway, if they follow the general Omega-3 trend, may prove on balance to be protective. Deficit of ALA and increased LA [99] so indirectly also AA, would result in increased COX and LOX12/15 action on LA and AA, so increased LA and AA oxylipins including HODEs.

Effect of Dietary ALA on Brain Function

Consistent with the argued importance of ALA and/or DHA to brain function including energetics, rats fed a high-LA safflower diet over two generations had significantly lower brain ATP concentration (78 %) than those fed on ALA-rich perilla diet [100], and altered learning behaviour.

ALA has been shown to have anticonvulsive properties in both animals and humans. In rats, an LA/ALA (25/70 %) mix had a similar anticonvulsant effect to a ketogenic diet, but limited effect on the brain phospholipid content, pointing again to roles for LA/ALA as peroxisomal sources of short fats and ACoA, and their direction likley by PPAR alpha and/or delta gene activation towards ultilisation as alternative fuel substrates in the brain energy pathways, rather than as a structural substrate [101].

A single "subchronic" injection of ALA into the bloodstream before induction of a stroke in mice reduced post-infarct ischaemic damage. Multiple pre-stroke ALA treatments improved survival by a factor of 3 at ten days, increased neurogenesis, enhanced brain plasticity and were significantly antidepressant. Other papers cited by the same authors have found ALA-promoted neurogenesis, synaptogenesis, the survival of immature neurons, and improved cerebral blood flow [102]; the immediacy of effect of a single subchronic injection of ALA, combined with wider observed effect but no signficant change in phospholipid compostion suggests the effect is not due simply to increased availability of EPA and DHA in plasma, but that ALA crosses the blood–brain barrier and has significant physiological impact, likely mainly but not exclusively through indirect pathways including the following: competition with LA for COX and LOX enzymes so reduction in oxidative stress including production of likely 'anti-infammatory' ALA related oxylipins; improvement in antioxidant protection and increased energy provision though activation in the brain astrocytes by ALA of PPAR alpha and delta beta-oxidation pathways, peroxisomal beta-oxidation of ALA to short chain fats and ACoA so improvement in energy availability, consequent displacement of PPAR gamma related peroxisomal beta oxidation including inflammatory pathway activities, uprating of antioxidant production pathways including of catalase, and generally alteration of gene activation.

Activation of PPAR alpha pathways may constitute '*a novel preventive and/or therapeutic tool against neurodegenerative diseases*' [103]. The above suggests dietary ALA may have greater potential in the brain as a fuel and inflammatory moderator than is currently appreciated.

DHA in the Brain

Brain development and function are extremely sensitive to, and dependent on, nutrient intake including LA, ALA, and/or the downstream product of ALA, DHA. Those with genetic polymorphisms for low desaturase conversion of ALA to DHA, as seen including amongst some American Indians, Continental Indians, and those of 'Celtic' descent, may have an obligate genetic evolutionary likely shoreline origin related requirement for pre-formed DHA intake, which particularly in pregnant women will be further

exacerbated by common "Western" dietary imbalances and deficiencies, which are known to inhibit desaturase function.

The lower rate of peroxisomal oxidation of DHA compared to EPA may help explain DHA's greater presence in structural brain lipids and differing outcomes in neurological related trials using EPA and DHA.

Alzheimer's; PPAR Gamma, PPAR Alpha, OA, POA, Mead Acid, SCD1; Imbalances

Alzheimer's is a flagship neurological condition. Imbalances in brain lipid composition; loss of LA; increased endogenous brain denovo lipid [104] and cholesterol production; increase in SCD1 desaturation activity [73] leading to higher brain levels of mono and polyunsaturated Omega-7 and Omega-9 fats including mead acid; and brain changes including; intracellular lipid deposition including cholesterol, increased oxidative stress [105], cardiolipin lipid species changes and imbalances, and mitochondrial dysfunction, have been linked to diseases of cognitive impairment including depression [106, 107] and Alzheimer's [108].

These Alzheimer's-related structural and energy pathway changes in the brain, within a wider context of raised "Western" dietary-related oxidative and so LDL related stress, are consistent with the excess activation by LA oxylipins of the PPAR gamma-related metabolic astrocyte and likely oligodendroycyte peroxisomal brain pathways; including uprated SCD1 [109] and HMGCoA reductase; leading to peroxisomal beta-oxidation of LA, and the consequent ACoA product being directed to substrates for cell maintenance, including production of PA, SA, Omega-7 and Omega-9 lipids and cholesterol; as well as concomitant increases in peroxisomal-related peroxide-based oxidative stress, including via the iNOS activation NO catalase inhibition pathway, together with wider oxylipin production; and likely brain energy deficit due to lack of PPAR alpha-related peroxisomal activity.

Astrocyte- and oligodendrocyte-related oxidative stress, likely peroxisomal in origin and predominately LA-based, has a role in Alzheimer's initiation and progression. Consistent with this PPAR gamma activity is increased, and PPAR alpha activity is reduced in Alzheimer's. NOS including iNOS is raised in Alzheimer's and related to amyloid placque; and as discussed, NO blocks catalase function and so uprating peroxisomally produced peroxide-related oxidative stress.

'*High-fat diets significantly increase the risk of Alzheimer's*'. '*4-Hydroxynonenal and acrolein*' (oxidised LA products), '*chronic low dose of* H_2O_2' (peroxide a product of peroxisomal beta-oxidation) and '*FFA induced, astroglia-mediated oxidative stress causes hyperphosphorylation of tau in neurons*' [110]. Saturated fats including PA will also, to varying lesser extents than LA and ALA be beta-oxidised by peroxisomes through the PPAR gamma pathways once they are activated including by LA HODEs.

The preferred substrate of LOX 12/15 is ALA, followed by LA, although AA is present in the brain structure in greater amounts; however, the presence of free LA and ALA may increase with brain damage or dysregulation, and will have preferential access to LOX12/15. The primary product of LOX12/15 oxidation of LA is 13HODE, which will also be formed by the action of the hydroxyl radical, a product of peroxide. LA oxylipin 13HODE will activate PPAR gamma, LA if present will be peroxisomally beta-oxidised leading through peroxide production to a feed forward oxidative stress cascade. Conversely, LOX12/15 null mice have lower oxidative stress in the brain [111]. 12/15 LOX brain activity is increased in stroke and Alzheimer's [112].

Increased LA oxylipins are seen in Alzheimer's, '*Total HODE levels* (in plasma and/or erythrocytes) *increased with increasing clinical dementia ratings*' [113], with 13HODE, 4HNE and MDA being linked to Alzheimer's [114]. Further '*Elevation of 4-HNE was related to the degree of cognitive impairment*' [115].

13HODE activates PPAR gamma which activates SCD1. Increased PPAR gamma activity would result in greater rates of peroxisomal beta-oxidation of LA, so comparative reduction in brain and wider LA levels, and promotion of both denovo lipogenesis and SCD1 activity. Increased SCD1 activity would result in desaturation of brain endogenous and exogenous dietary PA to POA, as well as increased OA and mead acid [116] in brain tissues, including in cardiolipin; intracellular lipid build was noted by Dr. Alois Alzehimer, interestingly '*inhibiting the rate-limiting enzyme of oleic acid synthesis* (SCD1) *rescued proliferative defects*' in Alzheimer's mice models [73].

Consistent with the greater peroxisomal beta-oxidation of LA and activation of SCD1, plasma '*levels of linoleic acid decreased and level of mead acid increased progressively from HC (Health Controls) to MCI (Mild Cognitive Impairment) to AD (Alzheimer's disease) patients*' [117]. In aged dogs '*Increased palmitoleic acid levels and desaturation index were positively correlated with increased reversal learning errors and decreased cognitive performance*' [118].

PPAR gamma may uprate HMGCoA reductase activity [119], which would result in increased cholesterol, as well as lipid build-up generally; consistent with this '*cholesterol abnormally accumulates in the dense cores of amyloid plaques in the brain of AD patients*' [120]. Conversely statins, HMGCoA reductase inhibitors as well as reducing cholesterol production, may reduce '*iNOS and reactive nitrogen and oxygen species*', so Alzheimer's risk [121], although results are mixed and actions of statins complex,

but this adds to evidence for the potential involvement of PPAR gamma lipid pathways and related gene set activation.

Increased oxidative stress, including due to; lack of accessible astrocytic PPAR alpha beta-oxidation pathway generated fuel substrate; LA oxylipin activated PPAR gamma-related diversion of LA and potentially to a lesser extent PA substrate to repair rather than energy, related lipid imbalances, including significant reduction in the degree of cardiolipin unsaturation [122], and oxidation of cardiolipin components, would result in damage to enzymes such as cytochrome C and mitochondrial DNA, together reducing brain including neuron energy availability [123]. These changes are all seen in Alzheimer's models.

Consistent with this PPAR alpha expression is lower in Alzheimer's patients. A number of studies suggest that PPAR alpha agonists have been associated with the reduced risk of dementias, including Alzheimer's. PPAR alpha '*plays a neuroprotective role in age-related inflammation, enhances memory consolidation and exert a neuroprotective action against Aβ-mediated toxicity* in vitro' [124].

As discussed, activation of PPAR alpha pathways will increase energy substrate availability, including short fats, ACoA and ACoA substrates, as well as activating a number of genes related to enhanced mitochondrial activity. PPAR alpha and delta activation will also likely improve antioxidant status and so reduce oxidative stress. PPAR alpha activation will by competition also redirect LA away from the PPAR gamma pathways to beta-oxdiation through the PPAR alpha pathways, so reduce LA substrate availability including for LOX12/15 and COX enzymes, hence inhibiting the LA oxidative stress related peroxisomal activation feed forward pathway.

Alzheimer's is also sometimes referred to as "Diabetes 3". '*More than 80 % of AD patients have type II diabetes*' [125], so impaired glucose function; related disruption of the neural energy pathways [126–128] will oblige increased brain selective lipid uptake and related peroxisomal beta-oxidation, to try and fill the impaired glucose function-related energy gap, but in the absence of PPAR alpha activation, and combined with an excessive amount of LA, in the presence of increased background dietary and consequent disease related oxidative stress, this additional lipid uptake will logically be proportionately more directed to the oxidative stress related PPAR gamma pathways than PPAR alpha energy related pathways.

LA also raises insulin independent of glucose and impacts beta-cell oxidative stress and lipid build-up, so has a role in diabetes, metabolic syndrome and insulin resistance. Further mitochondrial function '*depends on the availability of many essential vitamins, minerals and other metabolites*' [129] so will be impaired by a nutrient depleted preoxidised westernised diet.

Limited research suggests dietary MCTs and ketones may reduce the onset and/or the effects of Alzheimer's, which is consistent with brain glucose dysbiosis leading to a requirement for alternate fuels, and the use by the brain of short fats and ACoA produced through the PPAR alpha and potentially delta peroxisomal pathways, and co-activation of co-related energy production promoting gene sets. However excess dietary LA combined with pre-existing raised oxdiative stress will instead activate the brains astrocytic inflammatory PPAR gamma related pathways, and peroxisomal-beta oxdiation substrate will be directed towards brain endogenous lipogenisis rather than energy.

In summary, in the **context** of a Western nutrient-depleted, antioxidant compromised pre-oxidised Omega-3 poor diet, excess Omega-6 intake is a likely risk factor for Alzheimer's. Overprovision of LA including oxylipins, and lack of ALA, in brain astrocytes, oligodendrocytes and/or microglia; overactivation of PPAR gamma-related peroxisomes; under activation of PPAR alpha-related peroxisomes; excess oxidative stress due to lack of antioxidant capacity, iNOS activation, NO-based catalase inhibition, excess peroxide, so increased oxidative stress; [130] the increased presence of HODEs and systemic oxidative stress due to metabolic syndrome; increased requirement for peroxisomal activation to supply ACoA and MCFs as mitochondrial energy substrate due to metabolic syndrome related energy deficits leading to over-activation of the PPAR gamma related peroxisomal beta-oxdiation pathways; and lipid imbalances including of cholesterol, including reduction in unsaturation and oxidation of cardiolipin so mitochondrial dysbiosis, and increased unsaturated omega 7 and 9 fats in brain phospholipids; are significant likely LA/western-diet-related factors in dementias including Alzheimer's.

Wider Functional and Behavioural Implications of Lipid and Wider Redox Imbalances

Western arguably dietary-related degenerative neurological conditions at their most profound manifestation include Alzheimer's, and at a lower but no less individually and societally important levels encompass; neurological decline, suboptimal brain formation, early puberty and related brain development change, IQ loss, increased aggression and "territoriality", and loss of capacity for abstract thought and empathy, with all the wider downstream societal implications they have. Detrimental declines in these 'conditions' are logical consequences of over or imbalanced activation of redox pathways caused by basic nutrient damage deficits and imbalances including in essential fats LA and ALA.

The brain is the most energy demanding, nutrient, Omega-3:6 lipid, oxidative stress sensitive organ in the body. LA and its oxidised products, as key reproductive messaging systems and controllers including of steroid

hormones, have a crucial part to play in human behaviour and physiology, including aggression and territoriality, as also reflected in rutting animal behaviour.

Thought-provokingly in 2015 over a million children in the England are reported at some level to be receiving special educational needs support, over 200,000 having specific 'education health and care plans'. Most worrying and fundamental of all, there is a nascent trend for infants of mothers at greatest risk of nutritional degradation deficiencies and imbalances, having impaired neurological capacity, manifesting behavioural disorders and potentially even having smaller brains [131].

The first casualties of nutrient-depleted pre-oxidised Omega-3:6 imbalanced diets are likely loss of IQ, abstract thought and crucially empathy. What is the future for individuals nations and more widely humanity, if increasing numbers of humans are more aggressive and territorial, have falling IQs and depleted capacity for abstract thought and empathy? '*The greatness of humanity is not being human but humane*' Gandhi.

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The Roles of Linoleic and Alpha-linolenic Acid, Their Oxylipins and the PPAR Alpha-, Delta- and Gamma-Related Peroxisomal Pathways on Obesity in the Context of a "Western" Diet

31

Robert Andrew Brown

Terms	
AA	Arachidonic acid (Omega-6, 20 carbon derivative of LA)
ACoA	Acetyl coenzyme A (raw material for the energy/cholesterol pathways)
AGE	Advanced glycation end product (non-enzymatic covalently bound sugar to protein or lipid.)
ALA	Alpha-linolenic acid (Omega-3, 18 carbon plant-based polyunsaturated fat)
ATP	Adenosine triphosphate (enzyme used as an energy carrier)
CB1	Endocannabinoid receptor type 1 (cannabinoid receptor activated by endocannabinoid neurotransmitters including anandamide)
CD36	Cluster of differentiation 36 (fatty acid translocase receptor)
COX	Cyclooxygenase (enzyme catalysing oxidation of fatty acids)
CPT1	Carnitine palmitoyltransferase (acts as shuttle mainly for long-chain fats C:16–18 into mitochondria.)
DHA	Docosahexaenoic acid (Omega-3, 22 carbon derivative of ALA)
EPA	Eicosapentaenoic acid (Omega-3 fatty acid C20:5)
FAS	Fatty acid synthase (enzyme system to make palmitate)
GLA	Gamma-linoleic acid (Omega-6 fatty acid C18:3)
HMGCoA	3-hydroxy-3-methyl-glutaryl-CoA (found in two forms such as reductase and synthase. Reductase regulates cholesterol production, whereas synthase regulates HMGCoA production. HMGCoA is substrate for ketones or cholesterol.)
HSL	Hormone-sensitive lipase (different forms mobilise lipids from triglycerides and esters including from adipose tissue.)
iNOS	Inducible nitric oxide synthase (inducible isoform involved in stress response in macrophages microglia and other tissues)
LA	Linoleic acid (Omega-6, 18 carbon plant-based polyunsaturated fat)
LOX12/15	Lipoxygenases (enzymes catalysing oxidation of multiple lipid-based substrates)
LPL	Lipoprotein lipase (mobilises lipids from chylomicrons, VLDL, LDL, at the vascular face and intercellularly.)
MCF	Medium chain fat (medium chain fatty acid between C:6 and C:12)
MCT	Medium chain triglyceride (triglyceride containing fats between C:6 and C:12)
NAFLD	Non-alcoholic fatty liver disease (fat deposition in the liver not due to alcohol)
NO	Nitric oxide (an important signalling messenger and oxidant)
OA	Oleic acid (Omega-9, monosaturated fat C18:1)
OLR1	Oxidized LDL receptor 1 (receptor for oxidised LDL sometimes called LOX1)
PA	Palmitic acid (saturated fat C:16)

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POA	Palmitoleic acid (monounsaturated fat.)
PPAR	Peroxisome proliferator-activated receptor (3 forms such as alpha, gamma and delta.)
RXR	Retinoid X receptor (receptor that hosts PPARs in conjunction with other activators including retinoids)
SCD1	Stearoyl-CoA desaturase (delta-9-desaturase a key to formation of OA)
SREBP-1c	Sterol regulatory element-binding transcription factor 1 (a protein regulating lipid and glucose metabolism.)
4HNE	4-hydroxynonenal (exclusive Omega-6 fats peroxidation aldehyde)
HODEs	Family of oxidation products of LA
9 HODE	9-hydroxy-10E, 12Z-octadecadienoic acid (major LA oxidation product of LOX12/15, COX, photo-oxidation and autoxidation.)
13HODE	13-hydroxy-9Z, 11E-octadecadienoic acid (major LA oxidation product of LOX15/15, COX, photo-oxidation and autoxidation.)

Western processed nutrient depleted preoxidised LA rich diets are obesogenic

Western, linoleic acid (LA) and carbohydrate calorie-rich, alpha-linolenic acid (ALA) deficient, nutrient-depleted, highly oxidised inflammatory diets, lead to increased oxidative stress, which combined with excess intake of LA, a deficit of Omega-3s, lowered antioxidant capacity and with excess intake of easily oxidised sugars amplify natures' signalling systems that trigger fat storage, putting fat deposition mechanisms into overdrive.

One of the consequences of the high levels of LA, in the context of a nutrient insufficient processed pre-oxidised Western diet, is significant fat gain. Factors contributing to the risk of obesity are complex and multiple, including genetics, epigenetics [1], exercise, lifestyle, declining food quality and likely ability to sense combined with inbuilt programmed mechanisms to hunger for, seek out and consume foods normally associated with a high nutrient content, namely plant reproductive tissue-related foods that in nature are generally seasonal, but which are rich in LA, sucrose, glucose, fructose, carbohydrates, antioxidants and minerals.

Synergistically two elongated LA oxidised Omega-6 AA products are the endogenous activators of the CB1 cannabinoid receptors, helping drive hunger and fat deposition [2]. Mechanisms that may increase hunger so drive food consumption also exist for fructose and glucose, and potentially minerals as well.

Moderating LA intake and increasing ALA intake, and intermittent energy deficit stress through exercise or short-term fasting, combined with a nutrient dense diet high in antioxidants and low in pre-oxidised products, particularly in the obese, may reduce inflammation and oxidative stress, redirect substrate away from repair and related fat depostion pathways towards energy production pathways, reduce mitochondrial damage and dysfunction and so limit adipose tissue gain and comorbid conditions, as seen in the use of intermittent short-term fasting in diabetes [3]. Exercise without dietary change and calorie restriction is not an automatic route to weight loss.

Adipose Tissue—Evolutionary Rules

LA and ALA Cannot Be Made by Mammals so Are Essential Nutrients

Humans do not possess the necessary desaturates, so LA and ALA cannot be made in the body; the intake required to avoid deficiency is probably between 0.5 and 1 %, respectively [4], with more required in pregnancy.

Intake of LA and ALA in "Native" and "Westernised" Diets

Intake of LA and ALA in non-"Westernised" diets as determined by natural availability would have ranged between 1 and 4 % of calories and have been more or less in balance [5–7]. Between 1909 and 1999, in the USA, dietary daily PUFA availability has increased from 11.7 to 34.3 g [8], forming 2.5 % of energy intake [9] in the 1900s, rising to 7.5 % in 1989 [10], with higher amounts in some populations [11].

ALA intake in the same time frame has decreased [12]. Intake of green plant foods that are low in fat, but contain mainly ALA and with lesser amounts of LA, has also decreased. The replacement of green material in livestock feeds, by LA-rich ALA-poor grain, reduces the availability of ALA in both ruminant and non-ruminant meat, as well as in eggs.

In non-ruminants, LA is actively stored in adipose tissue in proportion to dietary intake, and would have followed seasonal trends and likely been between 1–4 % of adipose tissue. Due to grain becoming the primary feedstuff, the LA content of chicken fat has risen to as much as 30 % in some instances; 11 % LA and more is seen in pork; the total fat content of industrially farmed animals has also hugely increased.

Wild grazers from warm climates had fat mass averaging 1–3 %, as against 20–40 % fat mass in domesticated industrialised species [13], and greater proportions of ALA in tissue than domesticated cattle, 1–4 % [14]; they also have much lower proportions of adipose tissue to overall mass.

Industrial processing of oils and foods also reduces ALA content. Growing plants in greenhouses with low UV exposure might also reduce ALA content as well as plant phyto-antioxidants.

Vegetable oil consumption has increased considerably (see Fig. 31.1) [9]. Many vegetable oils contain significant percentages of LA. They are cheap and widely consumed, and often their presence is not immediately evident, because they are used as part of food preparation processes, such as frying, roasting and baking. Overall, LA intake, including in non-ruminant animal fats, strongly correlates with obesity (see Fig. 31.2) [15].

Professor Lands, a pioneer in this field, has been steadfastly vocally advocating the danger of postprandial oxidative stress from an eicosanoid perspective (see his diagram 5–3, page 38 of this reference) [5], and the need for a reduction in LA intake generally, for 50 years, and his perspective has been championed by Capt. Joseph R. Hibbeln, MD, Acting Chief NIAAA NIH, who has provided the vision for some fundamental and unfashionable research into the effects of excess LA, when set in the context of a "Western diet".

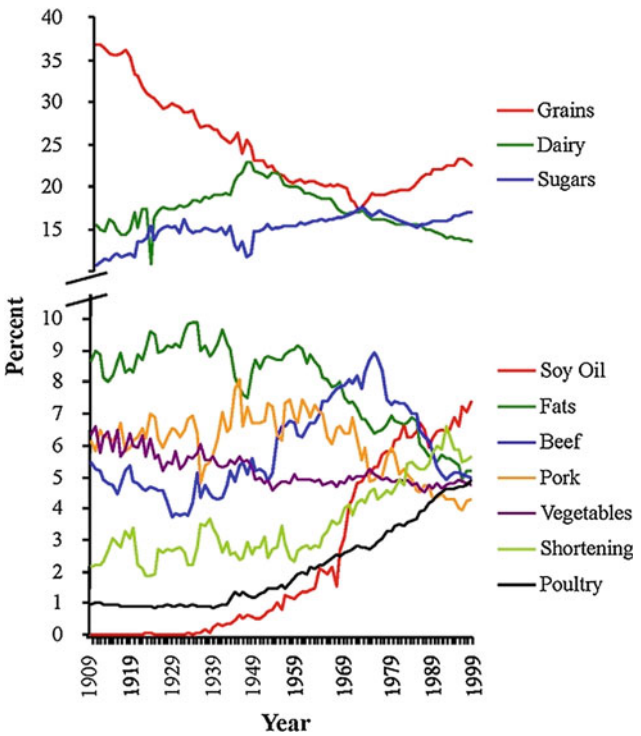

Fig. 31.1 "Major sources of calories between 1909 and 1999. Fats included shortening, butter, lard, margarine and beef tallow. Soybean oil was considered separately from other oils because of its disproportionate contribution. Dairy included all milk, buttermilk, condensed milk, cream, sour cream, yogurt, cheese and eggnog. Butter was not included in the dairy category to avoid double counting" from "Changes in consumption of Omega-3 and Omega-6 fatty acids in the United States during the twentieth century" [9] with very grateful thanks to the authors for the permission: Blasbalg T, Hibbeln J, Ramsden C, Majchrzak S, Rawlings R. Am J Clin Nutr. 2011 May; 93 (5):950–62

LA Ultimate Controller of Reproduction and Fat Deposition?

Human adipose tissue strongly accumulates LA; we and animals are arguably programmed to accumulate LA because it is essential for reproduction, and both relatively scarce in concentrated form as in plant reproductive seeds and nuts, and a seasonal resource in nature; unlike ruminants which concentrate LA and ALA from grass and its nascent seeds, we have no mechanism to limit LA adipose uptake from dietary intake because it was never needed.

In wild grazers from temperate climates, where food availability is governed by sunlight as well as rainfall, so fecundity of the environment, fat mass and lipid composition including LA content changes are still seasonal and key to reproductive capacity [16].

A nutrient-depleted Westernised diet leads to increased oxidative stress, which combined with excess intake of LA, a deficit of Omega-3s, lowered antioxidant capacity and excess intake of easily oxidised sugars amplifies natures' signalling systems that trigger fat storage.

In the absence of cyclical seasonal food availability cycles, periods of energy deficit, or the need to expend energy to gather food, these factors will inevitably lead to obesity. In some populations, obesity is set to become the norm.

Adipose Tissue—a Seasonal Storage Depot Primarily Directed to Support Reproduction and/or Hibernation

The likely primary evolutionary role of adipose tissue is to provide a nutrient reserve, for seasonally available externally or internally produced nutrients including LA, cholesterol [17, 18], lipid antioxidants, vitamin D [19] and iodine [20], primarily intended to support reproduction and related

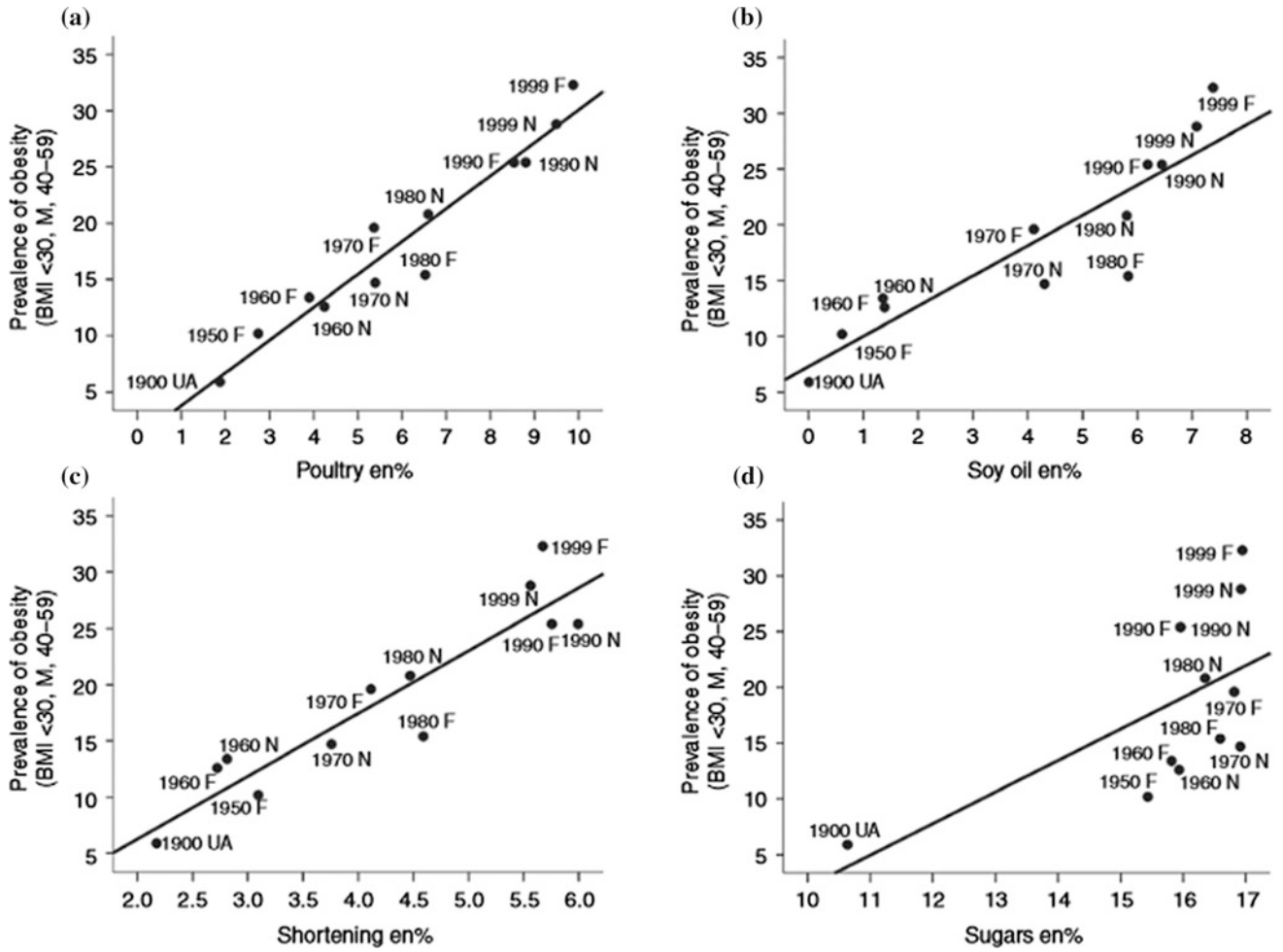

Fig. 31.2 "Dietary sources of linoleic acid (LA) and increasing prevalence rates of male obesity in the United States during the twentieth century. Prevalence of male obesity (40–59 years, BMI > 30) in each year is indicated by source: UA—Union Army Veterans, F—Framingham cohorts and N—NHANES cohorts. Scattergrams and univariate linear regression lines are indicated in each panel. Increasing prevalence rates of male obesity are positively correlated with apparent consumption of dietary sources of LA indicated in **a** poultry a per cent of energy (en%), ($r2 = 0.94$, $P < 0.000{,}000$), **b** soybean oil (en%) ($r2 = 0.82$, $P < 0.00005$), **c** shortening (en%) ($r2 = 0.86$, $P < 0.00002$) and **d** sugars (en%) $r2 = (0.37, P < 0.04)$ Animal diets of 1 en% and 8 en% LA were selected to model these changes" from "Dietary linoleic acid elevates endogenous 2-AG and anandamide and induces obesity" [15] with very grateful thanks to the authors for the permission: Alvheim A, Malde M, Osei-Hyiaman D, Lin Y, Pawlosky R, Madsen L, Kristiansen K, Frøyland L, Hibbeln J. Obesity (Silver Spring). 2012 Oct; 20(10): 1984–1994

behaviour, for example pregnancy and lactation in females, and rutting and display in males, as well as hibernation in relevant species. In a seasonal food environment in the absence of stored nutrients in adipose tissue the chance of a successful outcome to a pregnancy is low; an unsuccessful pregnancy is resource costly in genetic survival terms for both the mother and group.

Human also store fat-soluble nutrients in adipose tissue. Adipose concentrations of fat-soluble antioxidants, such as beta-carotene, vitamin K and vitamin D levels (4–2470 ng/g), vary massively between individuals [21]. Optimal nutrient storage quantities have yet to be determined.

Iodine and Other Fat-Soluble Antioxidants Are Delivered by LDL to Fat Tissue

LDL and likely other lipoproteins can carry iodine as well as plant-based lipid-soluble antioxidants. The iodine is presumably attached by electrophilic attraction, likely at double-bond sites. Iodine-supplemented thyroidectomised mice and humans have surprising quantities of iodine/iodide in the circulation.

Iodine is essential for reproduction including brain development and is a relatively scarce nutrient that is retained in low amounts by plants and concentrated by

grazers particularly in their milk; hunter-gatherers eat prey from nose to tail.

Increased intake of LA may increase the requirement for iodine, as first observed in puppies in 1921 by Mellanby and later by McCarrison in pigeons, at a guess due to lipid binding including in adipose tissue, reducing short-term iodine plasma availability and so thyroid uptake [22]. Consistent with other lipid-soluble nutrients, obese women had insufficient urinary iodide levels that were lower than controls −96.6 vs 173 µg/g [23].

Oxidised Product Reprocessing and Related Immune Function Centre

Adipose tissue, as well as being a major depot for fats in chylomicrons, is also a sink, store, 'immune'/reprocessing centre, and blood-level regulator for the contents of LDL including cholesterol and polyunsaturates, oxidised lipids and proteins, damaged pathogens including bacteria, AGEs and other detritus from the blood, thereby helping assist and protect vascular epithelial membranes from excess oxidative stress.

Obesity Is Signalled by Oxidative Stress

Obesity is signalled for by oxidative stress including through LA oxylipin and AGE activation of obesogenic pathways; oxidised products are delivered to adipose tissue likely in significant part by LDL, thereby including oxidised cholesterol, LA oxylipins and wider oxidised products attached to LDL.

In a pre-agricultural world oxidative stress levels at the vascular adipose cell interface will mirror the rising availability of seasonal products that are oxidation prone, namely sugars including glucose and fructose, and polyunsaturated fats found in plant reproductive-related material, which together form the basis for a wide range of obesogenic bioactive oxidised products, including oxylipins and AGEs.

Delivery Mechanisms

In addition to LDL a number of other potential mechanisms exist for delivery and export of lipids, together with related substrates destined for adipose storage, including; VLDL, HDL [24, 25] chylomicrons, albumin and via free fatty acids.

Following delivery, subsequent tissue uptake of fats across vascular membranes by LDL, chylomicrons and albumin occurs by different mechanisms. LDL is endocyted and unpacked within the relevant cell, and some VLDL and albumin are also endocyted. Most chylomicrons VLDLs and albumin have the fat abstracted at the vascular bloodstream interface by enzymes such as lipoprotein lipase, so not all of the content will necessarily cross into the adipose cells.

Delivery of Lipids to Fat Cells by Chylomicrons

The majority of the triglycerides, 60–80 % originating in chylomicrons, end up initially in adipose tissue [26–28]. Due to LPL and/or HSL preferences for fats with more double bonds, lipids in chylomicron with more double bonds, such as ALA, as well as EPA and DHA and their oxidised products, rather than being taken up by fat tissue, may well be directly preferentially reincorporated in albumin for onward delivery to other tissues, which is consistent with them being found in relatively low amounts in adipose tissue in those on normal diets.

LA is less rapidly taken up by lipolysis by adipose tissue, than shorter fats such as palmitic acid, and fats with more double bonds such as ALA, helping explain why; LA and OA (as well as to a lesser extent stearic acid); and OA to a greater extent than LA; influence the size [29] and quantity [30], and form a significant proportion, of the fatty acid chylomicron remnants taken up by the liver.

Delivery of Lipids Oxidised Material and Cellular Detritus to Fat Cells by LDL

An LDL receptor, CD36 null mouse, had a 40 % decrease in fat mass and was protected against weight gain [31], suggesting that LDL is a significant mechanism for accretion of fats by adipose tissue, as well as being an important delivery system to cardiac tissue.

OA is an obligatory and important component of LDL; SCD1 null mice cannot make OA, so their capacity to produce LDL is significantly inhibited, hence on a low fat carbohydrate rich diet, with no access to dietary OA; but not on a high-fat diet containing OA, are obesity resistant [32]. The resistance to obesity of SCD1 null mice adds to the evidence that delivery of lipids by LDL plays an important part in fat accretion and so obesity.

LDL has a particular role, because it is a carrier of significant amounts of LA, fat-soluble antioxidants including vitamin D, oxidised lipoproteins, oxidised lipids generally, iodine, cholesterol and associated oxidised plasma detritus including pathogens, and because LDL is endocyted into cells intact, so the entire LDL contents are destined for a mix of storage, substrate for expansion of adipose cell structures including vascular and related tissues to accommodate additional fat, or where necessary 'immune' related reprocessing by macrophages. Endocytosis of LDL provides an

alternative mechanism to supply LA to cells including adipose tissue that circumvents the relatively poor lipolysis of LA from chylomicrons. LDL contains higher levels of LA and OA, the preferred substrate of the peroxisomes, compared to VLDL [33, 34], and lower levels of saturated fats including C:16 PA.

Rates of Mobilisation of Fats Out of Adipose Tissue and Subsequent Beta-Oxidation

Fats are mobilised from adipose tissue by 4 enzymes [35, 36], much is still unknown [37], and species differences may exist. Rates of mobilisation of fats in and out of lipid stores increase with decreasing chain length and increase with the number of double bonds; also, Omega-3s with the double bond closer to the methyl end is more effectively mobilised than an equivalent Omega-6, so of the 18 carbon fats, ALA is preferentially mobilised; ALA likely better than GLA and significantly more than LA, and the order for EFA is "*C20:5, n-3>C20:4,n-6>C18:3,n-3> C18:2,n6>C22:6,n-3*" [38].

Consistent with this in humans, following an overnight fast, ALA was significantly better mobilised better into plasma from adipose tissue than other fats, followed in descending order by DHA, POA, EPA and AA [39].

The body location pattern of fat tissue deposition in those with analbumenia suggests that albumin may have important roles in reverse transport of lipids from adipose tissue. It is not clear if HDL also plays a significant role in the reverse transport of lipids back into the circulation for delivery to tissues.

LA Roles in Adipose Tissue Accretion

Evidence and biology suggests rising LA intake, particularly if oxidised, factors in and is reflected in human obesity trends. Oxidised LA products the HODEs are the primary endogenous activators of PPAR gamma, referred to as a master regulator of fat deposition, and activator of related peroxisomes. ACoA produced by peroxisomal beta-oxidation of fats will be directed by PPAR gamma activated genes towards lipid creation and depostion. Peroxisomes produce peroxide, thereby increasing oxidative stress and oxidised LA products including the HODEs, hence through PPAR gamma activation upregulating fat depostion, thus creating a positive feed-forward cycle.

LA-related oxidative stress increases LDL production by the liver by uprating lipid production-related genes, including increasing PPAR gamma and SCD1 activity, as well as synergistically assisting onward delivery by increasing activity of LDL receptors such as CD36 and ORL1, which are particularly active in tissues rich in peroxisomes, including adipose tissue, cardiac tissue and insulin beta cells, and logically in time may be shown to have a significant role in lipid delivery to the brain. LDL is particularly rich in the LA PA and POA lipid substrates preferred as beta-oxidation substrates by peroxisomes, with ALA being the top preference.

As an evolutionary strategy to signal for fat accumulation, the synergistic use of plant reproductive-related nutrients, most potently LA but also simple carbohydrates and sugars, which signal a fecund environment, through their oxidised bioactive messengering properties, makes sense:

- LA oxylipins including LA 13HODE uprate PPAR gamma and insulin, and reduce leptin, thereby inducing obesity; PPAR gamma peroxisome activation will result in ACoA production, which will be directed to substrate, including lipids and cholesterol to support new tissue creation and immune function, and can be used for storage;
- AA drives appetite and fat deposition through downstream endocannabinoids including anandamide (see Fig. 31.3) [15];
- glucose and to a lesser extent LA drive deposition via activation of insulin pathways;
- sucrose "induces" LOX12/15 [40] activity, so 13HODE formation and so PPAR gamma activation; and
- fructose is primarily directed to fat creation.

Prior to the 1950s, stored LA in human adipose tissue averaged may be 4 %. Since the 1950s, the LA content of body fat has risen from 4 % the upper seasonal amounts seen in wild herbivores to 10 % and up to 25 % in groups with very high LA intakes.

Consistent with the relevance of LA intake to obesity, in the USA, strong correlations exist between the proportion of LA in adipose tissue and dietary intake of LA (see Fig. 31.4) [41, 42], including from chicken intake and soy oil consumption.

Hibernators Store Very High Levels of LA Prior to Hibernation, Which May Have a Role in Lowering Metabolism

Monogastric seed-eating hibernators such as marmots store very high levels of LA prior to the hibernation, but will not hibernate if fed ALA, the need for low ALA suggests that mechanisms may exist by which LA can moderate or inhibit energy production pathways, and conversely ALA uprates energy production.

In hibernation, if LA oxylipin-promoted PPAR gamma-related peroxisomal beta-oxidation is a primary source of substrate for both energy and repair, potentially, high-level

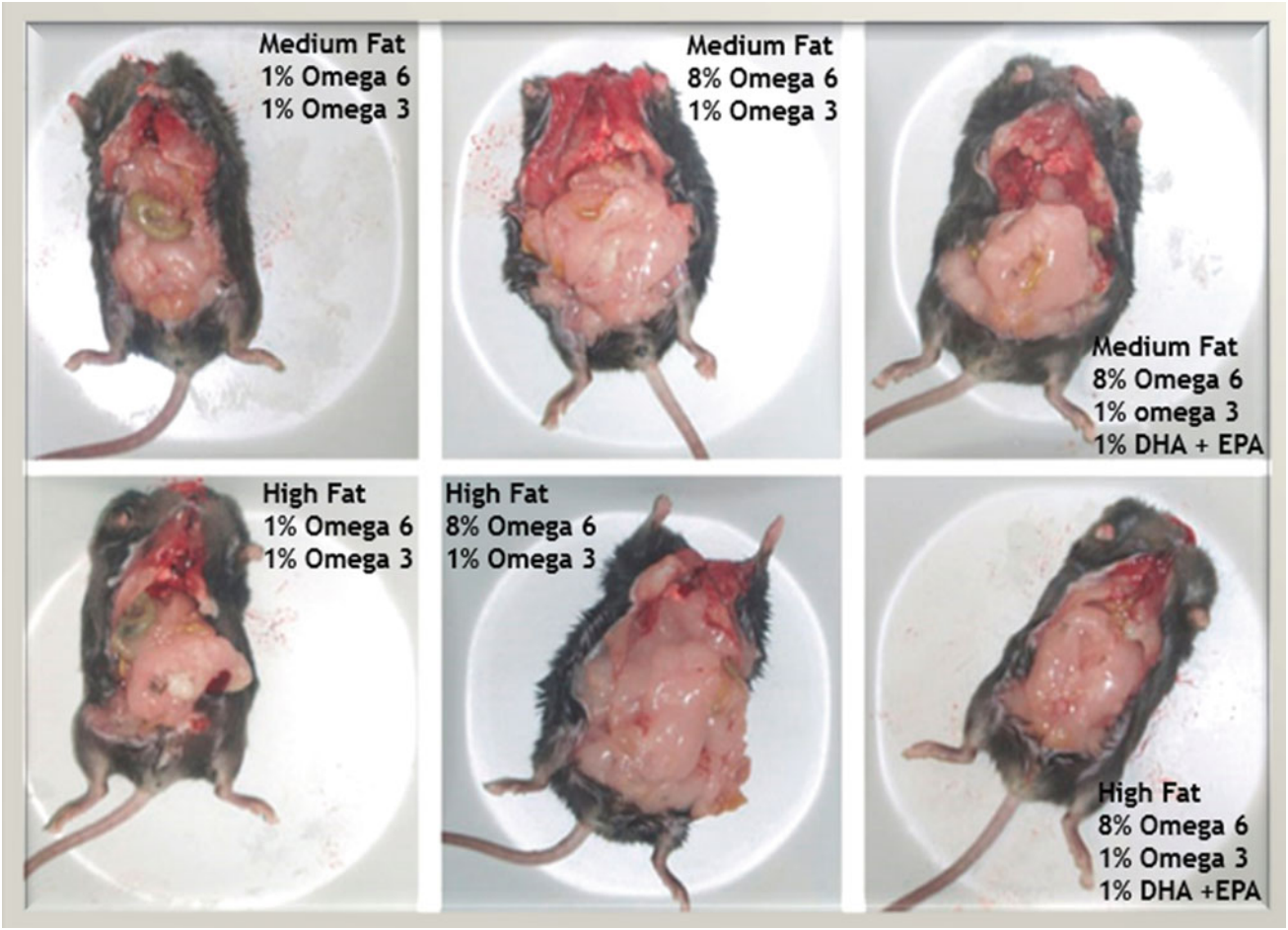

Fig. 31.3 "Reducing dietary LA to 1 en% prevents adipose tissue accumulation and reverses the obesogenic effects of a high-fat (60 en%) diet. Animals fed 8 en% LA accumulated more fat than animals fed 1 en% LA. The addition of 1 en% n-3 EPA/DHA to 8 en% LA diets prevented the increase in adipose tissue seen in animals fed 8 % energy LA. The obesogenic properties of a high-fat diet (60 en% fat) were reversed by selective reduction of LA from 8 to 1 en% and replacement by greater saturated fat. The animals shown are representative for the animals in each dietary treatment. *Upper row* isocaloric medium-fat diets of 35 en% fat, *lower row* isocaloric high-fat diets of 60 en% fat. The fatty acid composition, not total fat calories, determined the obesogenic properties of the diets. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid" of "Dietary linoleic acid elevates endogenous 2-AG and anandamide and induces obesity" [15] with very grateful thanks to the authors for the permission : Alvheim A, Malde M, Osei-Hyiaman D, Lin Y, Pawlosky R, Madsen L, Kristiansen K, Frøyland L, Hibbeln J. Obesity (Silver Spring). 2012 Oct; 20 (10): 1984–1994

activation of PPAR gamma pathways by redirecting lipid beta-oxidation through the peroxisomes, and a significant proportion of the ACoA product to repair and maintenance, would reduce mitochondrial fuel substrate availability thus lower mitochondrial ATP production, as well as increasing tissue levels of endogenously produced Omega-7 and Omega-9 fats and synergistically lowering LA, which will impact on cell membrane including cardiolipin composition, thereby potentially further reducing ATP-related metabolism, as well as directing energy to peroxisomal based thermogenesis.

Effect of LA on Metabolism Including in Metabolic Syndrome Through Induction of Mitochondrial Inhibition

A combination of excess LA, preferential lipolysis of it from adipose tissue, lack of activation of the PPAR alpha pathways by energy deficit stress or Omega-3 ALA, over-activation of PPAR gamma-related pathways, increased oxidative stress and creation of substrate including cholesterol and lipids to support tissue repair means that mitochondria and wider cells are exposed to 24/7/365 oxidative

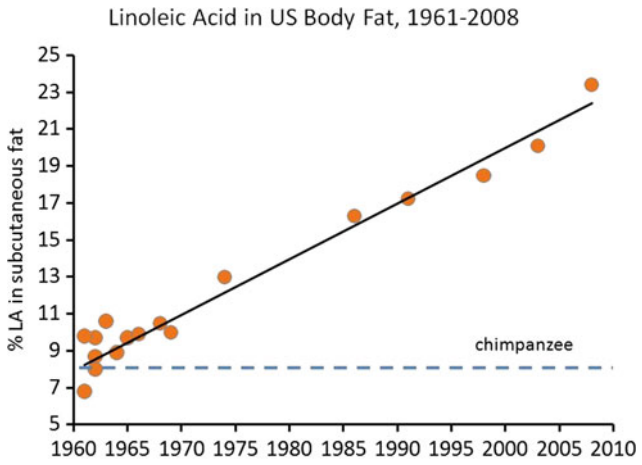

Fig. 31.4 A thought-provoking graph, which has been revisited and added to by Guyenet in a recent paper [41, 42]. The inclusion of the chimpanzee data by the author was indicational only; also, the chimp may have been captive. The graph titled "Linoleic Acid in US Body Fat, 1961–2008" is from his blog, with very grateful thanks to the author for the permission: Guyenet S. Whole Health Source Blog Seed Oils and Body Fatness—A Problematic Revisit http://wholehealthsource.blogspot.com/2011/08/seed-oils-and-body-fatness-problematic.html

stress and oversupply of lipid substrate for cellular maintenance leading to inflammation, immune activation, lipid compositional change, mitochondrial dysfunction and ultimately metabolic syndrome-related obesity.

Activation of Oxidised LDL Receptors in Adipose Tissue

LDL receptors such as CD36 and OLR1 are activated by oxidised LDL. OLR1 is additionally activated by AGEs, apoptotic cells and gram-positive and gram-negative bacteria, as well as by PPAR gamma. PPAR gamma "master controller of adipogenesis" is activated by oxidised LA products such as the HODEs, which are delivered by LDL.

Adipose Tissue—A Store of and Regulator of Blood Cholesterol?

As discussed LDL is a major lipid delivery system to adipose tissue. Significant cholesterol from LDL is stored in fat tissue. In obese humans, adipose tissue contains up to half of the cholesterol in the body [43]. This suggests a need to also consider adiposity when assessing fasting blood cholesterol levels and oxidative stress status.

As noted, fat and epithelial cells may act as a sink that helps regulate levels of oxidised and non-oxidised LDL, as well as cholesterol levels in the blood. Interestingly, HDL may also be involved in adipose lipid transport both to and from adipose tissue.

PPAR Gamma Master Adipogenic Controller

LA oxylipins, the HODEs, are the primary endogenous activators of PPAR gamma. During significant activation, PPAR gamma signals for tissue creation including adipogenesis, related immune-type function, and fat and cholesterol production and deposition.

PPAR gamma-related peroxisomes pathways are active in adipose tissue, will likely have roles in immune function and provide a pathway for creation of substrate for repair and to support oxidative stress production including phagocytosis.

LA in Fat Tissue Relevance of PPAR Gamma, LA, HODEs

PPAR gamma, LA and LA products, such as HODEs, synergistically in concert drive LA and wider fat deposition, including oxidative stress-based messengering, increased insulin production, reduced metabolic rate, increased fat and cholesterol production, Omega-7 and Omega-9 desaturation, increased LDL creation transport storage and vascular uptake, and other related mechanisms:

- Oxidised LA products such as 9 and 13 HODE, with the Oxo-HODEs, are the primary endogenous activators of PPAR gamma [44], which is considered a master controller of adipogenesis [45], and possibly obesity [46].
- PPAR gamma promotes, preadipocyte differentiation, cell growth and related activity.
- PPAR gamma crosstalks with insulin and thyroid signalling [47].
- PPAR gamma interacts with LOX15 pathways [48].
- PPAR gamma inhibition reduces obesity [49]. PPAR gamma null adipose tissue mice display diminished weight gain [50].
- PPAR gamma promoters increase obesity.
- PPAR gamma has roles in inflammation increasing expression of genes related to immune function, and tissue destruction, creation and repair, influences macrophage function and is associated with uprating of iNOS activity.
- PPAR gamma is also activated to a lesser extent by AA products such as 15-HETE and 15-dPGJ2.
- PPAR gamma promotes lipoprotein lipase activity, thereby increasing adipose fat uptake rates in rats [51].
- LA in cardiolipin, oxidised in situ to 13 HODE and derivative 4HNE, inhibits ATP production, slowing

metabolism and diverting energy resources that would otherwise be used for ATP creation, to fat production, as seen in metabolic syndrome.

- Increases in LA oxylipins uprate; SCD1 activity so OA and POA production; HMG-CoA reductase so cholesterol production and LDL output by ultimately promoting and facilitating creation in the liver of the major elements of LDL.
- Oxidised LDL receptors such as CD36 and OLR1 are both active in adipose as well as in cardiovascular tissue.
- PPAR gamma dramatically increased the activity of OLR1 [52], so likely the uptake of oxidised LDL including negative atherogenic subtypes.
- PPAR gamma increases CD36 expression. CD36 takes up unoxidised LDL in significant amounts, as well as oxidised LDL. CD36 appears to have a significant role in delivery of fats by LDL to adipose tissue and insulin beta cells, logically having a role in obesity, and adipose immune function. A CD36 null mouse has 40 % less fat [31]. Interestingly in the fed state, as much as 70 % of energy uptake of the heart is via LDL receptor CD36.
- PPAR gamma activation interacts with leptin, which also interacts with CD36. Leptin levels are doubled in an LDL receptor CD36 null mouse, which has impaired fat uptake, a 40 % decrease in fat mass, and is protected against weight gain [31]. Inhibition of LDL uptake also decreases oxidised product uptake, so reducing oxidative stress, LA oxylipin adipose tissue activation and adipogenesis.
- Adipose tissue acts as a depot for oxidised lipids and cholesterol products, helping regulate oxidised products, and cholesterol levels, in the bloodstream, but at the cost of increased long-term oxidative stress potential. In the obese, increased storage of oxidised product, and/or immune activity, may be factors in increased fat cell death rates and related macrophage activity [53].
- Expression of genes relating to fat uptake including CD36 may differ between genetic groups [54], potentially meaning that some groups are selectively more sensitive to the effects of dietary LA. Polymorphisms could be an evolutionary adaption, to optimise fat stores for successful pregnancy.
- In excess, COX2, 13HODE, 4HNE and other oxidised products of LA, and via activation of PPAR gamma [55], will promote angiogenesis [56–58], inflammation and the new tissue growth required to increase adipose cell tissue storage capacity.
- Oxidised AA products are endogenous cannabinoid activators, thereby promoting appetite, activating adipose CB1 receptors and encouraging fat deposition [2]. It would make evolutionary sense that the copious presence of seasonal otherwise scarce nutrients, essential to reproduction, promotes mechanisms that increase appetite. Interestingly, LA is also central to reproduction and energy storage in plants.
- In rats, in a study by Lai, LA strongly stimulates insulin secretion by factors of between fourfold and tenfold in a dose-dependent manner. "*All the results suggested that unsaturated fatty acids stimulated insulin secretion and additively increased glucose-induced insulin secretion in the perfused rat pancreas*". "*Linoleic acid alone stimulated a 391 % increase in the peak insulin concentration compared with the baseline in the rats fed a normal diet*". Long-term intake led to depressed insulin function [59] (see Figs. 31.5 and 31.6). It would make sense that LA costimulates insulin release with glucose, given insulin increases fat deposition and that glucose and LA are seasonally available coproducts in some plant reproductive materials.
- LA is a preferred peroxisomal substrate, and LA oxylipins, the HODEs, are the primary endogenous activators of PPAR gamma-related peroxisomes. Peroxisomal ACoA production can be used to form malonyl-CoA (Randle cycle), thereby blocking entrance of long-chain fats into the mitochondria including of beta-insulin cells as well as impacting insulin

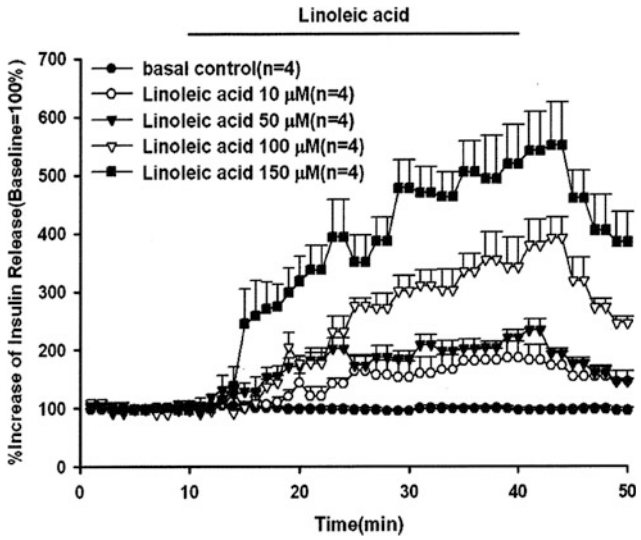

Fig. 31.5 "Effect of linoleic acid on insulin release. In pancreatic perfusion experiments, an equilibration period of 20 min preceded time 0. After a baseline period of 10 min, linoleic acid (10, 50, 100 and 150 μM) was administered for 30 min followed by basal medium (KRB) perfusion. *The horizontal line* indicates the presence of linoleic acid. Values are means ±SE (n = 4). Baseline effluent concentrations of insulin were 4172 ± 277, 1660 ± 352, 1910 ± 172, 1199 ± 127 pg/ml and 4271 ± 1739 pg/ml for the control group and 10, 50, 100 and 150 μM linoleic acid groups, respectively" from "The Natural PPAR Agonist Linoleic Acid Stimulated Insulin Release in the Rat Pancreas" [59] with very grateful thanks to the authors for the permission: Lai M, Teng T, Yang C. J Vet Med Sci. 2013 Nov; 75(11): 1449–1454. Diabetes Obes Metab. 2012 Nov;14 (11):1010-9

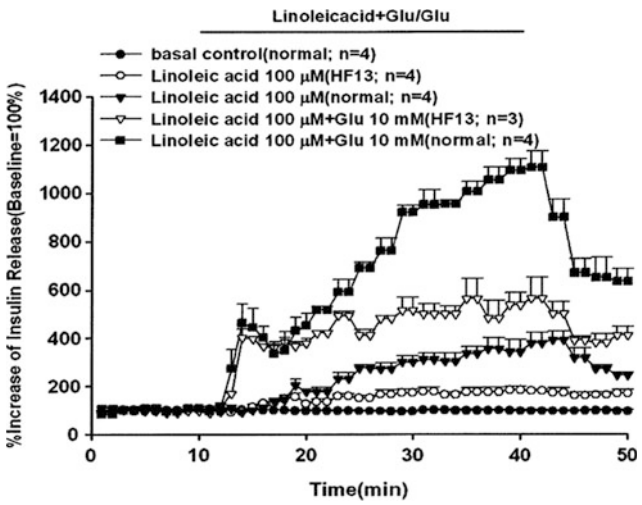

Fig. 31.6 "Effect of linoleic acid with or without glucose on insulin release in rats fed a high-fat diet for 13 weeks (HF 13). In pancreatic perfusion experiments, an equilibration period of 20 min preceded time 0. After a baseline period of 10 min, 100 μM linoleic acid with or without 10 mM glucose was administered for 30 min followed by a basal medium (KRB) perfusion. *The horizontal line* indicates the presence of linoleic acid and glucose. Values are means ±SE (n = 4). Baseline effluent concentrations of insulin were 4172 ± 277, 6463 ± 2373, 1199 ± 127, 3178 ± 318 pg/ml and 1626 ± 298 pg/ml for the control, linoleic acid (high-fat diet), linoleic acid (normal diet), linoleic acid with glucose (high-fat diet) and linoleic acid with glucose (normal diet) groups, respectively" from "The Natural PPAR Agonist Linoleic Acid Stimulated Insulin Release in the Rat Pancreas" [59] with very grateful thanks to the authors for the permission: Lai M, Teng T, Yang C. The Natural PPAR Agonist Linoleic Acid Stimulated Insulin Release in the Rat Pancreas. J Vet Med Sci. 2013 Nov; 75(11): 1449–1454. Diabetes Obes Metab. 2012 Nov; 14 (11):1010–9

regulation through related increases in peroxide so oxidative stress levels.

- Long-term high LA feeding, and/or release of stored LA from adipose tissue, may cause insulin insensitivity by a number of mechanisms, including long-term over-activation of insulin production, oxidative damage to beta cells including loss of mitochondrial function and beta-cell lipid build-up.

ALA and Relevance of PPAR Alpha

LA GLA and ALA in that order, followed likely by POA and PA, are the preferred substrates of the peroxisomes and, due to differences between mitochondrial and peroxisomal enzymes pathways [60], are not optimal fuel in the mitochondria, but are preferential substrates in peroxisomes.

PPAR Alpha Is Activated Primarily by Energy Deficit Stress Including Exercise and Fasting

Energy deficit in fasting, or during exercise [61], strongly activates genes related to lipid metabolism, including PPAR alpha and delta peroxisomal related energy pathways, promoting the beta-oxidation of fats, particularly ALA and LA, as well as other substrates such as prostaglandins and oxidised lipids, to short fats and ACoA, which can both ultimately be used to fuel mitochondria. Acute exercise raised PPAR alpha for 24 h; several exercise training sessions lead to sustained increase [62].

Energy deficit induction of PPAR alpha and delta pathways promotes the direction of the ACoA substrate produced by peroxisomal beta-oxidation of ALA and LA, to energy production, by uprating energy production-related genes in energy demanding tissues such as the liver, heart and brain.

Roles of PPAR Alpha and Differences with PPAR Gamma

Differences between PPAR alpha and gamma include the following:

- In contrast with PPAR gamma, activation of PPAR alpha is associated with increased energy output.
- PPAR alpha activation is associated with HMG-CoA reductase downregulation, but HMG-CoA synthase upregulation, thereby promoting ketogenesis rather than cholesterol pathway activity [63].
- Peroxisomes shorten long fats to MCFs, which are a preferred mitochondrial fuel, and are probably used as such, both in energy-related PPAR alpha activities and in PPAR gamma repair scenarios.
- MCFs in some tissue may enter mitochondria without carnitine.
- Peroxisomal product ACoA can be used to make malonyl–CoA, thereby inhibiting carnitine and creating a feed-forward loop increasing peroxisomal beta-oxidation of long-chain fats to short-chain fats and ACoA, which can then be directed to either energy, or repair, and/or reinforcing the malonyl-CoA blocking of carnitine.
- ACoA, when produced by PPAR alpha-stimulated peroxisomes, is directed by synergistic increases in activity of genes promoting mitochondrial-related activity, to mitochondrial energy pathways, rather than to tissue repair substrate.
- Products of ACoA include acetate and malate, which with short fats, open up the potential to alternative

mitochondrial fuel pathways by passing pathway blockages, including of CPT1 pathways, by malonyl-CoA and/or glucose pathway inhibition.
- PPAR alpha null mice on a high-fat diet have difficulty in maintaining energy in fasting, developing fatty liver and hyperlipidaemia [64].
- In rabbits, ALA from flaxseed is associated with increased leptin production, which may help regulate appetite [65].

ALA in Human Fat Tissue

In contrast to LA, limited ALA is stored in human adipose tissue, usually under 1 %; adipose tissue of American postmenopausal women contained 0.77 % ALA; in comparison, LA content was 17.23 %. Radiolabelled ALA studies in rats showed a high proportion (60 %) was beta-oxidised to carbon dioxide within 24 h. In humans, between 16 and 20 % was converted to carbon dioxide in 12 h.

In humans, dietary ALA increases ALA levels in plasma, LDL and HDL [66], which ultimately must end up in tissue to be stored, incorporated or beta-oxidised. ALA will alter cell membrane composition and cell function.

Dietary ALA will also improve the ALA, EPA and DHA profiles of plasma [67, 68]. ALA at high intakes may also increase ALA in adipose tissue [69, 70]. Balancing LA to ALA intake is known to alter cellular membrane tissue phospholipid composition, so function including that of muscle and nerves [71].

The lack of ALA in adipose tissue is due to; low dietary intake and availability in dietary triglycerides compared to LA, low dietary intake of green material, high utilisation by peroxisomes in the liver, low availability for incorporation into LDL and preferential lipolysis release from adipose tissue. Adipose ALA might be higher in humans on less industrialised diets. Interestingly, the ALA content of adipose tissue is much higher in some monogastric animals such as 'wild' horses.

Many questions remain to be answered about the role of ALA in energy pathways in different species, and the implication of ALA deficiencies in humans; these issues assume greater importance given western dietary changes and those imposed on our livestock,

ALA in Monogastrics and Other Animals

Significant amounts of ALA are seen in monogastric animals, chickens [72], rabbits, horses and pigs [73], if fed forage and/or ALA supplemented feed [74]. Ruminants however will have lower LA and ALA in adipose tissue than non-ruminants as they are metabolised by gut bacteria, reducing availability for storage. It appears that ALA storage in ruminants is more dependent on the phenolic content of pastures, which alter rumen bacterial function, than ALA content [75].

Adipose tissue of grass-fed horses, non-ruminant monogastrics, contained 17 % ALA and 4 % LA; in comparison, adipose tissue in oat-fed horses contained 2 % ALA and 22 % LA. High levels of ALA in adipose tissue were also seen in other equine studies, including ALA of 24.3 % and LA of 7.6 % in grass-fed Galician horses [76], genetic relics of the Iron Age.

Galician horses allowed to freely graze pasture and woodland had significantly more PUFA in intramuscular dorsal fat 40.7 % compared to those fed partially on concentration 25.2 % [77]. However as in humans, only trace amounts of ALA were found in ruminant brains. A significant proportion of the dietary ALA of grass-fed horses logically originated in green material mainly from galactolipids, with some in phospholipid form.

In rats, supplementation with ALA will address cardiac tissue *n* 3:6 imbalances [78], bringing ratios from 10–13 to 2–3, whilst DHA brings ratios back closer to 1:1.

The accumulation of ALA in mares' milk (38.4 %% ALA Erasmus p. 229) is also highly thought-provoking. Frozen animal tissue from the Upper Palaeolithic and Neolithic shows similar ALA trends; adipose tissue contained significantly more ALA than LA [79]. Interestingly, it has been suggested that in the 1960s, human milk had an LA: ALA ratio of 3:1 [80] (Dhopeshwarkar p. 68).

Linseed in feed significantly increases ALA in chicken [81]. ALA intake in rabbits increases ALA content in muscle [82] and in retroperitoneal adipose tissue. In pigs fed linseed, ALA in back fat increased from 0.87 to 4.9 %, presenting opportunities for improving ALA in the food chain through supplementation of monogastrics. The rise of ALA in pigs begs the question if a similar effect may be seen in humans, and if so at what intake and in what form?

Potential Use of Peroxisomal Beta-Oxidation of ALA to Provide Substrate to Mitochondria

In horses, monogastrics, during prolonged energy demand combined with inability to stop to feed, during flight or migration, given ALA is likely preferentially released from adipose and intramuscular fat tissue, is adipose-derived ALA significantly used via the peroxisomal pathways for energy production.

Humans might have a greater capacity to utilise the peroxisomal pathways for energy generation from stored polyunsaturated fat than currently contemplated. It is not

clear if significant ALA combined with reduced LA intake would appreciably change ALA in human adipose tissue [83, 84], or what ramification that may have.

ALA, LA and NAFLD

ALA is associated with increased beta-oxidation [85], reduced LDL synthesis [86] and lower NAFLD [87, 88]. In contrast, LA and its oxidised products, such as 9 and 13 HODEs and Oxo-HODEs, are associated with increased NAFLD, early-onset non-alcoholic steatohepatitis and type 2 diabetes in adolescents [89]. Lowering of oxidised LA lipid products reduced NAFLD [90]. LA is also associated with increased LDL production, increased vascular LDL uptake and net lowered plasma levels.

ALA Uprated Metabolism, Thermogenesis and Reduced "Feed Efficiency" in Livestock

ALA-fed animals also tend to exhibit lower "feed efficiency", thereby reducing weight gain, which is consistent with ALA at higher intakes possibly uprating metabolism including thermogenesis through peroxisomal pathways. Peroxisomes produce heat substrate peroxide and ACoA, rather than the ATP and CO_2 produced by mitochondria. ALA in adipose tissue falls during weight loss [91]. Dietary ALA may in some circumstances assist weight loss and has potential health benefits.

Dietary Absence of ALA

If ALA is not in the diet, it is also not available for onward conversion to EPA and DHA. Imbalance in the intake of dietary LA:ALA, poor desaturase function, other dietary imbalances and/or low EPA DHA intake will result in Omega-3: Omega-6 imbalances in plasma and tissues, including cardiac phospholipids and mitochondria. Levels of ALA in many of the foods commonly consumed are low (see Fig. 31.7).

Oxidative Stress and Obesity

Obesity and oxidative stress are cofactors in many Western diseases, including impaired neurological development and function, and in particular will damage the pancreas and thyroid, leading to further imbalances in metabolic signalling, and also impact the PPAR gamma-related RXR transcription pathways, which also interact with the insulin and thyroid receptors.

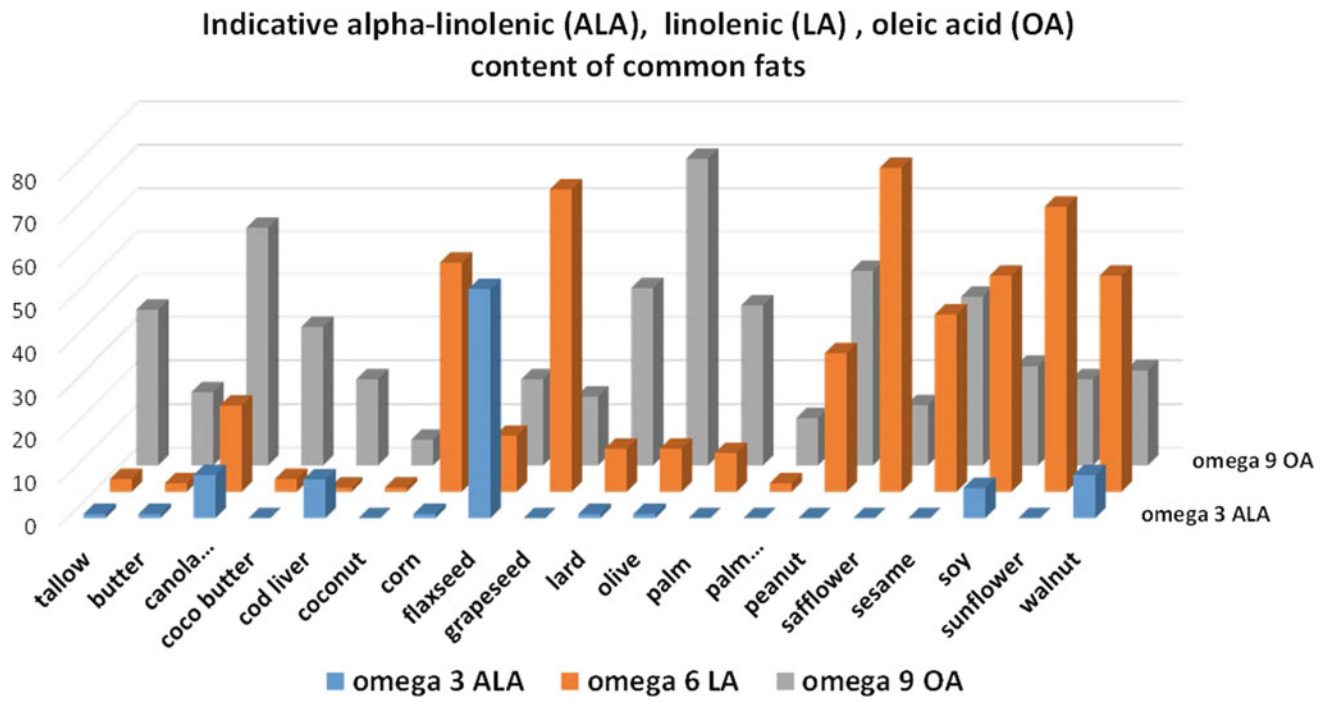

Fig. 31.7 The efficient growing and extraction of seed oils, combined with market demand, have led to the significant increases in dietary intake. Seed oils often contain a large percentage of LA. Many contain trace amounts of ALA, but few contain significant amounts. The content of ALA, LA and OA in oils will vary. The figure for cod liver oil includes the long-chain Omega-3s EPA and DHA

Ultimately excessive oxidative stress will lead to LDL derived accumulation of oxidised detritus, in adipose tissue, as well as other tissue including beta cells, and impair the ability of fat cells to mature and function, leading to deposition of fats randomly into other tissues including intramuscularly, as seen in ageing [92].

On the other hand, fresh dietary antioxidants imported into adipose tissue by LDL may help to protect stored polyunsaturates and cholesterol from further oxidation and potentially reduce release of LA oxylipins and oxysterols back into the bloodstream during fat lipolysis, and so begin to break the self-reinforcing cycle of oxidative stress-based obesity, but in their absence, the cycle of oxidative stress and fat deposition will continue unbroken.

ALA as a Competitive Substrate for LOX 12/15

LOX pathways are active in adipose-related tissues and have a role in obesity including being "*causally related to the development of insulin resistance in obesity* via *generation of proinflammatory metabolites*" and are increased in diabetes. LOX 12/15 metabolites were measured in subcutaneous and omental fat; "*Linoleic acid (LA) showed 10-fold higher values compared with AA and 100-fold higher than DHA*". 13HODE(S) was found to be the most abundant metabolite in adipose tissue [93].

ALA followed by LA in an animal model is the preferred substrate of LOX12/15, will displace LA LOX12/15 products and also lead to some displacement of COX AA prostaglandin products. ALA supplementation, or release from tissue, by competitive reduction of the capacity of LOX12/15 and COX enzymes to oxidise LA, and formation of oxidised products of ALA, will alter cellular function including LA-related peroxisomal inflammatory, immune-related pathways, and adipose-related fat deposition.

Given ALA is the preferred substrate of the LOX 12/15 enzymes and preferred fuel of the peroxisomes, dietary ALA deficiencies are likely to have significant physiological effects; excessive dietary LA intake is likely to magnify those effects.

LA Oxylipins the HODEs Primary Endogenous Activators of PPAR Gamma Are Associated with Obesity and Diabetes

LA oxylipins the HODEs are the primary endogenous activators of PPAR gamma, a master controller of obesity; PPAR gamma will upregulate VDLD and so LDL production and increase adipose uptake of minimally and highly oxidised LDL through activation of LDL receptors CD36 and highly oxidised LDL through OLR1, promoting obesity.

The obese have higher levels of 9 and 13 HODE than controls; levels fall with weight loss [94]. "*The basal concentrations of TBARS, 9-HODE, 13-HODE, … in the obese were significantly greater than those in normal subjects*" [94].

A Mediterranean diet reduced 9 HODE in premenopausal women in a "*dietary quality*"-related relationship; vegetables, whole grain, fruits, nuts, lower meat consumption and higher Omega-3s have significant effect; the Omega-6 intake was fairly consistent between the groups, possibly around 6 % of calories, which is lower than many [95]. Interestingly Italian centenarians had low 9 and 13 HODEs [96].

Adipose Tissue, Macrophage Content and Related Immune Including Cytokine Signalling Capacity

Adipose tissue provides a reprocessing facility for oxidised material, pathogens and general polar detritus delivered by LDL together with lipids, iodine, and lipid-soluble antioxidants to adipose tissue, and endocyted by activation of related LDL receptors.

The obese likely have higher levels of oxidised lipids in their fat stores, particularly LA and cholesterol, which will be released during weight loss, but lower stores of, so release of antioxidants [97].

Lean adipose tissue contains around 10 % macrophages, whereas in the obese "*inflamed M1-type macrophages, make up as much as 50–60 % of adipose tissue stromal cells*" [53]. As discussed, HODEs promote PPAR gamma activation. PPAR gamma is "*robustly expressed*" in macrophages [98, 99].

Excess oxylipins and lack of antioxidants will increase adipose immune function including monocyte infiltration and inflammatory cytokine production, leading to systemic body-wide inflammation in epithelial tissues including those related to the heart and lungs [100], as well as uprated iNOS activity and related NO production so diminished catalase, thereby increasing peroxide presence, so creating self-reinforcing oxidative messaging cascades and signalling to add to fat stores, which will be sustained by LA and related oxidised product release from adipose tissue even after the reduction of current dietary LA intake. Adipose oxidised LA release, in the absence of adequate antioxidants, will perpetuate or indeed increase oxidative stress, creating a self-reinforcing oxidative stress-based feed-forward plant reproductive material LA- and glucose-related cycle of increased fat deposition and inflammation.

Correlation Between Obesity and Oxidative Stress

Oxidative stress is clearly a cofactor in Western disease and central to the mechanisms of adipogenesis and fat deposition signalling. "I*n nondiabetic human subjects, fat accumulation closely correlated with the markers of systemic oxidative stress. These data are in good agreement with recent studies* [101] (Framingham) *suggesting that systemic oxidative stress correlates with BMI*" [102].

A study in children suggests obesity is associated with circulating oxidised LDL, which was significantly higher in the obese compared to those of normal weight [103]. Oxidative stress was also raised in those with metabolic syndrome [104] and in obese Cameroonian subjects [105]. Antioxidant status showed an inverse relationship with adiposity in 3042 adults from Greece [106].

Fructose Synergy with LA and Fat Deposition

Fructose in quantity is only found in seasonal plant reproductive material usually with LA glucose and carbohydrates. The Western diet includes large amounts of refined LA, glucose–fructose (in pure or sucrose form) and carbohydrates.

Fructose intake above immediate glycogen need is primarily directed to fat storage and in animal models is more effective at increasing fat deposition than glucose [107]. Fructose in synergy with LA oxylipins and PPAR gamma-related pathways increases expression of lipogenesis-related genes, including SCD1, SREBP-1c, and FAS, and in synergy with LA appears to promote increased LDL production, supporting LA export from the liver to wider circulation and adipose tissue [108, 109].

Evidence that fructose promotes obesity in humans is less clear [110], although indications begin to emerge that excess fructose consumption is a wider health issue [111]. Fructose may also factor in oxidative stress. A small randomised paediatric pilot study using a low fructose diet compared to control saw a reduction in levels of oxidised LDL [112].

Fructose is much more easily oxidised than glucose and may increase baseline blood oxidative stress levels [113], oxidation of the components of LDL and internal and exogenous dietary AGEs so plasma AGEs [114]; oxidised LDL receptors CD36 and OLR1 are activated by AGEs; high AGEs are a health issue, for example in ageing and chronic kidney disease [115].

LA and Glucose in Nature Are Found in the Same Foods, but Available Independently in Refined Food; the Metabolic Consequences

Evolutionary metabolic design, based on normal food availability and oxidation states, has led to the creation of a regulatory system, for the direction of substrate to energy or lipid creation that is based on both glucose (as sucrose or simple carbohydrate) and LA, being seasonal, found together as cocomponents in plant reproductive material and present in a limited range of proportions. The body is not expecting either glucose or LA in significant long-term disproportion or imbalance, or for them to be heavily pre-oxidised, or for them to be without the accompanying nutrients and antioxidants found in nature.

Arguably, the disassociation in refined food of the supply of LA and glucose, combined with heavy oxidative stress, lack of antioxidant capacity, and excess supply of either one or both, are factors in metabolic dysbiosis including obesity, diabetes and metabolic syndrome.

LA and glucose, directly or indirectly, impact both peroxisomal and mitochondrial beta-oxidation pathways. Peroxisomes activated by PPAR alpha, gamma and delta are present in beta-insulin cells, and in wider tissues including muscle.

- PPAR gamma is increased under hyperglycaemic and hyperlipidemic conditions, including through activation by LA HODEs.
- PPAR alpha is increased in fasting and exercise.

Both in wider tissue and beta insulin cells the control of mitochondrial beta-oxidation of fatty acids, and glucose-derived pyruvate, is linked, and likely mainly controlled through the production of ACoA, a substrate for malonyl-CoA, which binds to and inhibits CPT1, thereby restricting entry of long-chain fatty acids into the mitochondria (Randle cycle). ACoA is produced via pyruvate from glucose but also by the peroxisomal beta-oxidation of fats:

- ACoA produced by PPAR gamma-related peroxisomes will be directed by related gene activation to repair and renewal, including lipid and cholesterol production, as well as malonyl-CoA production. Excess LA oxylipins the HODEs are the primary activators of PPAR gamma. Insulin increases PPAR gamma expression [116].
- ACoA produced by PPAR alpha- and delta-related peroxisomes will be directed by related gene activation into

the energy pathways for beta-oxidation; PPAR alpha synergistically uprates CPT1 activation and gluconeogenesis pathways [117].

In the preagricultural world, where LA and glucose are found together in quantity in seasonal plant reproductive related food, when plant reproductive related dietary glucose availably seasonally falls. So conjointly will dietary availability of LA, and vice versa, as a result ACoA derived from both; glucose metabolism; and LA via peroxisomal oxidation; will fall, food energy intake will be again redirected away from seasonal fat deposition towards the energy pathways in part through the lifting of malonyl-CoA inhibition of carnitine and so again allowing mitochondrial access to long chain fats for mitochondrial beta-oxidation.

In contrast, in the modern world of refined foods, dietary change may result in a glucose fall, but LA levels could remain high due to independent consumption of refined oxidised LA sources, and or industrialised LA rich non-ruminant meats, which would have been virtually impossible in a natural dietary scenario where foods are never split into their component parts, and animals never raised almost exclusively on grains, as happens in modern food refining and processing, and agriculture.

LA oxylipins the HODEs will drive the PPAR gamma-related peroxisomal beta-oxidation production of ACoA, which will keep malonyl-CoA levels high, resulting in continued insulin resistance, even following a fall in glucose. Arguably, ACoA derived from PPAR gamma-related peroxisomal beta-oxidation is a missing piece in the jigsaw of insulin resistance pathways.

Both Glucose and LA Raise Insulin

In the preagricultural world, large amounts of oxidised LA in the diet would be a reflection of high levels of plant reproductive material and accompanying carbohydrates, and well-nourished animal-based foods. Excess calories would be available, and fat accretion in a time of plenty would be a sensible strategy.

As might be expected based on the above scenario, LA (and other lipids) stimulates insulin release, albeit to a lesser extent than glucose. Long term in excess, a mix of dietary LA intake and/or release of LA from LA-rich fat cells will contribute to permanently higher LA in plasma, so stimulation of beta cells and consequent stimulation of insulin release which will enhance fat storage and/or contribute to insulin resistance.

Beta-Cell-Related Insulin Resistance, INOS, NO, PPAR Alpha, Delta and Gamma, and LA Excess

Factors connected to Western diet, such as excess oxidative stress, low antioxidant status [118], mitochondrial damage, imbalances in fat metabolism, excess peroxide, LA oxylipins 13HODE and 4HNE, combined with low glutathione [119], and lack of PPAR delta and alpha [120] activation have all been implicated in loss of beta-cell function. Beta-cell malfunction is associated with oxidative stress, lipid accumulation and mitochondrial damage [121].

Insulin beta (and thyroid) cells have restricted catalase productivity [122], making them particularly susceptible to oxidative stress including iNOS-related NO catalase inhibition. Low selenium [123, 124], and cysteine [125, 126], would compound low beta-cell (and thyroid) antioxidant capacity by inhibiting glutathione production [127, 128]. Peroxisomal peroxide production, combined with low catalase output, is likely an important factor in lipotoxic damage of insulin beta cells [129].

iNOS appears central to beta-cell function including the regulation of insulin release [130], and possibly through NO-related blocking of catalase so increased peroxide availability. iNOS and NO also participate in apoptosis [131]. Could PPAR gamma stimulation of iNOS and related NO blocking of catalase explain lower catalase production as observed in 'stressed' beta cells.

LDL receptor CD36 is active in beta cells [132, 133] and, as in the heart and adipose tissue, likely is an important LDL transporter during the postprandial state, so significant amounts of the intact content of LDL, including oxidised LA products, such as 4HNE and 13HODE and lipid-soluble antioxidants, will be endocyted, so linking beta-cell oxidative status to that of the wider bloodstream and hence ultimately to dietary factors, including oxidised lipid and lipid-soluble antioxidant intake. Further, 13 HODE and related oxylipins in LDL would promote PPAR gamma rather than PPAR alpha activation in beta cells.

PPAR gamma in excess would likely direct ACoA produced by peroxisomal oxidation of fats supplied by LDL including LA, to the production of lipids and cholesterol, and likely long-term result in lipid accumulation [134], increased oxidative stress, and damage to beta-cell mitochondria via cardiolipin oxidation, which are hallmarks of insulin beta-cell degeneration [135].

Conversely, PPAR alpha and delta activation would be protective. In beta cells, in common with the wider body, PPAR alpha is increased during fasting. In beta cells, in common with the wider body, short-chain fats and ACOA

produced by PPAR alpha- and delta-related peroxisomes are a possible source of "non-damaging" fuel for pancreatic cell mitochondria. PPAR delta which appears central to the response of insulin cells to unsaturated fats is protective [136, 137], displays synergy with the RXR receptors, decreases fat accumulation so lipotoxicity [138] and increases mitochondrial function [139].

In contrast long-term constant LA oxylipin PPAR gamma activation in beta-insulin cells, increased intracellular beta-cell lipid deposition, increase peroxide tone combined with LA oxylipin-induced oxidation of cardiolipin, will lead to beta-cell dysbiosis.

Role of Malonyl-CoA, and ACoA in Regulation of Insulin, and Partition of Fats to Peroxisomal Beta-oxidation

"*Lipid metabolism in the beta-cells is critical for the regulation of insulin secretion*" [140]. PPAR gamma-related peroxisomal activities, production of ACoA, raised malonyl-CoA and related raised peroxide-based oxidative stress, may factor in control of insulin production. Increase in malonyl-CoA precedes insulin production, increasing threefold on exposure of cloned beta-insulin cells to glucose. Genetic inhibition of malonyl formation reduced glucose-stimulated insulin secretion (GSIS) by 40 %.

It has been suggested that "*malonyl-CoA mediates the switch from fatty acid catabolism to lipid synthesis during glucose stimulation of beta-cells*" [141]; possibly of significance LA oxylipin-stimulated PPAR gamma-related peroxisomal beta-oxidation product ACoA would logically be directed by PPAR gamma-related gene activation to lipid including cholesterol production, contributing to beta-cell lipid accumulation. MCF products of peroxisomal beta-oxidation would lead to cellular inclusion of MCF as observed.

It may be that malonyl-CoA production consequential on PPAR gamma ACoA production during peroxisomal beta-oxidation of lipids, as well as that following glucose metabolism, and peroxisomal peroxide iNOS activation so NO blocking of catalase, are regulatory factors in insulin production control, as well as factors in the raised oxidative stress levels that underpin diabetes [142].

"Mitochondrial" Wider Tissue Insulin Resistance: Restricted Long-Chain Lipid Access to Mitochondria by CPTIA Malonyl-CoA Randle Cycle-Related Blocking

LA oxylipins (through PPAR gamma activation of peroxisomal beta-oxidation of LA to ACoA) and insulin/glucose pathways (via pyruvate and ACoA) [143] **both** increase malonyl-CoA. Reduction of insulin following falling dietary glucose levels, without a corresponding reduction in oxidised LA delivered by blood lipoproteins from dietary sources, or adipose storage, will mean that lipids are still prevented from entering the mitochondria by malonyl-CoA blocking [144] (Randle cycle). Where long chain fats are prevented from entering the mitochondria they may be directed to peroxisomal beta-oxidation, which in the case of PPAR gamma related activity will increase net peroxide so oxidative stress. Insulin production or release appears to require the presence of oxidative stress.

Consequently in those with high LA fat deposition, and/or high LA intake, combined with high oxidative stress due to Western refined food intake, dietary glucose and related insulin reduction will not result in normalisation of the uptake of lipids by the mitochondria, because LA oxylipin HODE based PPAR gamma-driven malonyl-CoA production will block long-chain lipids from entering the mitochondria, thereby ensuring through stimulation of PPAR gamma related peroxisomal oxidation continued mitochondrial related "insulin resistance".

Further as discussed, raised LA in plasma, of dietary, or adipose origins, delivered by LDL to beta-insulin cells, will raise insulin independent of glucose intake. The effects of high LA in adipose tissue will take years to adjust, the half-life of LA in adipose tissue being measured in years, the impact of which will be exacerbated by lack of ALA, low adipose antioxidant status and low plasma lipid-related antioxidant status.

Half-Life of Adipose Tissue and Implications for Long-Term Oxidative Stress

Based on the kinetics of LA, the half-life of LA in adipose tissue is 1–2 years or more; it takes 3–4 years to equilibrate significant dietary changes in LA intake [145, 146], varying by tissue; for example the half-life of breast tissue is months rather than years.

The long adipose tissue half-life of LA, possibly around 600 days, and so in the severely obese, potential ongoing release of LA and its oxidised products from adipose tissue over several years (even following dietary LA intake reduction), unless ALA:LA balances, antioxidant and pre-oxidised food intake are fully addressed, has significant implications for long-term Omega-3:Omega-6 blood lipid profile, so the potential even on calorie restricted diets of LA rich adipose tissue in the obese to create ongoing long-term oxidative and inflammatory LA based stress, which in the absence of adequate ALA and antioxidants and/or exercise and/or short term fasting will continue to promote oxidised LA related signalling for fat deposition.

This adds to the rationale for supplementation of ALA through high ALA seed or oil, to provide competitive substrate for the LOX12/15 enzyme, and substrate to stimulate PPAR alpha-related peroxisomal beta-oxidation.

The LA content of non-adipose tissues also varies with dietary intake, resulting in marked changes in the lipid composition of the "*small intestine, liver, testes, skeletal muscle and sciatic nerve*". The rate of change of phospholipids will be faster, but is still ultimately dependent on the balance and amount of LA and ALA in blood lipids.

Competition for Plasma Nutrients by Adipose Tissue—An Under-recognised Issue in Obesity

Adipose tissue requires nourishment and maintenance and can exceed 50 % of body mass in obese subjects; it is designed to acquire and store lipid-soluble nutrients to meet its optimal carrying capacity, which is as yet undetermined.

Competitive accretion by adipose tissue depletes serum levels of fat-soluble antioxidants in the obese. High levels of adipose tissue will impose significant additional metabolic nutrient demands on obese patients, who are somewhat ironically are already likely eating low nutrient content, highly processed and pre-oxidised, Western calorie dense diets.

Consequently, the obese likely have both lower intake/levels of and a higher functional need for fat-soluble or attached antioxidant factors, including vitamin D, iodine, and lipids such as beta-carotene [147] and vitamin E [148], to meet the greater metabolic needs of larger and more numerous fat cells, including as substrate for fat cell tissue creation, and to power increased immune activity.

"*Studies of extremely obese adults undergoing bariatric surgery have identified a wider array of pre-existing nutritional deficiencies prior to surgery*" "*Several large cross-sectional studies of obese and overweight children indicate that they may have lower concentrations of antioxidant vitamins, retinol and beta-carotene (vitamin A), as well as alpha-tocopherol (vitamin E)*" [149–151].

LA and Weight Loss in the Obese

Weight loss in the obese may require a combination of a nutrient-rich diet, calorie restriction and exercise. Over 8–10 weeks, 40 % calorie restriction in mice on a high-fat diet with a significant LA and lard component lost fat mass, experienced decreased oxidised lipid levels in the liver and reduced cytokines in circulation, compared to controls [152].

This again emphasises that health, including cardiac issues surrounding LA, is not simply about excess, but also is impacted by wider lifestyle factors including calorie restriction and exercise.

In the obese, a more rigorous reduction of LA, and inclusion of ALA, as well as EPA and DHA, exercise and calorie restraint, and nutrient-rich diet, including antioxidant factors, would likely be required to offset the diet-related inflammatory and obesogenic oxidative stress effects of the release of stored and oxidised LA and cholesterol from adipose tissue.

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32 The Crucial Relevance of ALA and LA as Primary Peroxisomal Beta-Oxidation Substrates, of Oxidised LA as the Primary Endogenous Activator of PPAR Gamma, and Energy Deficit as the Primary Activator of PPAR Alpha

Robert Andrew Brown

Terms	
AA	Arachidonic acid (omega-6; 20 carbon derivative of LA)
ACoA	Acetyl coenzyme A (raw material for the energy/cholesterol pathways)
ALA	Alpha-linolenic acid (omega-3 18 carbon plant-based polyunsaturated fat)
APOE	Apolipoprotein E (lipid transport signature protein)
ATP	Adenosine triphosphate (enzyme used as an energy carrier)
CD36	Cluster of differentiation 36 (fatty acid translocase receptor)
COX	Cyclooxygenase (enzyme catalysing oxidation of fatty acids)
CPT1	Carnitine palmitoyltransferase (acts as shuttle mainly for long chain fats C:16-18 into mitochondria)
DHA	Docosahexaenoic acid (omega-3 22 carbon derivative of ALA)
EPA	Eicosapentaenoic acid (omega-3 fatty acid C20:5)
GSH	Glutathione(s) (a very important antioxidant family)
GLA	Gamma-linoleic acid (omega-6 fatty acid C18:3)
HMGCoA	3-hydroxy-3-methyl-glutaryl-CoA (Found in two forms reductase and synthase. Reductase regulates cholesterol production. Synthase regulates HMGCoA production. HMGCoA is substrate for ketones or cholesterol)
HSL	Hormone-sensitive lipase (different forms mobilise lipids from triglycerides and esters including from adipose tissue)
iNOS	Inducible nitric oxide synthase (inducible isoform involved in stress response in macrophages/microglia and other tissues)
LA	Linoleic acid (omega-6 18 carbon plant-based polyunsaturated fat)
LOX5	Lipoxygenase (enzyme catalysing oxidation including AA and EPA)
LOX12/15	Lipoxygenases (enzymes catalysing oxidation of multiple lipid-based substrates)
LDLR	Low-density lipoprotein (LDL) receptor (LDL receptor for minimally oxidised LDL)
LPL	Lipoprotein lipase (mobilises lipids from Chylomicrons, VLDL, and LDL; both at the vascular face and intercellularly)
MCAD	Medium-chain acyl-coenzyme A (dehydrogenation of fats C:6-12 in mitochondria and present in inner mitochondrial membrane)
MCT	Medium-chain triglyceride (triglyceride containing fats between C:6 and C:12)
MCF	Medium-chain fat (a fat between C:6 and C:12)
NO	Nitric oxide (an important signalling messenger and oxidant)

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OA	Oleic acid (omega-9 monosaturated fat C18:1)
OLR1	Oxidized LDL receptor 1 (receptor for oxidised LDL sometimes called LOX1)
PA	Palmitic acid (saturated fat C:16)
PPAR	Peroxisome proliferator-activated receptor (3 forms alpha, gamma, and delta)
ROS	Reactive oxygen species (reactive molecules containing oxygen)
SA	Stearic acid (saturated fat C:18)
SCD1	Stearoyl-CoA desaturase (delta-9-desaturase so key to formation of OA)
SOD	Superoxide dismutase (reduces superoxide to oxygen or peroxide)
Wy14643	PPAR alpha activator (activates PPAR alpha-related peroxisomes)
4HNE	4-Hydroxynonenal (exclusive omega-6 fats peroxidation aldehyde)
9HODE	9-hydroxy-10E, 12Z-octadecadienoic acid (major LA oxidation product of LOX12/15, COX, photo-oxidation and autoxidation)
13HODE	13-hydroxy-9Z, 11E-octadecadienoic acid (major LA oxidation product of LOX12/15, COX, photo-oxidation and autoxidation)

The Crucial Relevance of LA and ALA to Peroxisomes

LA and ALA are central to peroxisomal function. Peroxisomes and their products are underappreciated partners fundamental to healthy function of energy pathways, oxidative pathways, immune function including likely supporting macrophage phagocytosis, detoxification, and lipid including cholesterol production pathways.

Crucially, LA and ALA are the preferred beta-oxidation substrates of peroxisomes:

- Oxidised LA products, such as the HODEs including 9 and 13HODE and oxo-HODE, in the context of a 'Western diet' are often the most common oxylipins in plasma, and the primary endogenous activators of PPAR gamma.
- The main PPAR alpha activators include; energy deficit stress, primarily activity including exercise or fasting; and/or secondarily 'excess' (greater amounts than needed for tissue maintenance) dietary including oxidised omega-3s, for example, large amounts of dietary ALA from grass in monogastrics; long chain omega 3s, including DHA from the food chain in marine mammals, which cannot be metabolised through mitochondria; It would make physiological sense to metabolise 'excess' dietary polyunsaturates to energy, both to make best use of available resources and to provide a mechanism to prevent excess build up in tissues.
- A diet rich in unoxidised and oxidised LA, low in lipid soluble dietary antioxidants and related antioxidant nutrients including those required for glutathione function, low in omega-3s and replete with refined easily oxidised carbohydrates, will both activate PPAR gamma-related peroxisomes and provide them with significant amounts of a preferred fuel, promoting reproduction pathway-related immune repair activities and adipose deposition, synergistic production of lipids and cholesterol, increased oxidative stress, and diversion of energy resources from ATP production to maintenance substrate and lipid deposition.

Peroxisomes

Some Basic Features and Roles of Peroxisomes

Peroxisomes are multifunctional organelles of great physiological importance and essential to development and health. They '*harbour*' about 50 different enzyme activities. [1] Peroxisomal malfunction has severe functional consequences, which manifest in a variety of health conditions, including Zellweger syndrome, with widespread and significant effects, including risk of early mortality, impaired cerebral development, functional issues, and deficient energy production.

There are a wide range of peroxisomal activators with different activation capacities, some of which are common between PPAR alpha, delta, and gamma, peroxisome types, and some of which are applicable to specific PPARs.

Peroxisomes are fundamental to healthy function of energy pathways have much greater metabolic influence on energy pathways than realised. In rat peroxisomes, whilst much more numerous in rat liver, beta-oxidise a significant percentage of OA and palmitate accounting for 20–35 % of oxygen consumption, giving an indication of their potential importance to energy pathways. [2] Also interestingly peroxisomes pathways that assist mitochdondrial energy production, or lipid creation and related pathways, by providing substrate, may through catalase have the ability to reduce oxygen requirements by recycling peroxide, raising interesting questions. Further aerobic and anaerobic pathways are more diverse and complex than generally portrayed [3].

Where mitochondrial beta-oxidation produces ATP, peroxisomal beta-oxidation produces heat, MCFs, ACoA and peroxide; consequentially activation of peroxisomal energy pathways through fasting will increase calorie expenditure through heat 'wastage' without increased activity and potentially without commensurate oxygen consumption (see below), which is of potential relevance to weight loss strategies.

Different Roles of PPAR Alpha Gamma and Delta Related Peroxisomes

Taking a broad overview, PPAR alpha-, delta-, and gamma-related peroxisomes have different functional effects, '*distinct transactivation characteristics and display preference for distinct subsets of target genes*', [4] and in future may be found to have different and distinct tissue- and function-related structures.

All three are active in some tissues, for example, the placenta [5, 6], but in most tissues the activity of one is often dominant

- PPAR alpha peroxisomes pathways are activated by energy demand stress, including fasting and exercise, [7] to a lesser extent by omega-3 activators including, ALA, [8] excess DHA, and potentially by some oxidised fats. PPAR alpha is found primarily in tissues with high energy requirements, including the liver, heart, [9] kidney, and brain. In humans, PPAR alpha has been described as '*a master regulator of hepatic lipid metabolism*', and '*at the centre of a regulatory hub fatty acid uptake, fatty acid activation, intracellular fatty acid binding, mitochondrial and peroxisomal fatty acid oxidation, ketogenesis, triglyceride turnover, lipid droplet biology, gluconeogenesis, and bile synthesis/secretion*' [10]. PPAR alpha promotes a number of energy-related gene pathways, which increase energy production and mitochondrial function. Crucially, PPAR alpha promotion may also correspondingly promote antioxidant pathways to help ensure energy substrate producing beta-oxidation pathways are protected from oxidative stress as far as is possible, [11] further the action of excess catalase on peroxisomal beta-oxidation product peroxide could produce and so recycle oxygen as well as water. There is significant PPAR conservation between species particularly of PPAR alpha [12]. The ability of Inuit with a CPT1 variant, who are not in ketosis and have limited dietary glucose; and ability of breast fed infants also not in ketosis; to largely fuel themselves from fats; suggest that peroxisomal energy pathways as a significant source of MCF and ACoA consitute the body's prevalent energy substrate source at least in some tissues such as the brain, during short-term glucose deficiency, rather than the ketones pathways.
- PPAR delta is ubiquitous and predominant in skeletal tissue, but less is known about PPAR delta, which appears to have roles in promoting energy pathways including thermogenesis, [13] detoxification, antioxidant support, and given its ubiquitous nature might have a role in prostaglandin disposal, which would logically moderate inflammation. PPAR delta peroxisomes are more often associated with ALA than LA. Assuming PPAR delta-related peroxisomes behave like those activated by PPARs alpha and gamma, they will produce peroxide, catalase, short fats, and ACoA. The PPAR delta pathways are presumably at least partially directed to supporting the energy pathways, possibly primarily in muscle tissue; however, much is unknown.
- 'PPAR' gamma peroxisome pathways appear to be primarily associated with omega-6 oxidised products, and 'reproductive'-related activities including tissue creation repair and recycling, macrophage- and microglial-related activity, and adipose tissue promotion [14]; they are found primarily in epithelial, gut, [15] adipose, and immune cells, including monocytes, macrophages, and microglia. PPAR gamma activates a wide range of gene pathways, many related to fat metabolism, and includes FABPs, leptin, LPL, SCD1, APOE, CD36, LDLR, and OLR1, which form a part of an extensive list by El Akoum [16]. He describes PPAR gamma as 'at the *Crossroads of Health and Disease:.A Masterchef in Metabolic Homoeostasis*'. PPAR gamma, but not alpha or delta, is obligate for pregnancy and key to placental development; its absence results in absolute foetal mortality. It also has '*a pivotal role in controlling wound macrophage clearance of apoptotic cells to ensure efficient skin wound healing'* [17]. Oxidised LA products, including HODEs, are the primary endogenous activators of PPAR gamma, simulating increased peroxisomal activity and/or numbers. Other lesser activators exist including some natural dietary products. Related pathways synergistically activated by oxidised products of omega-6 and/or PPAR gamma, such as OLR1 also have roles in repair, including in angiogenesis, immune function, and regulation of levels of oxidised products in the blood.

Overall Oxidation Rates of Fats

The 'overall' rules for the rate of combined peroxisomal and mitochondrial metabolism of fats from a body wide perspective appears to be, rates increase with;

- declining chain length;
- increasing numbers of double bonds;
- and omega-3s are preferred substrates over omega-6s.

The above summary represents a mix of both peroxisomal and mitochondrial oxidation, but mitochondrial and peroxisomal pathways, enzymes and transport mechanisms so substrate preferences are different.

This also reflects the preferences of LPL and HSL, so allowing the management of lipid levels in tissues to avoid uncontrolled lipid build-ups. It would make sense that there was a synergistic relationship between them as to uptake and release rates.

Preferred Substrates and Rates of Beta-Oxidation of ALA GLA LA and Other Fats by Peroxisomes

The preferred substrates for peroxisomal beta-oxidation are ALA > GLA > LA. Rates of peroxisomal beta-oxidation of other fats are dependent on a mix of factors including chain length, number of double bonds, and double-bond position. ALA is the prime preferred substrate and more actively beta-oxidised than LA by a wide margin. [18]

The relative rates of peroxisomal beta-oxidation at high lipid concentration by solubilised liver peroxisomes were approximately ALA:36, LA:21, OA:15, and AA:4 [18]. (Hiltunen data abstracted from diagram 2) The rate of beta-oxidation rises with concentration.

The presence of increasing numbers of double bonds improves oxidation rates. Conversely, increasing chain length decreases beta-oxidation rates. Oxidation rates consequently depend on the mix of these factors. The rate of oxidation of OA was a little lower than that of DHA, although Bourre reports 18 carbon fats are all better metabolised than longer polyunsaturated fats. The peroxisomal oxidation rate of OA is lower than that of linoleic. Saturated fats are also beta-oxidised by peroxisomes, palmitic acid better than stearic acid, but at much lower rates than LA and ALA [19].

In rat liver, the order of preference of peroxisomes for beta-oxidation of polyunsaturated fats is ALA› GLA› LA› EPA› DHA› AA. This order is consistent with observations in humans that ALA is more highly metabolised than longer unsaturated fats and is not found in significant amounts in adipose tissue; and preferential peroxisomal beta-oxidation would explain why ALA results in a higher resting metabolic rate, despite being a poorer energy substrate than saturated fats in mitochondria.

The relative abilities of peroxisomes and mitochondria to better metabolise different fats may be in part a consequence of the cross membrane transport pathways available to them, [20] as well as enzyme differences [21]. For example peroxisomes can beta-oxidise DHA but mitochondria cannot, mitochondria efficiently oxidise short fats C:4 and above, but peroxisomes do not; these functional synergies point to an evolutionary symbiotic relationship between peroxisomes and mitochondria, which are both found in plants, and plants in turn are significantly dependent on 18 carbon fats, the most common terrestial lipids.

Wider Functional Roles of Peroxisomes

Importantly, peroxisomes can metabolise fats including; branched and long and very long chain fats; alcohol; bile acids; prostaglandins [22] (Masters p. 101) and related substances; xenobiotics; dolichols; polyamines; hexose monophosphate; and other compounds including oxylipins. They are also fundamental to cholesterol production, synthesis of ether lipids, the purine pathways, uric acid disposal, and DHA synthesis. (Masters p. 101–141) More generally, they are important in removal of toxins as well as the production of catalase and peroxide.

Potential Mitochondrial Damage from Reliance on LA for Fuel During Conditions of Oxidative Stress

During the conditions of oxidative stress in a diabetic animal model, peroxisomal and/or mitochondrial dependence on dietary LA as a fuel substrate results in mitochondrial damage, possibly consequent on peroxisomal peroxide related, combined with NO-related inhibition of catalase, damage to cardiolipin LA, and so mitochondrial malfunction [23].

Peroxisomes Produce Short Chain Fats and ACoA

Unlike in the mitochondria, in peroxisomes fats can leave the repeating beta-oxidation cycle at any point; so a range of fat lengths will be produced as end products of the beta-oxidation process. These MCFs are not found in significant amount in tissue or adipose storage, are not well oxidised by peroxisomes, and can enter mitochondria in some cells without carnitine, so likely generally are beta-oxidised locally by mitochondria.

Each peroxisomal fat shortening carbon removal cycle also produces one ACoA; a raw material for acetate, malate [24] and potentially other energy substrates, as well as ketones; which can be fed into the mitochondrial energy pathways in times of shortage such as fasting, or alternatively be used for creation of lipids and cholesterol to support repair and maintenance. The process of channelling ACoA to cholesterol production happens in the peroxisome [25].

Potential Restrictions on Peroxisomal DHA Beta-Oxidation

Given the importance of DHA to the brain and reproduction, a facility to inhibit DHA peroxisomal beta-oxidation when DHA levels in the body were low as in famine, and to promote it when plentiful as in the diet of marine mammals, would make evolutionary sense.

Potential Energetic Advantages Including Altered Respiratory Quotient of Metabolism of Lipids via the Peroxisomal in Combination with Mitochondrial Pathways

The combination of peroxisomal beta-oxidation of lipids and subsequent mitochondrial oxidation of peroxisomal beta-oxidation products, may offer a more efficient beta-oxidation pathway than the use of the mitochondrial pathway alone. Interestingly, migrating birds and humming birds [26] appear to preferentially use polyunsaturated fats and also use of other fats including potentially palmitic acid for fuel, logically via PPAR alpha peroxisomes pathways, which also would likely concomitantly increase antioxidant production thereby protecting against energy production-related oxidative stress. Humming birds, which have very high energy demands, were observed to have relatively high rates of peroxisomal activity compared with fish, despite subsisting on plant based food, supporting the position that energy demand is a more potent activator of PPAR alpha pathways than Omega 3 lipids.

PPAR alpha-activated peroxisomal beta-oxidation and related catalase recycling of peroxide to oxygen and water might impact respiratory quotient, so be a factor that allows marine mammals to make longer energy intensive dives, including by reducing the need for oxygen and mitochondrial thermogenesis. Fish oil feeding in rats resulted in lower oxygen extraction whilst maintaining cardiac contraction [27] potentially due to the ability of catalase to reduce peroxide produced by peroxisomal beta-oxidation to oxygen, so recycling it. Recycling of oxygen and thermogenesis could also facilitate energy intensive flight during bird migration at cold high altitudes.

These peroxisomal pathway traits may well to some extent have human application. There are anecdotal suggestions by those interested in 'low-carb diets' that 'low-carbers' have improved oxygen performance under water and during energy intensive climbing at altitude. Fasting studies looking at oxygen usage under exercise stress during long-term fast, compared to baseline, under equivalent exercise conditions, reported lower respiratory quotient, and V_{O2Max} [28]. Calculation of ATP production per unit of oxygen 'consumed' across all related pathways may assist determination of these complex questions [29]. The study considered ketones as fuels but not the impact of activation of PPAR alpha. Lipid intake was not specified as to type.

Mice treated with cyanide, a mitochondrial likely primarily cytochrome C inhibitor, with co-administration of a PPAR alpha proliferator, but not in controls, recover normal oxygen consumption rates within 20 min, which is intriguing [30, 31].

Peroxisomal Oxidative Combined with Blood Brain Barrier Lipid Preferences Largely Determines Structural Lipid Composition of the Brain?

The relative rates of peroxisomal and mitochondrial lipid beta-oxidation have wider reaching onward implications for metabolic pathways, and particularly so in the brain where the blood brain barrier can selectively restrict or prevent fat uptake.

Importantly in the brain, selective capacity of fats to cross the BBB, combined with low peroxisomal preference for beta-oxidation of longer saturated fats, middling preference for medium saturated fats such as palmitic acid, and maximum peroxisomal preference for polyunsaturated fats, combined with low brain CPT1A and/or high malonyl-CoA so restricted neuronal and astrocyte mitochondrial long chain fat uptake and beta-oxidation, may explain the content balance and mix of structural brain lipids (with the exception of cardiolipin), including the virtual absence in the brain lipid structure of LA, ALA, and EPA, and why the brain does not generally, beyond normal recycling maintenance, significantly beta oxidise its own structural lipid content for energy.

The rate, amount, and type of fat oxidised in the brain through the peroxisomal pathways will be governed by both lipid availability within the BBB and order of peroxisomal substrate preference. Research into the subject is limited.

Peroxisome Proliferation and/or Increased Size and Activity in Humans

Whilst peroxisomes are more active in rodents, potentially due to a seed-eating heritage, in humans monkeys [32] and pigs (61 %) [33] stimulation by PPARs also leads to not insignificant increases in peroxisome size, activity and/or numbers, [34–36] albeit to a lesser extent than rodents.

Different shapes of peroxisomes have been identified in humans including round, triangular, oval, and worm like, but research into their individual function appears limited. Peroxisomes in different tissues, for example, the brain liver kidney and intestines, can differ in size, may have tissue specific functionality, as well as responding to different PPARs. [37]

Given the differing roles of the PPARs, it seems likely that different peroxisome types respond more actively to different activating PPARs, rather than one peroxisome type having sufficient functional flexibility to respond differently to all three PPARs.

More research is needed to better determine the extent of changes in peroxisome numbers, size, type, and activity, and how they factor in human metabolic pathways including gene activation.

LA and ALA—Their Different Metabolic Roles and Activation Pathways

LA and ALA have different metabolic and physiological effects including energy production and tissue repair; broadly generalising, they predominately activate 'different' peroxisomes in different tissues; the nature of activation reflects the nature and function of the tissue;

- LA is targeted to repair and renewal; oxidised LA products are fundamental to reproduction, immune function and tissue repair including activating and fuelling PPAR gamma-related peroxisomal pathways, [38] with the proviso that during energy deficiency, excess LA will be directed to energy-related pathways [39] rather than inflammation, immune function, and repair.
- ALA is targeted to energy provision.

There is likely a functional crossover and interaction between the 'different' PPAR-related peroxisomes in tissues with both a high energy and repair requirement, for example, in the liver and a foetus.

PPAR Gamma Different Roles; Normal Physiological Activation V 'Excess' Activation

PPAR gamma, when activated in normal physiological circumstances during 'homestasis', will help regulate and reduce oxidative stress, but when highly activated and in the context of increased oxidative stress, including cytokine production, will enhance oxidative stress in a feed-forward cycle, including through peroxisomal beta-oxidation production of net peroxide in support of oxidative bursts in macrophages/microglia and related cells. The activation of the oxidised LDL receptors by PPAR gamma is arguably an interlinked mechanism in an LA-dependent web that helps regulate immune- and tissue-related destruction repair and creation.

PPAR Alpha Activation by Energy Deficit and to a Lesser Extent by Omega-3S

Fish birds and monogastrics as well as humans with high polyunsaturated fat intakes, high energy demands, and intermittent food intake need to sustainably utilise stored surplus lipid substrate including LA, ALA, DHA, and other stored lipids, as metabolic fuel. LA and ALA are poorly metabolised in mitochondria [24]; DHA cannot be metabolised in mitochondria, but all including PA, POA and OA to varying extents are efficiently metabolised by peroxisomes.

PPAR alpha is 'a *master regulators of hepatic lipid metabolism*', [40] most active in energy demanding tissues, with a major role in metabolic regulation. Consistent with the need for resource conservation during energy deficit, PPAR alpha activation downregulates '*growth tissue remodelling and cellular communication*', and conserves protein [41].

PPAR alpha activation-related brain and heart lipid remodelling through reduction of omega-3 and 6s, and replacement by endogenously produced omega-9 polyunsaturates, would have significant physiological effect including on behaviour, but this may be a slow process, as it is likely that most ACoA substrate will be directed to energy, rather that de novo lipid formation.

Over time, energy deficit-based diminution of; LA stores, substrates for the prostaglandin and HODE pathways, including downstream prostaglandin PGE2, would have physiological effects including through steroid hormone pathways on both cellular function and behaviour, including reducing reproductive capacity and related behavioural characteristics including aggression and territoriality.

Exercise or short-term 'fasting', through activation of PPAR alpha, possibly diverts excess dietary LA, or LA released from adipose tissue, to energy, via peroxisomal and mitochondrial activation, so making excess LA unavailable to overactivate PPAR gamma-related tissue repair, immune, inflammation, fat storage, and related pathways.

This further uprates metabolism; diverts energy to thermogenesis; increases antioxidant output; improves the fuel status of crucial energy hungry organs such as the heart and brain; and potentially reduces oxygen consumption; with significant and multiple implications for the control of western inflammatory-related conditions, including obesity, diabetes, cardiovascular, and some neurological diseases.

In those eating oxidative-prone LA-rich Western diets, with consequent high levels of stored oxidised LA in adipose tissue; during short-term fasting the release of oxidised products, including LA oxylipins from adipose tissue and

plasma, [42] combined with a dietary lack of balancing ALA and antioxidant factors, by maintaining LA oxylipin signalling for increased oxidative stress, consequent PPAR gamma activation, increased LDL uptake, and so ongoing inflammation could partially override the effects of PPAR alpha activation, leading to continued vascular membrane damage and adipose tissue deposition.

Peroxisomes; Peroxide Production, the Hydroxyl Radical and Oxidative Stress

Peroxide is a ubiquitous fundamentally important oxidative signalling messenger, which can cross cell membranes, travel significant distances, and be converted in situ to the hydroxyl radical. Peroxisomes are probably the major site of peroxide production as well as sources of nitrous oxide [43] and superoxide, heat but not energy. Peroxisomes are implicated in both control and initiation of oxidative stress, as well as important for the functioning of macrophages and microglia, and so health and disease [44].

PPAR gamma-related production of peroxide in excess of catalase in macrophages and microglia will activate LOX and COX enzymes and through 13HODE production promote wider feed-forward PPAR gamma-related oxidative activity and free radical production.

Interaction of peroxide with Fe2 + produces the hydroxyl radical, which is extremely powerful, fast acting, influential, indiscriminate, and potent, likely the most powerful and reactive form of oxygen, with strong affinity for unsaturated double bonds, so an aggressive oxidiser of polyunsaturated fats to oxylipins, including 9 and 13HODE [45]. Significant amounts of iron are present in peroxisomes and also in the catalase enzyme. As discussed, there are limited harm reduction antioxidant options to terminate hydroxyl oxidative cascades.

In contrast, activation of PPAR alpha and delta is associated with increased antioxidant function; in pigs' liver clofibrate resulted in higher catalase +41 % and lower peroxide −32 % [46].

Peroxisome Antioxidant Production Capacity Including of Catalase

Peroxisomes, in addition to making peroxide, are the body's primary source of catalase, contain enzymes to make SOD, glutathione, and other antioxidants, [1] so can have important roles in antioxidant protection as well as creation of oxidative stress. Catalase is the most effective antioxidant against peroxide [47]. Glutathione can also reduce peroxide but is not an effective substitute for it.

In humans, low physiological excess of peroxide signals for system regulation, including increased antioxidant production, for example, for control of vascular function; in contrast at higher levels peroxide signals for inflammation, new tissue, repair, fat accretion, and immune defence.

Endothelial cells exposed to LA, with or without the addition of vitamin E, were significantly larger [48, 49]. and contained more '*cytoplasmic-like lipid droplets*' compared to controls. Interestingly, those cells treated with vitamin E and LA also contained considerably more cellular debris.

Excess LA causing cell growth and at the same time damage is consistent with 13HODE-activated PPAR gamma pathways, leading to increased tissue repair and/or renewal. The regulation of peroxide production-based activation of PPAR gamma though; NO via activation of iNOS so blocking of catalase resulting in increase of peroxide levels, which in turn would increase 13HODE and so PPAR gamma and peroxisomal activity in a feed-forward loop; as exacerbated by low omega-3s and absence of energy deficit, so low PPAR alpha activation; would be of considerable importance in tissues with limited antioxidant protection that utilise peroxisomes equally for both energy production and tissue repair support, such as the brain, pancreatic beta cells, and thyroid. In such organs significant overactivation of PPAR gamma related pathways in the context of a western nutrient depleted preoxidised diet would arguably result in increased oxdiative stress, mitochodrial damage, energy substrate deficit, and intercellular lipid build up and/or imbalance, as seen in cardiac and insulin beta-cell dyfunction.

The heart would be particularly at risk of PPAR gamma-related oxidative stress, excess lipid production, and reduced mitochondrial efficiency because in the non-fed state, 70 % of fuel lipids and accompanying HODEs of dietary origin are delivered by LA-rich oxidation susceptible LDL. The overactivation of PPAR gamma increased iNOS activity so NO based catalase inhibition, and overactivation of the immune inflammatory pathways would be consistent with rising numbers of oxidative stress-related conditions seen in these organs.

The relevance of the differing effects of omega-3 and 6 on immune- and tissue-related creation function are evident in the results of the use of parenteral omega-3 rich lipid products, which reduce the incidence of liver inflammation and fibrosis in those with short bowel syndrome [50]. Further, omega-3 rich parental nutrition may reduce cancer-related cachexia, [51] and interestingly in initial reports it has been shown, in combination with 'chemo', to reduce secondary liver cancer in pancreatic cancer patients. (ISSFAL presentation 2015)

As in all things balance is the key; an excess of omega-3s conversely may inhibit immune response capacities [52].

Role of Peroxisomal Peroxide Production in Conjunction with INOS Produced NO in Phagocytosis?

During immune and related induced oxidative stress, including activation of PPAR gamma by LA oxidised products, the HODEs, the excess of peroxisomal beta-oxidation product peroxide over catalase due to blocking of the haem-based catalase enzyme by NO, produced consequent on synergistic, including cytokine and direct or indirect peroxide [53] activation of iNOS, contributes to oxidative stress, arguably including that necessary for macrophage phagocytosis. Peroxisomal activation, so peroxide production and consequent 13HODE creation, would create a positive feed-forward loop for increased PPAR gamma and peroxide induced iNOS activation. Overactivation and feed forward would contribute to oxidative breakdown including sepsis.

Mammals—Peroxisomes; PPAR Gamma and Cellular Creation Maintenance and Repair

Peroxisomes responsive to PPAR gamma are mainly present in epithelial-related tissues with high cell turnover, growth or renewal requirements, and/or immune related function [54]. PPAR gamma impacts regulation of multiple cells and processes including macrophages, leucocytes, dendritic cells, B cells, T cells, and neutrophils [55]; wound healing including angiogenesis, collagen formation, granulation, and apoptosis clearance; as well as having a role in adipogenesis, vascular plaque and foam cell formation [56, 57].

Peroxisomes, potentially particularly PPAR beta-related peroxisomes, also degrade prostaglandins and oxylipins, and in doing so would help moderate inflammatory messaging. Energy deficit, LA intake reduction, and omega-3 sufficiency would also moderate PPAR gamma-related activity including iNOS, LOX 12/15, COX, and consequent oxidative stress levels.

Capacity of Peroxisomal Beta-Oxidation Products to Support Mitochondrial ATP Production as Alternative Fuel Substrates to Glucose and/or Ketosis

The level of peroxisomal beta-oxidation contribution to the energy and repair pathways is greatly underappreciated. In scenarios of energy exertion, famine, fast, and glucose depletion, peroxisomal beta-oxidation provides short fats and ACoA, which can be a substrate for acetate, malate other pathways, as well as potentially for ketone production or gluconeogenesis so providing mitochondrial fuel; ACoA can also be used as a substrate for tissue maintenance including for lipid and cholesterol production [58] (Salway p. 36). Thus, peroxisomes can help both fuel and maintain the mitochondria and other tissues, including importantly those of the brain.

Neonates thrive, as well as running and building energy demanding brains, on breast milk, which is low in glucose and carbohydrates and high in fats; they are not generally in ketosis adding to evidence that fat pathways other than those involved in ketosis have central roles in the energy pathways.

PPAR alpha-related beta-oxidation is also likely increased in some human genotypes, including Inuit with a CPT1 malfunction. Carnitine enzyme CPT1A is an important transfer mechanism of fats chain lengths C:6-C:20 into the mitochondria [59]. Some Inuit have a CPT1A [60] polymorphism; theirs is '*broken*', [61] consequently they cannot efficiently transfer fats and crucially the common longer chain dietary fats C18-C20 into the mitochondria for fuel.

Other carnitine acyltransferase exist for shorter 'medium' chain fats, and MCFs in some tissues do not require carnitine, providing alternative mitochondrial lipid fuel supply routes

A 'defective' CPT1 transport mechanism for long fats into the mitochondria would require those Inuit to significantly rely on peroxisomal beta-oxidation as a source of short fats; and ACoA as substrate for acetate, malate, and/or ketones to fuel mitochondria [62].

These Inuit lived primarily on marine foods, which contain limited glucose, but some glycogen. [63] Despite limited glucose and diminished capability to transport long chain fats into the mitochondria for beta-oxidisation, research suggests that northern Inuit are not regularly in ketosis, [64, 65] yet on their traditional diet are healthy.

The key to healthy survival of CPT1A impaired Inuit may be their marine diet high in omega-3s including EPA and DHA, 'low' in LA, and rich in minerals, iodine and antioxidants. Given the inability of all mitochondria to metabolise DHA, and the inability of this group to import long chain fats into mitochondria, it would make sense if these Inuit peroxisomally oxidised the common 16 and 18 carbon dietary fats and polyunsaturates as well as longer fats including DHA to provide alternate mitochondrial fuel substrates, including MCFs and ACoA.

PPAR alpha [66] energy-related peroxisomes, and mitochondrial energy-related pathways including MCAD, are activated by fasting, high energy demands, high protein, and omega-3s. Energy requirements would be increased by cold temperatures and the Inuit hunter-gatherer lifestyle; increased peroxisomal activity including thermogenesis might be a useful adaptation to a very cold environment and diet rich in marine food. This adaption would also have been useful for those living in such conditions during periods of glaciation.

The historic reports of lack of cardiovascular disease and Western oxidative stress-related conditions in Inuit on traditional diets, suggests in the presence of excess omega-3 fats and adequate nutrients, and likely extensive reliance on peroxisomal activation for energy substrate supplies, in an environment that often demands an active lifestyle, does not lead to abnormal peroxide-related oxidative stress.

The shortest fat product of peroxisomal oxidation is C:8 an MCF. Ketogenic/ MCT diets are an accepted treatment protocol helping bypass some types of mitochondrial-related dysfunction [67]. HMGCoA synthase, which supports ketone production, was increased, and the HMGCoA reductase, the basis of the downstream activation of the cholesterol pathway, was reduced in chickens that fed a high omega-3 diet [68]. PPAR alpha has been observed to increase HMGCoA synthase, conversely in a PPAR alpha null mouse ketones and a number of mitochondrial fat metabolising enzymes are greatly reduced [69].

In contrast to a 'ketogenic' Inuit type omega-3 nutrient rich diet, 'ketogenic' 'paleo' type diets high in omega-6 may increase oxidative stress [70]. Rats on a ketogenic diet containing 18 % linoleic acid had significantly increased 4HNE, lower GSH, and 'NRF2', a key oxidative stress response transcription factor [71].

'Ketogenic', sometimes referred to in loose terms as 'Paleo' diets, I postulate are less likely to increase oxidative stress if containing adequate omega-3 and limited omega-6, and/or if resulting in periodic energy deficit due to calorie restriction, exercise, or part day fasts

High omega-6 content may be a reason why ketogenic diets trials in cancer patients have not clearly shown positive outcomes; however, the subject is complex [72]. The use in cancer including brain cancer and/or conceivably intermittent fasting to potentially moderate peroxisomal pathways so gene activation, including through changing the balance between PPAR alpha/beta and PPAR gamma pathway activation, potentially of ketogenic diets combined with high omega-3 looks a more promising avenue of research.

Peroxisomes, PPAR Alpha, Energy Production, Malonyl-CoA

PPAR alpha-activated peroxisomes, likely with PPAR delta-related peroxisomes, are found in tissues with high energy demands, including the liver, cardiac tissue, and brain. PPAR alpha activation resulted in significant functional changes in several hundred genes in both mice and humans, '*top 50 of most highly induced genes . . . includes genes involved in mitochondrial fatty acid oxidation and ketogenesis, peroxisomal/microsomal fatty acid oxidation, fatty acid binding and activation'* [40].

Peroxisomes provide MCFs down to C:8, as well ACoA as a basis for other substrates that are effective fuel for mitochondria. Fats under 12 carbons, at least in some tissues, do not require carnitine to transport them into the mitochondria, so bypassing faulty fat transfer pathway CPT1A defects, or malonyl-CoA carnitine inhibition. In the rat hearts, radiolabelling showed peroxisomal beta-oxidation was a primary source of acetyl-CoA, and malonyl-CoA [73]. A lack of peroxisomal function in cardiac tissue results in cardiomyopathy.

The ACoA produced by peroxisomal beta-oxidation is in part directed to make malonyl-CoA, which inhibits carnitine-related transport of fats into the mitochondria. The effect of malonyl-CoA on fat transport varies between tissues, being ten times higher in the heart than liver. Malonyl-CoA also appears to have particular relevance in the regulation of beta insulin cells.

Malonyl-CoA blocking of CPT1A activity and the take up of long fats into the mitochondria would make more fat available for peroxisomal oxidation, and so providing a feed-forward mechanism shunting energy pathways towards more efficient mitochondrial fuels, by encouraging peroxisomal conversion of long fats to short fats, rather than direct mitochondrial beta-oxidation of longer fats including polyunsaturates [74].

Shunting of fats via malonyl-CoA creation through peroxisomal beta-oxidation pathways offers metabolic advantages, including thermogenesis, potentially reduced oxygen consumption/recycling, additional options to fuel mitochondrial beta-oxidation, and/or bypasses enzyme blockages, as well as in parallel uprating antioxidant production and reducing LA substrate available for excess activation of the PPAR gamma-related pathways.

Peroxisomes and Calories

Activation of peroxisomes so production of heat rather than energy from calories, and greater mitochondrial operational efficiency on short fats may account for the reported greater effectiveness of some low carbohydrate calorie restricted diets.

In a calorimetric trial comparing a high fat–low carbohydrate to an equicaloric high carbohydrate diet, the high fat 'low-carb' diet resulted in greater resting calorie expenditure; about 250 calories a day, an amount equivalent to one hour's exercise [75]. The paper does not provide an analysis of the fat, omega-3:6 intake and balance, timing of meals, or provision or inter-meal snacks, so information as to 'fasting' status. An increased metabolic rate in the absence of additional exercise suggests that calories are going into heat, so still conforming to principles of conservation of energy.

Omega-3 ALA supplementation in chicks increased growth and protein tissue content, but feed efficiency was reduced; similar outcomes were seen in other animal feeding studies.

However, results of fat vs carbohydrate diet studies are inconsistent [76]; a lack of activation of PPAR alpha pathways on diets that are borderline in calorie terms, but rich in LA and oxidants, poor in antioxidant factors, deficient in omega-3s, including ALA, and lacking exercise or intermeal fasts may still signal for fat deposition.

The preferential metabolism of ALA is supported by research using an American diet-based study, which demonstrated that over a 9 h time frame, radiolabelled ALA is less well metabolised than C12, a significant constituent of coconut fat; but significantly better metabolised than C16, and other 18 carbon fats including LA [77]. Addition of ALA to such diets may help mobilise PPAR alpha energy-related pathways.

ALA would also help balance the release of LA from fat stores so improving the LOX12/15, COX, LA/ALA oxylipin plasma product profile and increase antioxidant production so helping control oxidative stress, reduce inflammatory and signalling for lipid deposition, and likely help sustain and fuel effective mitochondrial function.

As discussed in relation to Inuit, PPAR alpha and delta energy deficit related activation, combined with high omega-3 intake, may be a key to reducing peroxisomal-related oxidative damage, and increasing resting energy expenditure including thermogenesis; more research is required.

Thermogenesis Temperature Adaption and Hibernation

The existence and development of desaturase enzymes for insertion of multiple double bonds in simple carbon chains, greater membrane flexibility, and lower temperatures of solidification allowed adaptation of life to a greater range of temperatures, thereby to diurnal land-based existence.

Plants that are cold resistant have higher proportions of ALA in their membranes, [78] as do plants exposed to longer hours of light [79]. Longer hours of light occur in summer at higher and colder latitudes.

Polyunsaturated fat adaption to cold is also seen in marine organisms, including bacteria, fish, and marine mammals. Marine organisms from higher latitudes generally contain more long chain polyunsaturated fats, particularly omega-3s. In humans, remarkable feats of survival of cold by Inuit, including surviving for several days in freezing conditions even after immersion, have been reported.

Interestingly marmots, hibernating vegetarians, store very large amounts of LA in their fat tissue prior to hibernation, but will not hibernate if fed large amounts of ALA [80, 81]. Gerbils, also hibernators, have been shown to increase peroxisomal and reduce mitochondrial activity in hibernation [82].

PPAR gamma-related peroxisomal oxidation in hibernation and/or torpor possibly would provide; thermogenesis; temperature control; short fats for limited ATP needs; ACoA as substrate for tissue repair; oxidative peroxide for signalling and immune function; reduced mitochondrial activity; lowered oxygen requirement, and mechanisms for metabolism of waste and toxic metabolic products.

Does PPAR gamma-related peroxisomal thermogenesis with tissue-dependent assistance from PPAR alpha help explain warmth generation during tissue repair and assist temperature control in sleep? [83] Conversely, PPAR alpha would primarily provide energy and increased mitochondrial activity, which is not conducive to torpor; this may explain why LA but not ALA induces hibernation.

PPAR Delta; Antioxidant Promotion; Detoxification; Enhanced Energy Production

This overview focuses on PPAR alpha and gamma rather than delta, because they are more clearly linked to LA and ALA, more extensively researched, and information on PPAR delta is limited.

A role for PPAR delta as a promoter of antioxidant production would fit with reports that PPAR delta reduces inflammation, and it being involved in the energy and detoxification pathways. PPAR delta activity appears complex and intertwined [84]. PPAR delta is activated by omega-3 polyunsaturated fats to a much greater extent than PPAR alpha and interestingly by 4HNE [85].

PPAR delta appears to have roles in detoxification, its activation by oxidative product produced in the presence of toxicants such as 4HNE, is logical; might it also have a role in disassembling so regulating prostaglandins. A system ‘designed’ to support detoxification, antioxidant function, and promote energy pathways ideally would also synergistically increase antioxidant production as energy output increased. Consistent with this, genetically altered mice with cardiac tissue expressing additional PPAR delta function, uprated antioxidant enzyme function SOD by 50 % and catalase by 75 %. Efficiency of cardiac function was improved with reduced, fibrosis, and mitochondrial dysfunction. Despite increased energy output, no rise in oxidative stress was seen.

Conversely, PPAR delta deletion causes cardiac energy dysfunction. Further, PPAR delta appears essential to mitochondrial function and biogenesis [86]. PPAR delta agonists; enhanced fatty acid oxidation; increased thermogenesis; reduced triglycerides; increased HDL, and glucose function [87]. In humans, a PPAR delta agonist uprated energy pathways in a similar way to exercise [88].

Stored LA, Impact of Peroxisomal Pathways, Dieting and Oxidised Stress

Stored LA and its oxidised products are released at times of energy deficit. Those with high percentages of stored LA in adipose tissue may have constant or intermittent exposure to unnaturally high levels of stored LA and oxylipins, and/or in situ oxidised LA products in plasma components including LDL, which will impact all cellular processes. High LA may also result in imbalances in desaturase function. The constant plasma presence of LDL that is high in LA may also be a factor in insulin resistance.

Regulation of peroxide-related oxidative stress by glutathione pathways will be inhibited if the required dietary nutrients including selenium and cysteine are not present in the diet in sufficient quantities.

Those who are dieting and particularly obese may need higher intakes of ALA, as well as the long chain omega-3s, to balance the daily release of LA including its oxidised products from adipose tissue. Exercise without dietary change or calorie restriction is not an automatic route to weight loss.

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Effects of Omega-3 Fatty Acids in Myocardial Infarction

33

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Introduction

Cardiovascular disease (CVD) is a global health problem, leading to the cause of mortality and morbidity worldwide [1]. Myocardial infarction (MI) is defined pathologically as myocardial cell necrosis caused by significant and sustained ischemia [2]. MI results from either coronary heart disease, which implies obstruction of blood flow due to plaque in coronary arteries, or much less frequently, to other obstructing mechanisms (e.g., spasm of plaque-free arteries). Plaque is a consequence of atherosclerosis, which is a major contributor to the pathogenesis of CVD. To reduce CVD risk, lifestyle modifications, such as smoking cessation, moderate alcohol consumption, exercise, stress reduction, weight control, and diet, are needed. Furthermore, these factors contribute synergistically to prevent CVD [3–6]. Regarding diet, several authors have attempted to clarify the mechanisms responsible for the atheroprotective effects of n-3 polyunsaturated fatty acids (n-3 PUFAs). Dietary n-3 PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are present in fish meat and oils. Furthermore, the percentage of EPA plus DHA in erythrocyte membrane fatty acids is an index of systemic n-3 PUFA status, the omega-3 index [7]. Asians consume more fish than Caucasians and have omega-3 indices more than two times greater. Nevertheless, CVD occurs in Asians, although its prevalence is lower. The mechanisms responsible for the anti-atherosclerotic and anti-thrombotic effects of n-3 PUFAs have not been determined, but several studies conducted over the last three decades have attempted to clarify the mechanisms involved. N-3 PUFAs lower serum triglyceride levels, improve endothelial function, have anti-thrombogenic effects, reduce adhesion molecule and inflammatory mediator levels by regulating their gene expressions or by promoting their transformations to downstream metabolites, and increase the stability of atherosclerotic plaque [8–11]. Ever since it was reported in 1970 that Greenland Eskimos have a low cardiovascular (CV) mortality rate, epidemiologic studies have suggested that n-3 PUFAs play a role in the prevention of CVD [8]. Recent intravascular ultrasound studies have shown inverse associations between n-3 PUFA blood levels and the development of vulnerable plaque and atheroma progression in patients that attained low-density lipoprotein cholesterol goals of less than 100 mg/d on statin therapy [12–14]. Furthermore, several trials have shown significant CV benefits for dietary n-3 PUFAs in patients at high risk of CVD, such as in patients that experienced MI within 3 months of study enrollment [15–17], although it should be added that in these studies, the anti-inflammatory effects of n-3 PUFA were not found to be strong enough to reduce neo-intimal proliferation. This chapter reviews the role of n-3 PUFAs in MI. Trials undertaken to examine the MI preventative effects of n-3 PUFAs are listed in Table 33.1.

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Table 33.1 Clinical trials on n-3 polyunsaturated fatty acids in the cardiovascular disease

Study	Participants	Omega-3 Dose (g/d)	Control	Follow-up	Primary outcome	RR (95 % CI)	No. of events in intervention group	No. of events in placebo group
DART study [18]	2033 Pts with MI	Either no counseling or counseling (1) reduced fat + increased PUFAs intake (2) intake of 200-400 g/wk of fatty fish (3) increased intake of cereal fiber	Standard care	2y	All-cause mortality			
Singh et al. [19]	240 Pts with suspected AMI	Omega-3 FA (1.8 g/d), EPA (1.08 g/d), and DHA (0.72 g/d) or mustard oil (2.9 g/d)	Placebo	1y	Total CV events (non-fatal MI, cardiac death)	0.71 (0.48–1.05)	30(24.6)	41(34.7)
GISSI-P trial 2002 [20]	11,324 Pts with MI	Omega-3 FA (1 g/d) EPA (0.85 g/d), DHA, vitamin E (300 mg/d)	Standard care	3.5y	CVD death, non-fatal MI, non-fatal stroke	0.80 (0.68–0.94)	556(9.8)	621(11.0)
OPACH Group Svensson et al. [21]	206 Pts with chronic hemodialysis	Omega-3 FA (1.7 g/d)	Placebo (olive oil)	2y	Total CV event, death AMI, angina pectoris, stroke, TIA, peripheral vascular disease	1.04 (0.71–1.48)	62(60.2)	59(57.3)
JELIS trial Yokoyama et al. [22]	18,645 Pts with hyperlipidemia	1.8 g/d of EPA with statin	Standard care	4.6y	All-cause mortality, cardiac death, SCD, MI, stroke	0·81 (0.69–0.95)	262(3.5)	324(4.4)
GISSI-HF Tavazzi et al. [23]	6975 Pts with CHF	Omega-3 FA (1 g/d)	Placebo	3.9y	All-cause mortality, cardiac death, SCD, MI, stroke	0.91 (0.83–0.99)	955(27)	1014(29)
Matsuzaki et al. [24]	3664 Pts with prior CVD, TC ≥ 250 mg/dL	1.8 g/d of EPA with statin (pravastatin 10 mg/d or simvastatin 5 mg/d)	Statin alone	4.6y	Major CV events	0.77 (0.63–0.96)	158(8.7)	197(10.7)
Omega trial Rauch et al. [25]	3851 Pts with AMI in the past 3–14 days	Omega-3 FA (1 g/d), EPA (0.46 g/d), and DHA (0.38 g/d)	Placebo (olive oil)	1y	SCD	1.20 (0.98–1.48)	182(9.5)	149(7.9)
Alpha Omega. Kromhout et al. [26]	4837 Pts with MI	Omega-3 FA (0.4 g/d), EPA (0.23 g/d), and DHA (0.15 g/d) ± ALA	Placebo (margarine) ± ALA	3.7y	Major CV events (fatal or non-fatal CV events, PCI, CABG)	1.02 (0.89–1.17)	336(14.0)	335(13.8)

(continued)

Table 33.1 (continued)

Study	Participants	Omega-3 Dose (g/d)	Control	Follow-up	Primary outcome	RR (95 % CI)	No. of events in intervention group	No. of events in placebo group
SU.FOL.OM3 trial Galan et al. [27, 29]	2501 Pts with MI, UA, ischemic stroke within 12 month	Omega-3 FA (0.6 g/d), EPA, and DHA ± vitamin B	Placebo ± vitamin B	4.7y	Major CV events (non-fatal MI, stroke, CV death)	1.06 (0.78–1.44)	81(6.5)	76(6.1)
Eussen et al. [30]	4153 Participated in the Alpha Omega trial among statin nonusers	1) 400 mg/d EPA–DHA only, 2) 2 g/d ALA only 3) EPA–DHA + ALA	Placebo (margarine) ± ALA	3.7y	Major CV events (fatal CVD, non-fatal MI, cardiac arrest, stroke, cardiac interventions)	0.48 (0.22–1.06)	9(9)	20(18)
ORIGIN trial 2012 [31]	12,536 Pts at high risk for CV events with IFG, IGT, or DM	Omega-3 FA (1 g/d), EPA (0.46 g/d), DHA (0.38 g/d), insulin glargine	Placebo (olive oil), standard care	6.2y	All-cause mortality, cardiac death, MI, stroke	0.98 (0.87–1.10)	574(9.1)	581(9.3)
The risk and prevention study 2013 [32]	12,513 Pts with multiple CV risk factors	Omega-3 FA (1 g/d), EPA + DHA 0.9:1–1.5:1	placebo (olive oil)	5y	Death or hospitalization from CVD	0.97 (0.88–1.08)	733(11.7)	745(11.9)

Intervention Studies

The DART Study

The early Diet and Reinfarction Trial (DART) was a randomized secondary prevention trial based on long-term dietary intervention in 2033 men with a history of MI [18]. Patients were randomized to receive either no counseling or dietary counseling according to 1 of 3 dietary strategies: (i) reduced total fat intake with increased PUFA intake, (ii) increased intake of fatty fish (200–400 g of fatty fish per week, which provided an additional 500–800 mg/d of n-3 PUFAs), or (iii) increased intake of cereal fiber. Patients in the group that received counseling on reducing fat intake and increasing PUFAs did not show any difference in mortality compared with the group that received no counseling or the other two experimental groups. Conversely, those that received counseling on increasing fatty fish intake achieved a reduction in all-cause mortality of 29 % as compared with those that received no counseling. However, long-term dietary interventions did not change the incidence of reinfarction. Nevertheless, this study showed for the first time that an increase in fatty fish consumption by patients with a history of MI decreases all-cause mortality.

Singh et al.

In a small randomized, placebo-controlled trial, the effects of treatment with fish oil on cardiac events were compared in 122 patients treated with fish oil containing EPA + DHA (1.8 g/d, respectively) (group A), 120 patients treated with mustard oil (20 g/d) containing α-linolenic acid (2.9 g/d) (group B), and 118 patients treated with a placebo (group C) [19]. After 1 year of treatment, total cardiac events were significantly less in the fish and mustard oil groups than in the placebo group (25 and 28 % vs. 35 %, $p < 0.01$). As was observed in the DART trial, non-fatal MIs were also significantly lower in the fish and mustard oil groups than in the placebo group (13.0 and 15.0 % vs. 25.4 %, $p < 0.05$). Furthermore, the fish oil group also showed significant reductions in cardiac death, total cardiac arrhythmia, left ventricular enlargement, and angina pectoris as compared with the placebo group.

The GISSI-Prevenzione Study

The GISSI-P (Gruppo Italiano per lo Studio della Streptochinasinell' InfartoMiocardico—Prevenzione) trial randomized 11,324 patients, who were enrolled within 3 months of acute MI, for treatment with 850 mg/d of n-3 PUFAs, vitamin E, both, or placebo [20]. Notably, the findings of this study showed that n-3 PUFAs reduced total death by 15 %, CV death by 30 %, and sudden cardiac death by 45 %. However, no additional benefit was observed with respect to the incidences of non-fatal MI or stroke. The limitations of this trial included the absence of blinding, the absence of a placebo group, low use of conventional medical therapy, such as cholesterol lowering agents (28.6 %) or angiotensin converting enzyme (ACE) inhibitor/angiotensin II receptor blocker (ARB) (40.9 %), and a low rate of coronary revascularization. This large prospective trial supported the hypothesis that the benefit conferred by n-3 PUFAs was mainly due to their antiarrhythmic effects.

Svensson et al.

Svensson et al. conducted a randomized, double-blind, placebo-controlled trial to test the effect of n-3 PUFAs on the secondary prevention of CV events in hemodialysis patients [21]. The primary endpoint was a composite of mortality and total CV events. In total, 206 patients were assigned to n-3 PUFA group ($n = 103$) or a control group ($n = 103$) and followed for 2 years. During follow-up, 121 of the 206 (59 %) reached the primary endpoint. Although n-3 PUFA were found to have no significant benefit on CV events or death (62 vs. 59), a significant risk reduction was observed in terms of the incidence of MI in the n-3 PUFA group, (4 vs. 13; $p = 0.036$). Despite being limited by a comparatively small number of patients and a large number of withdrawals, it was shown that n-3 PUFA significantly reduced the risk of MI as a secondary prevention.

The JELIS Study

The Japan EPA Lipid Intervention Study (JELIS) showed that long-term supplementation with EPA at 1.8 g/day reduced a composite endpoint of sudden cardiac death, MI, unstable angina, and revascularization by 18 % in Japanese patients with hypercholesterolemia [22]. In patients with a history of coronary artery disease (CAD, 14,981), EPA treatment diminished major coronary events by 19 % (secondary prevention subgroup 158 (8.7 %) in the EPA group versus 197 (10.7 %) in the control group; $p = 0.048$). In patients with no history of CAD, EPA reduced major coronary events nonsignificantly by 18 % [104 (1.4 %) in the EPA group vs. 127 (1.7 %) in the control group; $p = 0.132$]. Of note, since this study population probably had high serum levels of EPA because of high fish consumption in the Japanese population, primary endpoint incidence was lower than in other study. Although in the study cohort more than 80 % of patients had no history of prior CAD, the investigators found a significant reduction in

the primary endpoint in secondary prevention groups but not in primary prevention groups, which was explained by a decrease in the atherosclerotic process.

The GISSI-HF Trial

The Gruppo Italiano per lo Studio della Sopravvivenzanell' Infartomiocardico (GISSI) trial was a randomized, double-blind, open-label, placebo-controlled trial that was undertaken in Italy with a focus on patients with congestive heart failure (CHF) [23] and provided stronger evidence on the beneficial CV effects of n-3 PUFAs in CHD [20]. In this study, the efficacy of 1 g/d of n-3 PUFAs and vitamin E on mortality was investigated in 11,323 Italian patients with a recent history of MI. Over a 3.5-year follow-up, n-3 PUFA administration significantly reduced CV death, non-fatal MI, and stroke by 20 % (95 % CI, 6–32 %; $p = 0.006$). In disagreement with the results of the GISSI-Prevenzione trial, some recent randomized controlled trials (RCTs) did not demonstrate the benefits of n-3 PUFAs on fatal CV events. In the GISSI-HF trial, patients were randomly assigned to 1 g/d of n-3 PUFAs ($n = 3494$) or placebo ($n = 3481$) and followed for an average of 3.9 years. Primary end points were death and admission to hospital for CVD. Nine hundred and fifty-five (27 %) patients in the n-3 PUFAs group and 1014 (29 %) patients in the placebo group succumbed to all-cause death [hazard ratio (HR), 0.91; 95.5 % confidence interval (CI), 0.833–0.998]. In addition, 1981 (57 %) patients in the n-3 PUFA group and 2053 (59 %) patients in the placebo group died or were admitted to hospital for CVD (HR 0.92; 99 % CI, 0.849–0.999). The treatment with n-3 PUFA was well tolerated, and gastrointestinal discomfort was the most frequent adverse event in both groups (3 %). Although the advantage was small (8–9 % of primary end points), it should be noted that according to these results for every 100 patients with CHF, 1 g/d of n-3 PUFAs could save 2.61 lives and avoid 4.72 CVD events.

JELIS Trial Subgroup Analysis

The secondary prevention findings of the JELIS trial were reported in 2009 [24]. Thirty-six hundred and sixty-four patients with prior CAD and a total cholesterol level of ≥250 mg/dL were enrolled and followed for 4.6 years. Patients were randomized to receive either statin alone (control group; $n = 1841$) or 1800 mg of EPA + statins (EPA group; $n = 1823$). The incidence of major adverse coronary events (MACE) was lower in the EPA group (8.7 vs. 10.7 %, HR 0.77; 95 % CI 0.63–0.96, $p = 0.017$). Of 1050 patients with a history of MI, the incidence of MACE in the EPA group (15.0 %) was lower than in the control group (20.1 %, HR 0.73; 95 % CI 0.54–0.98; $p = 0.033$). Notably, patients with prior MI achieved a 27 % risk reduction, and those that had undergone prior intervention (percutaneous transluminal coronary angioplasty or coronary artery bypass grafting) achieved a 41 % risk reduction. These results suggest that combinations of conventional treatment and EPA administration should be considered for the secondary prevention of CAD, and that, n-3 PUFAs should also be considered for this purpose.

The Omega Trial

The Omega study was a multicenter, randomized, double-blind, placebo-controlled trial that was conducted to test the effects of highly purified n-3 PUFAs administered at 1 g/day for 12 months (the EPA/DHA ratio differed from that used in the GISSI-Prevenzione trial) on the incidence of sudden cardiac death among 3851 patients that had experienced acute MI during the previous 3–14 days [25]. Secondary endpoints included major cerebrovascular and CV events. In this trial, no difference was observed between rates of primary (1.5 vs. 1.5 %; $p = 0.84$) or secondary endpoints (major cerebrovascular and CV events, 10.4 % vs. 8.8 %, $p = 0.1$; revascularization in survivors, 27.6 vs. 29.1 %, $p = 0.34$) in the placebo and n-3 PUFA groups. However, the incidence of sudden cardiac death or a CV event was less than one-half of that expected, which made the trial underpowered with respect to the detection of the cardioprotective effect of n-3 PUFAs. In addition, angiotensin converting enzyme inhibitors or angiotensin receptor blockers, β-blockers, statins, and clopidogrel were prescribed to 91, 94, 94, and 88 % of patients, respectively, and this may have contributed to the low rate of CV events observed and thus reduced the benefit due to n-3 PUFA treatment.

Alpha Omega Trial

The results of the Alpha Omega Trial were published [26] a few weeks after the results of the Omega Trial, and like the Omega Trial, this trial reported neutral results for EPA/DHA (doses were lower than in the GISSI-Prevenzione trial). The Alpha Omega Trial was a multicenter, double-blind, placebo-controlled study that tested the effects of margarine enriched with n-3 PUFAs on CV events among 4837 patients (60–80 years) with a history of MI. Patients were randomly assigned to one of the four treatments for 3.3 years: (A) 0.4 g/d of a combination of marine n-3 PUFAs of (EPA + DHA), (B) 2 g/d of the plant-derived n-3 PUFA (α-Linolenic acid), (C) marine (0.4 g/day) + plant-derived (2 g/day) n-3 PUFAs, or (D) placebo. Neither plant- nor

marine-derived n-3 PUFAs decreased the primary endpoint and the rate of major CV events, including cardiac interventions (HR with EPA + DHA = 1.01, 95 % CI 0.87–1.17, $p = 0.93$; HR with α-linolenic acid = 0.91, 95 % CI 0.78–1.05, $p = 0.20$). Furthermore, secondary endpoints were not decreased by n-3 PUFAs. However, because of the design of the Alpha Omega Trial, it is difficult to interpret its results. This was a factorial trial and thus only allowed two-way comparisons of patients that received treatment or did not receive treatment. In comparison with EPA + DHA versus not EPA + DHA, 2404 patients with EPA + DHA at 400 mg/d plus α-linolenic acid at 1008 mg/d (total of 1408 mg/d of n-3 PUFAs) were compared with 2433 patients with α-linolenic acid at 984 mg/d (total of 984 mg/d of n-3 PUFAs). In comparison with α-linolenic acid versus not α-linolenic acid, 2409 patients treated with α-linolenic acid at 2 g/d plus EPA + DHA of 201 m/d (total 2201 mg/d of n-3 PUFAs) were compared with 2428 patients with EPA + DHA at 196 m/d (total of 196 mg/d of n-3 PUFAs). Thus, the influence of n-3 PUFAs was not compared with a real 'placebo' but with patients treated with a smaller amount of n-3 PUFAs. In the case of marine n-3 PUFAs, there was a very small difference in the total amount of n-3 PUFAs administered to the groups (1.4 g vs. 1.0 g, respectively). In addition, the lack of effectiveness of n-3 PUFAs in the Omega and Alpha Omega studies compared with the GISSI-Prevenzione trial might have been due to the more up-to-date treatments (statins) administered to participants in accord with current clinical guidelines for post-AMI patients [27]. More specifically, the proportion of patients treated with in GISSI-Prevenzione trial (29 %) was substantially lower than in the Omega (94 %) and Alpha Omega (85 %) trials. However, it should be added a recent report described clinical synergism between the effects of n-3 PUFAs and statins for most CV outcomes [28].

The SU.FOL.OM3 Trial

The results of the SU.FOL.OM3 trial were published in 2010 [29]. During a mean follow-up of 4 years in 2501 CHD patients, treatment with n-3 PUFAs (600 mg/d) did not show a protective effect on the incidence of non-fatal MI. However, the relatively modest number of events (157 events in 2501 patients; 6.3 %), the administration of a low daily dose of n-3 PUFAs, and a 15 % reduction in the expected rate of CV events unfavorably affected the statistical power of this trial and biased study results. Nevertheless, n-3 PUFAs were found to have no significant effect on MI risk; the number of non-fatal MI events was 32 (2.6 %) in the n-3 PUFAs group and 28 (2.2 %) in the placebo group (HR, 0.88; 95 % CI, 0.53–1.46). Eussen et al. [30] tested the statin-specific effects of EPA + DHA and/or α-linolenic acid on major CV events as determined by the Alpha Omega Trial and found they differed between statin nonusers ($n = 413$) and statin users ($n = 3740$) in post-MI patients. In addition, the effects of an additional 400 mg/d of EPA + DHA, 2 g/d of α-linolenic acid, or both on major CV events were evaluated in these two patient groups. Four hundred and ninety-five (13 %) statin users and 62 (15 %) statin nonusers developed a major CV event. Among statin users, an additional dose of n-3 PUFAs did not decrease CV events (HR, 1.02; 95 % CI, 0.80–1.31). However, in statin nonusers, only 9 % of those that received (EPA + DHA) +ALA experienced a CV event as compared with 18 % in the placebo group (HR, 0.46; 95 % CI, 0.21–1.01). Furthermore, in post-MI patients not administered statins, low-dose n-3 PUFAs were found to have possibly prevented major CV events, which suggest that statin changes the effects of n-3 PUFAs on the risk of major CVD.

The ORIGIN Trial

The ORIGIN (Outcome Reduction with Initial Glargine Intervention) trial was a double-blind study with a 2-by-2 factorial design, and involved 12,536 randomized patients of mean age 64 years with type 2 diabetes, impaired fasting glucose, or impaired glucose tolerance [31]. In this omega-3 fatty acid trial, patients were randomly assigned to receive 900 mg of n-3 PUFAs daily ($n = 6281$) or placebo ($n = 6255$) and to receive either insulin glargine or standard care. The primary endpoint was CV death, and secondary endpoints included a composite of CV death, non-fatal MI, or non-fatal stroke, all-cause mortality, and arrhythmic death. The results of the ORIGIN trial showed that n-3 PUFAs did not reduce the rate of CV events in high-risk patients with prediabetes or diabetes after 6 years. CV deaths occurred among 9.1 and 9.3 % of those randomized to n-3 PUFAs or placebo, respectively. N-3 PUFA intake did not affect fatal or non-fatal MI, fatal or non-fatal stroke, or hospitalization for heart failure. A number of reasons might explain the neutral effect of n-3 PUFAs in this population of patients. Patients enrolled in this trial were taking cardioprotective drugs (ACE inhibitors or ARB 68.8 %, beta blocker 69.6 %, aspirin or antiplatelet agent 69.6 %, CCB 27.2 %, and statin 53.0 %). In addition, patients enrolled in other trials within 3 months of MI (the GISSI-Prevenzione trial) or with a history of heart failure (the GISSI-HR trial) were not confined to individuals with abnormal glucose, which is associated with a high risk of CHD. These results suggest that an n-3 PUFA dose of 1 g/d might have been too low to reduce triglyceride levels, which concurs with the triglyceride reduction observed in a similar study involving patients with diabetes, in which those receiving 2 g/d of n-3 fatty acids showed a reduction in triglyceride level of 0.09 mmol/L.

The Risk and Prevention Study

The Risk and Prevention Study was a double-blind, placebo-controlled trial that assessed the ability of n-3 PUFAs to prevent major coronary events in patients without a history of MI but at high CV risk who were being treated according to standards of care [32]. A total of 12,513 patients were enrolled, and 6244 were randomly assigned to n-3 PUFAs group and 6269 to a placebo group. The primary endpoint was a composite of death from CV causes or hospital admission for CV causes. The primary endpoint occurred in 1478 patients (11.8 %), including 733 of 6239 who received n-3 PUFAs (11.7 %) and 745 of 6266 who received placebo (11.9 %). The incidence of the primary endpoint was not significantly reduced by n-3 PUFAs, although plasma triglyceride levels fell significantly more in patients given n-3 PUFAs than in those who received placebo (-28.2 ± 1.3 mg/dL vs. -20.1 ± 1.3 mg/dL, $p < 0.001$). No significant benefit could be attributed to n-3 PUFAs with respect to reducing the risk of hospital admission for CV-related causes. No evidence was obtained to support the usefulness of n-3 PUFAs regarding the prevention of CV-related death or disease in patients with multiple CV risk factors or atherosclerotic disease, but with no previous history of MI.

Meta-analysis

Rizos et al.

These authors conducted a meta-analysis on 20 randomized, double-blind, placebo-controlled trials and included 68,680 patients who were evaluated for the effects of n-3 PUFAs on all-cause mortality, CV death, sudden cardiac death, MI or stroke [33]. The results failed to show n-3 PUFAs supplementation reduced the risks of all-cause mortality (relative risk, RR 0.96, 95 % CI 0.91–1.02), CV death (RR 0.91, 95 % CI 0.85–0.98), sudden cardiac death (RR 0.87, 95 % CI 0.75–1.01), MI (RR 0.89, 95 % CI 0.76–1.04) or stroke (RR 1.05, 95 % CI 0.93–1.18). Some reasons were offered to explain the neutral effect of n-3 PUFAs, namely a low mean n-3 PUFAs dose (1.51 g/d), small sample sizes, and the use of n-3 PUFAs from diverse sources [34].

Kwak et al.

Fourteen randomized, double-blind, placebo-controlled trials (involving 20,485 patients with a history of CVD) were included in the final analyses of this study [35]. Supplementation with n-3 PUFAs did not reduce the risk of overall CV events (RR, 0.99; 95 % CI, 0.89–1.09), all-cause mortality (RR, 0.96; 95 % CI, 0.90–1.02), sudden cardiac death (RR, 0.93; 95 % CI, 0.66–1.30), MI (relative risk, 0.81; 95 % CI, 0.65–1.01), or congestive heart failure (RR, 0.92; 95 % CI, 0.73–1.17). In subgroup analyses by concomitant medication use, no preventive effect was observed regardless of the use of lipid-lowering agents, while a minimal preventive effect (RR, 0.71; 95 % CI, 0.50–1.00) was associated with the use of antiplatelet agent (aspirin). Inclusion of small trials and lack of inclusion of GISSI-P (excluded for lack of double-blinding) and of DART and the inclusion of trials involving low doses of EPA/DHA (SU.FOL.OM3) could have contributed to the lack of benefit found for n-3 PUFAs.

Wen et al.

This study was conducted to determine whether n-3 PUFAs supplementation in CHD patients is associated with a significant reduction in the risks of death from cardiac causes, sudden cardiac death, and death from all causes. The authors showed that exclusion of the Alpha Omega Trial or of the Omega Trial led to a significant reduction in major CV events when meta-analysis estimates were computed for remaining studies [36]. Although the evidence obtained was insufficient to demonstrate n-3 PUFA supplements had a preventive effect against MACE in CHD patients, subgroup analysis demonstrated a reduction in the incidence of major CV events. Furthermore, subgroup analysis showed a significant major CV event reduction in patients that received >1 g of n-3 PUFA/d. On the other hand, supplementation for >2 years showed no more benefit than supplementation for <2 years.

Conclusions

Available published evidence on the effects of n-3 PUFAs show a statistically significant reduction in risk of MI (−25 %), but not in the risk of all-cause mortality [37]. Taken together, our observations suggested that (1) n-3 PUFAs are useful in specific clinical settings, for example, in post-acute MI patients with at high risk of arrhythmia due to residual ischemia or left ventricular dysfunction; (2) the clinical outcomes of n-3 PUFAs are unequivocal, because their effects appear to depend on clinical conditions and risk profiles. However, no definite cutoff dose has been established for n-3 PUFAs in the context of protection from CVD, because the validities of the dose–response models used to study the CV effects of n-3 PUFA remain controversial even when meta-analysis is used [37, 38]. Based on a review of the literature, the CV protective roles of n-3 PUFAs can be explained by atheroprotective effects and by other

mechanisms, such as the suppression of cardiac arrhythmias, reduced myocardial oxygen consumption, improved cardiac filling and myocardial efficiency, the regulation of insulin resistance, and decreases in heart rate or blood pressure [39–42]. Studies are needed to determine whether the addition of n-3 PUFA to statin therapy confers additional benefits in terms of reducing the atherosclerotic burden and the rate of major adverse cardiac event in CAD patients requiring stent implantation. Furthermore, the additive beneficial effects of n-3 PUFA on the regression of atherosclerosis and on clinical outcomes remain to be confirmed in patients on statin after stent implantation for CAD. In addition, large-scale studies are required to investigate the effects of n-3 PUFAs in different races and ethnic groups.

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Alternative Origins for Omega-3 Fatty Acids in the Diet

34

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Abbreviations

ALA	Alpha-linolenic acid
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FA	Fatty acid
FADS	Fatty acid desaturase
FAO	Food and Agricultural Organization of the United Nations
IMF	Intramuscular fat
LD	Linkage disequilibrium
LA	Linoleic acid
LC PUFA	Long-chain polyunsaturated fatty acids
NOS3	Nitric oxide synthase
SC	Short-chain
SDA	Stearidonic acid
USA	United States of America

Introduction

Omega-3 fatty acids are found in an array of plant and animal sources. Alpha-linolenic acid (ALA), the shortest fatty acid within the omega-3 fatty acid chain (Fig. 34.1), is present in an array of foods including flaxseed, walnuts and other vegetable seeds oils as well as fish, meat and poultry [1]. The subsequent fatty acids of the omega-3 pathway (Fig. 34.1) are less abundant but can also be found in some plant seed oils, fish and meat flesh [2, 3]. Omega-3 fatty acids, in combination with other unsaturated fatty acids, maintain the fluidity of the cell membrane and may be cleaved by enzymes such as phospholipase A_2, cyclooxygenase and lipooxygenase, activating inflammatory-associated pathways [4]. Importantly, the length and extent of unsaturation of omega-3 fatty acids affects these inflammatory-associated processes within cells. Anti-inflammatory mediators are cleaved from omega-3 fatty acids, while omega-6 fatty acids provide precursors for pro-inflammatory metabolites [4]. Presently, a majority of the health benefits associated with omega-3 fatty acids are attributable to the long-chain polyunsaturated fatty acids (LC PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [5].

The discovery of the importance of marine omega-3 fatty acids began in the 1970's following the observation of low rates of coronary atherosclerosis among Greenland Eskimos consuming a diet high in fish and whale blubber [6, 7]. These LC PUFA have shown health-promoting effects in regard to chronic inflammation, dyslipidaemia, allergic disease and some cancers [8–12]. The current dietary recommendations for fish-derived omega-3 fatty acids is 0.25 g per day of EPA plus DHA [13]. Marine LC PUFAs, EPA and DHA, are synthesised from shorter chain omega-3 fatty acids by desaturation and elongation by specific enzymes (Fig. 34.1). Although humans produce the enzymes required for these conversions, two enzymes in this pathway are insufficient, meaning direct sources of EPA and DHA are important [14]. Marine fish feed on algae, the primary producers of LC PUFA. As fish higher up in the food chain eat these fish, there is an accumulation of LC PUFA within fish flesh. Therefore, fish are a major dietary source of LC PUFA for humans.

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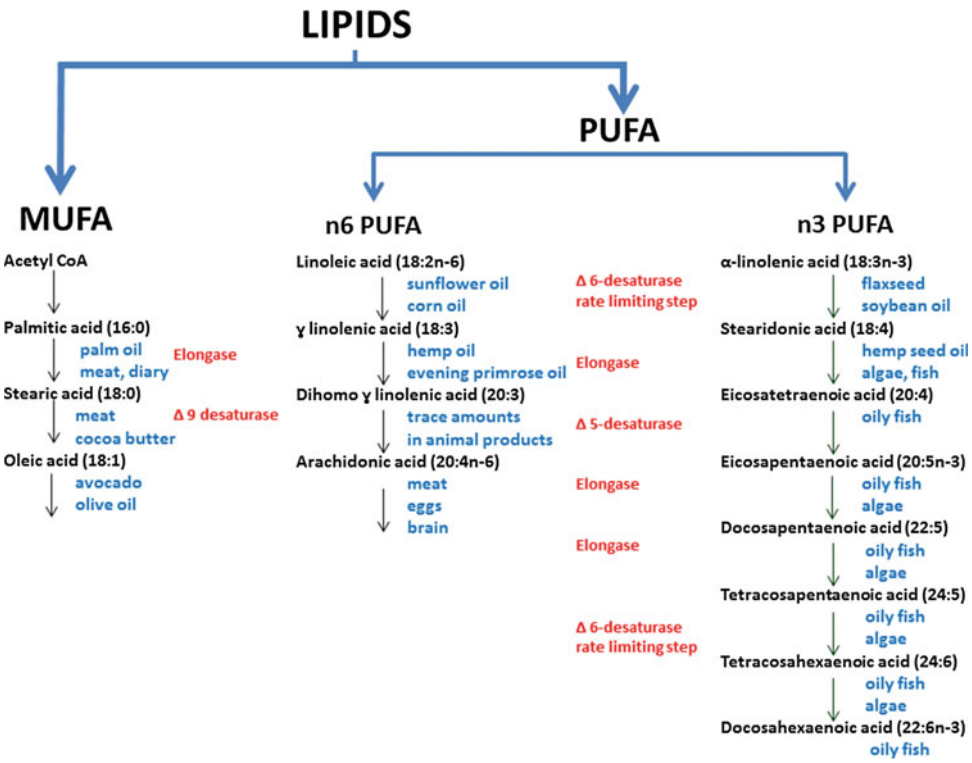

Fig. 34.1 The biosynthesis pathways of the omega-3, 6 and 9 family of poly- and mono-unsaturated fatty acids (copyright permission received [105]). The main dietary sources are shown in *blue text* and the enzymes in *red text*

Due to the benefits of including marine omega-3 LC PUFA in the diet, there is increasing demand and need for these oils. LC PUFA can be included in the diet by taking fish oil supplements or by directly consuming fatty fish. The current consumption or supplementation using either of these methods is still not at the recommended level [1]. Furthermore, the current demand for fish has led to overfishing of certain species, putting pressure on wild fish stocks [15]. Overfishing is increasingly becoming a concern for sustainability, which is heightened by changes in fish ecosystems and habitat loss [16, 17]. Fisheries not only supply fish for supplement production and fish consumption but also for fish feeds in order to maintain sufficient levels of EPA and DHA within fish flesh. Therefore, alternative fish diets are also explored. Although this may not directly affect the present fish oil consumption rates, the problem will develop over time as fish decline becomes more pronounced, unless more sustainable regulations are introduced. One explanation of the low fish oil intake in the population is the concern for the presence of metal toxins, such as methylmercury within fish flesh [18, 19]. Furthermore, vegetarians and vegans are not able to easily incorporate LC PUFA into their diet and require alternative sources for these important fatty acids [20]. These concerns indicate the need for alternative, safe and sustainable sources of dietary LC PUFA.

Plants may be genetically modified in order to express the enzymes involved in the synthesis of EPA and DHA [21]. These bioengineered oilseeds have the potential to replace fish-derived LC PUFA in the diet. Recent research within bioengineered plant seeds is promising, as recent studies demonstrate sufficient accumulation of EPA, as well as DHA, in engineered plant seed oils [22]. Additional explored alternatives include the implementation of LC PUFA into livestock meat and other animal products by introducing specific animal diets. Furthermore, this chapter will discuss the use of microalgae as a source of LC PUFA, a notable substitute for vegetarians and vegans. It is also important to note that algae can provide a source of protein and biofuel, highlighting it as a sustainable and multi-purpose resource. Additional research is required in order to develop high-DHA-containing algae supplements that are both affordable and palatable.

Why Do We Need Alternative Sources of EPA and DHA PUFA?

The Growing Concern of Overfishing and Habitat Loss

Data published by the Food and Agriculture Organization of the United Nations suggest that the world's fish supply has been growing steadily with a 3.2 % increase per year [23]. In 2011, about 130 million tonnes of the 154 million tonnes of fish produced were for human consumption. In regard to fish production, the two major sources of fish are aquaculture and capture fisheries. Aquaculture is the term used for farming of

aquatic organisms, including both invertebrate and vertebrate marine species. Although capture fisheries supply the majority of fish production worldwide, aquaculture is expanding exponentially [23]. The production of fish from these farms has increased dramatically in order to meet the demand for fish and fish oils and reduce the impact on wild fish stocks [24]. Furthermore, farmed fish require at least some proportion of fish products in their diets in order to maintain adequate levels of LC PUFA within flesh [4]. Therefore, an increase in aquaculture is met with an increased demand for fish.

As well as producing fish for human consumption and other non-food uses, some aquaculture farms provide fish to replenish stocks in the wild [25]. Nevertheless, the high demand for fish oils provides an important reason to develop alternatives sources of LC PUFA [21]. Aquaculture has removed some of the pressure from wild stocks; however, the destruction to habitats of wild fish and natural marine processes is a crucial aspect to consider [16]. Simple changes can have major effects, such as small changes in temperature. These small changes can influence distribution of certain species in the oceans and complete changes to food chain dynamics [26]. Additionally, due to trends in demand for certain fish species or populations, there are indirect effects on other species due to the introduction of imbalances within the aquatic food chain [17]. These imbalances and changes to natural habitats can lead to reductions in other fish species not currently exploited, compromising biodiversity. Although these fish farms reduce the impact on wild fish stocks, there is still the need for fish-based diets within these farms, suggesting the need for fish products is only increasing with the growth of aquaculture.

Pollution and Metal Toxin Accumulation

Mercury (Hg) occurs naturally in our environment and can be transmitted through the atmosphere as elemental or ionised mercury, Hg^{2+} and Hg^{3+}, respectively. The release of mercury into the atmosphere occurs from a variety of natural and anthropogenic sources, one being coal-fired power plants [18]. In the appropriate reducing environments, oceanic or wetland Hg^{2+} can form methylmercury (CH_3Hg). As a result, there is a rise in CH_3Hg, which then accumulates within flesh of marine or freshwater organisms, creating a direct route for human exposure. The presence of CH_3Hg within seafood can be especially unsafe for particular groups of people [19]. Extreme cases, such as the CH_3Hg poisonings of Minamata, Japan, in the 1950s, indicate the potential toxicity of CH_3Hg within fish and shellfish; however, the toxicity levels in most areas are considered to be relatively low in comparison [27].

Although it is generally accepted that CH_3Hg is detrimental to health, not all negative claims associated with CH_3Hg are well supported. Some studies suggest an effect of CH_3Hg exposure on cardiovascular disease risk factors, such as hypertension, although inconsistent data mean this association warrants further investigation [28, 29]. It is well established, however, that exposure to sufficient levels of CH_3Hg may influence the development of the foetus, particularly during brain development [30]. These reports motivated governments to improve the awareness of CH_3Hg contamination to prevent intoxication, especially among young children, pregnant women and women of childbearing age [31, 32]. Advisories also specify particular species of fish associated with high CH_3Hg contamination, usually large species like shark or swordfish [19]. These fish carry higher contamination risks due to their position at the top of the food chain, as they accumulate higher proportions of CH_3Hg by eating smaller contaminated fish.

Unfortunately, there is concern as to the effectiveness of governmental CH_3Hg warnings. For example, consumers partaking in a study in France demonstrated poor ability to recall high-risk species, which may reflect the lack of change in consumption levels of highly contaminated fish species [33]. However, others suggest advisories may be pushing too strongly on CH_3Hg contamination, which may account for an overall decrease in fish consumption and lead to too low intakes of LC PUFA [19]. For example, it is known that LC PUFA are very important during brain development of the foetus; however, a study by the FAO suggests that benefits of LC PUFA for pregnant women outweigh the risks of toxicity to the foetus [34]. Both Japan and the USA have observed a decrease in fish consumption over recent years, which may be a consequence of the heightened public awareness of fish toxicity [19].

It is necessary to emphasise the risks for particular groups or populations and also formulate the risks of not including LC PUFA in the diet. One study found that comparing risk and benefit of consuming fish is dependent on the species [35]. Therefore, it is also important to clarify which species are safe and which are most associated with high CH_3Hg contamination and should be avoided. Furthermore, prospective studies would give insight into potential effects of chronic, low-dose exposure of CH_3Hg on human health and is worth attention. Therefore, this knowledge could be applied to risk assessment advice.

Vegetarians and Vegans

Vegetarians and vegans have low intakes of LC PUFA [20]. However, both vegetarians and vegans have lower rates of cardiovascular disease and non-communicable disease, despite their low blood levels of EPA and DHA [36]. Therefore, it is not clear how necessary it is for vegetarians to include direct dietary sources of marine LC PUFA. Vegetarians generally exclude all meat and fish products. However, there exists a variety of different vegetarian and vegan diets. For

example, some vegetarians will include fish in their diet, but not meat, and some vegans may eat honey but not dairy products. Therefore, although vegetarians and vegans have low LC PUFA intake, the lower risk of non-communicable diseases may be related to other dietary sources [37].

Vegetarians and vegans often have diets with higher amounts of ALA compared to meat-eaters. Nevertheless, DHA exists at extremely low levels within plasma and blood cells of vegans and vegetarians, suggesting synthesis of LC PUFA is insufficient [36]. DHA is important for development of the foetus, as well as brain and visual development in children. A recent study determined benefits of DHA in infant formula on visual acuity [38]. Therefore, it is important for vegetarian and vegan mothers to include LC PUFA in their diet during pregnancy and lactation [39]. Furthermore, it has been known that DHA is associated with cognitive decline and depression among adults; however, the results have been inconsistent [40, 41]. Therefore, vegetarians and vegans would benefit by including LC PUFA in the diet.

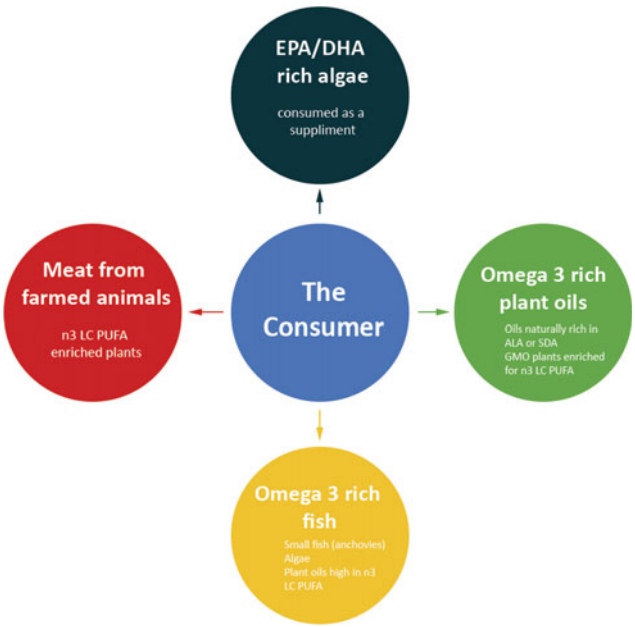

Fig. 34.2 Sources of omega-3 long-chain polyunsaturated fatty acids in the human diet. *EPA* eicosapentaenoic acid; *DHA* docosahexaenoic acid; *ALA* alpha linoleic acid; *SDA* stearidonic acid; *n3 LC PUFA* omega-3 long-chain polyunsaturated fatty acids; *GMO* genetically modified organism

Currently Explored Alternatives

Oily fish and certain algae are the only known naturally available sources of EPA and DHA, although short chain (SC) omega-3 PUFA can serve as a source of fatty acids for the conversion to EPA and DHA in humans and other animals (Fig. 34.1). Declining fish stocks compel us to consider alternative sources of EPA and DHA [42]. Alternative sources of LC PUFA that can be converted to EPA and DHA include plant sources such as flaxseed, echium and soya, among others [43, 44]; metabolically engineered plants such as *Camelina sativa* [45–48]; animal sources, for example milk, eggs and meat [49–51]; and algae [4] (Fig. 34.2). Metabolic engineering of crop plants to accumulate EPA and DHA at levels comparable to fish has been technically challenging. Genes associated with the entire omega-3 LC PUFA pathway have had to be introduced [52, 53], and in addition, it has been necessary to avoid the accumulation of unwanted C18 fatty acids [54, 55]. Approximately 65 % of consumers prefer functional foods over supplements [56], thus driving the design of omega-3-rich plant and animal products. In this section, currently explored alternatives to wild marine fish as a source of EPA and DHA will be explored.

Plant Sources of Omega-3 PUFA as an Alternative to Oily Fish

Plant sources of omega-3 PUFA are high in SC but are devoid of LC PUFA such as EPA and DHA [57]. However, these SC PUFAs can be converted to the desirable LC PUFA via the omega-3 PUFA pathway (Fig. 34.1). In this pathway, there are two conversion steps that are rate limited by the delta-6-desaturase enzyme in humans [14]. The efficiency of this conversion is partially determined by the genotype of the *fatty acid desaturase* (*FADS*) family of genes found on chromosome 11 [49, 58]. The following SNPs are known to influence omega-3 LC PUFA levels in red blood cells: rs1535 (in LD with rs3834458), rs174448, rs174537, rs174575, rs2727270 and rs10000778 [49, 58]. Similarly, it is believed that nitric oxide synthase (NOS3) may determine responsiveness to omega-3 PUFA, and carriers of the minor allele of rs1799983 show a greater benefit in response to omega-3 PUFA consumption than those with the major allele [59]. This association suggests that genotype does play a role in the efficiency of conversion of ALA within the omega-3 PUFA pathway and therefore may vary from one person to another and from one ethnic group to another.

Land plants do not typically synthesise LC PUFA but can be engineered to do so by introducing a suite of genes that can direct the synthesis of the precursors or the actual fatty acids of interest [48]. When designing such experiments, it is first necessary to identify the genes of interest, insert them into a suitable host plant that has good agronomical traits [57] and ensure they are under the control of suitable promoters that can direct expression to occur at an appropriate stage of development and in an apt part of the plant from which oil can be easily harvested, such as the seed [48]. In addition, it is important to minimise side products such as omega-6 fatty acids.

In response to the need to establish a sustainable source of LC PUFA, efforts have focused on modifying the composition of plant oils such that they contain LC PUFA [57]. The first reports on the bioengineering of stearidonic acid (SDA), EPA and DHA in oilseed plants took place in 1997, 2004 and 2002, respectively [60, 61]. It is possible to bypass the first rate limiting delta-6-desaturase step (Fig. 34.1) by consuming plants high in SDA, a precursor of EPA synthesis [4]. Plants are naturally low in SDA, and for this reason researchers have identified environmental conditions to optimise the production of SDA, as well as plants that can be modified to increase the production of SDA [62]. Although gastrointestinal side effects were noted in response to daily soybean oil consumption in humans, no increased effects were seen in response to soybean oil with enhanced levels of SDA [63] and no adverse effects were seen in a rodent model [64]. It seems prudent to investigate the safety profile of plant-based oils suitable for the increased production of precursors of EPA and DHA before fatty acid synthesis is enhanced. Despite the challenges associated with introducing a number of genes to modify the production of omega-3 PUFA as precursors of EPA or DHA synthesis in plants, this land-based approach looks promising [63]. A number of plant species have been investigated as potential sources of omega-3 PUFA or as models in proof of principle experiments to enhance the level of the longer chain PUFA.

Arabidopsis, otherwise known as rockcress, is a member of the family *Brassicaceae*. *Arabidopsis thaliana* was the first plant to be sequenced, and its biology has been well studied. Proof of principle with respect to metabolic engineering of oil production in vegetative tissues and the occurrence of bottlenecks has largely been established in Arabidopsis [52, 57, 65]. Arabidopsis is regarded as a model and has been used to test individual genes as well as gene complexes to modify oil production, particularly the successful accumulation of EPA and DHA [57, 65]. At present, proof of principle experiments are focused on the bioengineering of oil production in leaves [57]. As these principles are translated into other oil and biomass crops, so the attention on Arabidopsis will wane.

Glycine max, otherwise known as **soybean**, are grown for their protein (approximately 40 %) and oil content (approximately 20 %) [66]. In the past, soybean has been selectively bred based on its protein content; however, the focus has shifted more recently to its oil content. The naturally high linoleic acid (LA) and ALA content of soybean oil leads to oxidative instability [66]. Although oxidative instability can be addressed through partial hydrogenation, this process generates *trans* fatty acids and in order to avoid this problem, genetic strategies have been used to change the fatty acid profile of soybean oil [66, 67]. Pressure to address this issue increased with the requirement in 2006, of the USA Food and Drug Administration, to include the amount of *trans* fatty acids on food labelling, and food applications are the driving force behind the enhancement of the nutritional content of soybean oil [66]. As mentioned elsewhere in this section and in the section on "aquaculture" below, SDA-rich oils have been used in place of fish oils in fish feed. However, it is clear that plant oils, including soybean, that are rich in ALA and/or SDA cannot entirely replace fish oil in fish feed. For this reason, the focus has been on metabolic engineering for the development of soybean seeds that are enriched with EPA and DHA [66, 68], although much work has also focused on the identification of genes/mutations associated with oil yield [69, 70].

Echium oil is extracted from *Echium plantagineum* and appears to be used more as a supplement rather than a food. *E. plantagineum* originates from the Mediterranean region and has become an invasive weed in Australia and South America and was previously not regarded as a food source [71]. Echium oil maybe of value in aquaculture and farm feeds, as the seed oil contains 9–16 % SDA, which is much higher than many other plant sources, such as hemp seed [71]. Echium oil has been used in fish feed experiments to assess the impact of an SDA-rich oil on EPA and DHA levels in muscle tissue. Although an accumulation of SDA developed, the levels of EPA and DHA remained markedly lower than in those fish fed with fish oil [72, 73].

Flaxseed, otherwise known as linseed, is derived from the seeds of *Linum usitatissimum*. Flaxseed originates in India and is now grown throughout the world. Initially used only in the production of paints and industrial-type products, a portion of the seeds produced are now pressed at low temperatures under inert gas to prevent oxidation [74] and carefully stored so as to be suitable for human consumption [44]. Flaxseed oil (usually consumed as capsules or raw oil) is high in ALA (Fig. 34.1), which needs to be first converted to SDA before being enzymatically elongated to form EPA and DHA [44]. There is conflicting evidence regarding the impact of ALA-rich flaxseed oil on circulating EPA and DHA, with not all studies reporting a positive health response [75, 76], and a generally accepted inefficient conversion of approximately 10 % and 1 % of ALA to EPA and DHA, respectively [77].

Camelina sativa is a *Brassicaceae* oilseed with a short generation time [45]. Transgenic *C. sativa* (RRes_EPA and RRes_DHA) are a plant source of EPA and DHA, wherein the plant has been metabolically engineered to enhance its capacity for omega-3 LC PUFA synthesis in the seeds [65]. *C. sativa* was successfully engineered to amass EPA or EPA and DHA in the seed by utilising the endogenously accumulated high levels of ALA without the unwanted build-up of omega-6 fatty acids [65]. The RRes_DHA seeds on average contain 11 % EPA and 8 % DHA and the RRes_EPA seeds on average contain 24 % EPA, whereas "bulk fish oil" contains 13 % DHA and 13 % EPA. In addition,

Fig. 34.3 One of the authors in a field of rapeseed in South Canterbury, New Zealand (December 2014)

there is a decrease in the accumulation of oleic acid in both lines [65]. Such research has demonstrated that it is feasible to metabolically engineer crop plants to produce EPA and DHA at comparable levels to that found in oily fish [65].

Brassica napus, more commonly known as **rapeseed**, is used to produce food-grade canola oil (Fig. 34.3). Canola oil is a monounsaturated fatty acid that is high in oleic acid and is an attractive target for introducing a pathway for the production of DHA [48]. Modern food-grade oil varieties of rapeseed have the elongase genes knocked out, and this creates a perfect environment for LC PUFA biosynthesis [48]. Due to elongase gene inactivity, a pool of substrates accumulate and these substrates can be used as precursors for LC PUFA biosynthesis [48].

In addition to consuming plant sources of omega-3 PUFA, such sources can also be used to feed commercially farmed oily fish and thus reduce the depletion of ocean stocks, as well as to enrich the omega-3 LC PUFA in farm animals [4, 5]. Numerous crop species have undergone plant breeding experiments and metabolic engineering to enrich their fatty acid profiles with omega-3 LC PUFA, and numerous successes have been noted [5, 54, 69, 78].

Aquaculture and Fish Feeds

In order to meet the growing demand for world fish consumption and yet protect wild fish stocks from further depletion, fish production from commercial fish farms worldwide has increased by an average of 8.3 % annually over the past four decades [79]. Krill, anchovies and microalgae are often used as an omega-3 LC PUFA source in commercial fisheries [80, 81], and although the production of krill and algae may be sustainable, the present use of anchovies, sardines and mackerel is not [5, 82]. The replacement of fish oil in aquaculture is becoming increasingly important, as wild feed-grade fish stocks decrease [5, 83]. However, one of the problems associated with commercial fish farming is that fish cannot only be fed with plant-based oils, but also require fishmeal and/or fish oil in order to achieve fatty acid levels equivalent to fish caught in the wild [81]. Different species of fish vary in their capacity to adapt to a plant oil-based diet. For example, marine flatfish species such as Senegalese sole seem to adapt well to such diets, while marine carnivorous species such as gilt-head seabream, do not [84].

Although not widely researched, experimentation has been performed with the partial substitution of fish oil with beef tallow in the diet of fish. Pérez et al. found that gilt-head sea bream fed a mix of fish oil and beef tallow (60 g/kg) had comparable weight and EPA and DHA content to those fish fed entirely on a fish oil-based diet [85]. In contrast, those fish that received vegetable oil and beef tallow, and no fish oil, grew more slowly and had markedly lower levels of EPA and DHA [85]. Clearly, there is potential for the use of beef tallow as a partial replacement of fish oil in the diets of some fish species.

Coupland suggested that SDA-rich plant oils maybe useful to enhance the EPA and DHA levels of plant oil-fed fish, as

well as to enhance the levels of these two fatty acids in humans [62]. Cleveland et al. investigated the effect of plant oils rich in ALA or SDA as an alternative to fish oils in the diets of rainbow trout [86]. Although trout fed diets rich in SDA or fish oil were heavier than those fed high ALA diets or a mixed vegetable oil diet, the lipid source of EPA and DHA was reflected in the fish fillets [86]. The trout that received fish oil diets had significantly higher EPA and DHA flesh levels whereas there was no significant difference in the EPA and DHA levels in the flesh of the trout fed one of the three plant-based oil diets [86]. However, when rainbow trout were fed a fish oil finishing diet for four weeks following fish oil and plant oil-based diets, the difference in EPA and DHA values between the fish fed the altered diets was not significant [87]. Nonetheless, the trout fed the fish oil diet throughout the study had higher values of EPA and DHA [87].

When feeding Atlantic salmon various fish and vegetable oil-based finishing diets, Miller et al. [83] found no difference in growth rate. Consistent with the findings of Cleveland et al., Miller et al. found that the source of omega-3 PUFA did impact on the level of EPA and DHA in both the red and white flesh of the salmon. The fish oil-fed salmon had significantly higher levels of EPA, DPA and DHA than fish fed the vegetable oil-based diets [83]. However, fish fed an SDA oil-based diet had significantly higher levels of DHA in their flesh than those fed canola oil-based diets [83]. Nonetheless, none of the above mentioned plant-based diets were comparable to feeding fish on fish oil-based diets when analysing for EPA and DHA content.

Another species of fish, namely Senegalese sole, have been found to be well suited to aquaculture [84]. A fish oil diet was substituted with a plant oil-based diet for 140 days (followed by a fish oil diet of 26 days for all diet groups), and this change did not significantly alter the levels of EPA and DHA in the flesh [84]. When experimenting with the diets of the common carp, Schultz et al. found that carp receiving plant oil-based diets rich in SC PUFA had comparatively low LC PUFA flesh levels [88]. However, if a fish oil-rich diet was used as a 30-day finishing diet, the LC PUFA content was largely restored [88]. Clearly, fish oil finishing diets can help restore EPA and DHA levels, and further research is required to optimise the content and timing of such diets in different fish species in order to maximise the EPA and DHA levels in fish fillets.

Animal Sources of LC PUFA as an Alternative to Oily Fish

Most people do not consider animal sources, other than fish and seafood, as being a good source of omega-3 PUFA. It is not surprising that feeding domestic farm animals such as pigs a diet that includes fish oils increases the EPA and DHA content of the flesh, however, this is both expensive and unsustainable [89]. For this reason, plant sources of omega-3 PUFA have been tested in domestic animal feeds. For example, it is possible to supplement animal feeds with plant sources high in ALA or SDA and in doing so enhance the flesh levels of omega-3 PUFA [72, 90–92]. The fatty acid composition of animal products such as meat, milk and eggs reflects the relationship between tissue fatty acid biosynthesis and intake of fatty acids (lipids) [93]. This relationship is stronger in the monogastrics (e.g. pigs and chickens) than in ruminants (e.g. cows and sheep), and therefore, we are more likely to see a positive response when modifying the diet of the former rather than the latter [93]. Nonetheless, dietary interventions have been carried out in both groups so as to enrich milk, eggs and meat with LC PUFA [51, 72].

In pigs fed different quantities of flaxseed as a finishing diet (65 days prior to slaughter), the amount of EPA in the plasma, liver and kidney increased; the amount of DPA increased in the muscle, liver and kidneys, while the concentration of DHA increased in the plasma and none of these fatty acid levels increased in the subcutaneous fat [94]. In chickens fed a diet with and without linseed oil from day 12 of life to day 29 (at which point they were slaughtered), the fatty acid content of the chicken breast changed. These changes included an increase in the levels of ALA, EPA, DPA and DHA, while the levels of LA and AA decreased [91]. Seeing that the chickens did not receive a source of LC PUFA in their diets, one can conclude that the ALA was endogenously converted to EPA, DPA and DHA [91], but that this conversion rate was negatively influenced by the amount of ALA consumed [95].

Nudda et al. investigated the effect of a diet enriched for ALA on the LC PUFA content in lamb meat as well as carcass yield [96], as there is some disparity regarding timing of supplementation to achieve maximum benefit in ruminants [97–99]. The study was designed to provide information with respect to yield and ALA content of the meat (from lambs) and milk (from ewes). Supplementing the diet of ewes *prepartum* resulted in a statistically significant increase in DHA content of the intramuscular fat (IMF) of the lambs [96]. Although in all supplemented groups an increase in omega-3 PUFA was observed, ewes supplemented both *pre* and *postpartum* showed the highest levels in IMF [96].

As demonstrated by Matthews et al. and Haug et al, flaxseed oil can be used as an animal feed supplement in order to achieve raised levels of LC PUFA in pigs and chickens [91, 94]. It is also clear that this supplementation does not need to be for very long, as increased levels were achieved following a 65-day supplementation in juvenile pigs [94] and a 17-day supplementation in juvenile chickens [91].

Although flaxseed is the product that appears to be most commonly used with respect to dietary supplementation of fatty acids in livestock feed, other sources of omega-3 PUFA have been used. In contrast to flaxseed, echium oil is high in ALA and SDA and has been used to investigate the effect of a diet high in SDA fed to poultry [72]. The poultry were found to have a high total omega-3 PUFA level, with the exception of the fatty acid DHA [72]. A 50 % palm frond oil diet was also used to enhance the ALA levels of goats' meat when compared to goats fed a standard diet [90]. The goats' meat in turn was used to feed rats, wherein a significant increase in ALA and DHA together with decreased plasma cholesterol was observed [90].

In addition, milk products can also be enhanced with respect to their LC PUFA content. In the above mentioned study, milk from ewes supplemented with linseed increased in yield and in total MUFA, PUFA and omega-3 PUFA content [96]. Likewise, the diet of (human) lactating mothers can change the LC PUFA content of breast milk [100], and in much the same way, the LC PUFA content of the various milk products that are commercially available can be enhanced [99]. In a study by Weill et al, linseed fed to livestock resulted in increased omega-3 PUFA leading to a decrease in the omega-6: omega-3 ratio in butter, meat and eggs, with the highest reduction of 86 % found in the latter [101]. In addition, blood fatty acid levels were monitored in people who consumed the aforementioned products, and fatty acid profile was found to change such that the omega-6: omega-3 ratio decreased [101].

Although the inclusion of fish oils in the diet of chickens can be used to successfully raise the omega-3 PUFA content of eggs, one of the problems associated with such a diet is a fishy odour and/or flavour [102]. However, this can be overcome, and LC PUFA levels still increased by supplementing the diet of the chickens with linseed, whitebait and fructo-oligosaccharide (a prebiotic) [51]. Such a diet led to no significant change in flavour, and an increase in ALA, EPA and DHA as well as an increased egg weight [51].

Macro and Microalgae Sources of LC PUFA as an Alternative to Oily Fish

Marine microalgae are primary producers of the omega-3 LC PUFA, EPA and DHA [48]. Therefore, algae could offer a solution for non-fish eaters so that they too can gain the health benefits of these two essential fatty acids that cannot be obtained directly from any other vegetarian source [56]. Microalgae are an important source of LC PUFA and most produce either omega-3 or omega-6 fatty acids and not both. This suggests that the metabolic pathway is strictly controlled [103]. Microalgae produce omega-6 or -3 PUFAs in response to substrate preference of delta-6-desaturase for either LA or ALA [103]. However, when the substrate preference is similar, a molecular switch can occur. This switch from delta-6-desaturase to delta-15-desaturase (favours the synthesis of omega-3 PUFA) is controlled by genotype, and this control can be modified by temperature, where low temperatures induce omega-3 LC PUFA production [103]. This switch is not well understood and work is ongoing so as to maximise EPA and DHA production.

Fermentation of marine microalgae can produce algae oils with DHA values as high as 40 %, and these oils can then be used to supplement other foods that can then be sold as functional foods that are both palatable and varied sources of DHA [48, 56].There is evidence from the UK to support the view that approximately two-thirds of consumers prefer to eat functional foods rather than taking supplements, and they are prepared to pay 30-50 % more for the items in order to do so [56]. Ongoing research is required to develop products that are rich in EPA and DHA from algae that are both palatable, perceived to provide a health benefit and affordable. Nonetheless, algae supplements and pharmaceutical products do have a place in the market, particularly the administration of purified forms for the support of those with heart disease [104].

Conclusion

It is widely acknowledged that marine LC PUFA are important, healthful dietary components. Due to the inefficient conversion of SC omega-3 PUFA into the LC PUFA, EPA and DHA are considered semi-essential fatty acids for humans. Increasing world population and the growing awareness of the benefits of marine LC PUFA are driving the demand for these omega-3 fatty acids. These pressures are negatively influencing wild fish stocks through increasing fish supply from capture fisheries and higher production of fish within aquaculture farms, where fish diets are required to optimise the LC PUFA within fish flesh.

Plant sources of LC PUFA offer a promising land-based alternative to fish oils by introducing LC PUFA-producing enzymes into plant genomes. However, the success of these plant-based LC PUFA sources relies on the acceptance of genetically modified plants, which is sometimes met with disagreement. Alternatively, plants naturally high in SDA and other omega-3 fatty acids such as ALA may enhance diets of livestock and/or humans, in order to increase the levels of LC PUFA within animal meat, eggs and milk, or in breast milk with respect to humans. Macro and microalgae are suitable for vegetarians and vegans, a class of diet almost devoid of EPA and DHA. Furthermore, algae biomass can provide multiple products and large-scale production of algae is considered sustainable. Thus, there are multiple alternatives to fish oils that are currently being explored. It is likely that some or all of

these alternatives become increasingly used in future, in order to maintain adequate levels of fish stocks while keeping EPA and DHA sources within human diets.

Conflict of Interest The authors declare they have no conflict of interest.

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Maternal Long-chain Polyunsaturated Fatty Acids and Pregnancy Outcome

35

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Abbreviations

AA	Arachidonic acid
ALA	Alpha-linolenic acid
DHA	Docosahexaenoic acid
GDM	Gestational diabetes mellitus
IUGR	Intrauterine growth restriction
LA	Linoleic acid
LCPUFA	Long-chain polyunsaturated fatty acids
PUFA	Polyunsaturated fatty acids

Maternal Nutrition and Pregnancy Outcome

The nutritional status of the woman, prior to conception and during pregnancy, is recognized as an important determinant of pregnancy outcome. The pregnant mother provides nourishment for embryonic and fetal growth [1–4] and also prepares her body for labor and parturition [5].

The mother modifies her metabolism early from pregnancy to support the nutritional needs of the fetus [6]. Nutrients transported to the fetus are known to influence cell number and differentiation in the blastocyst that regulates fetal growth and organ development. However, maternal nutritional restriction can lead to fewer cells in the inner cell mass and cause blastocyst abnormalities [7]. Nutrient deficiencies also lead to serious complications of labor, preterm deliveries [8, 9] and contribute to high rates of maternal morbidity and mortality [10, 11]. It also leads to lower birth weight [12], restricted postnatal growth [13], altered organ/body weight ratios [14], and congenital malformations [8] in the offspring.

An adequate and balanced supply of both macro- and micronutrients is critical for maintaining pregnancy and appropriate fetal growth where macronutrients (carbohydrates, proteins, and lipid) provide energy for fetal growth while micronutrients play a major role in the metabolism of macronutrients and are involved in the cellular metabolism of the fetus [10, 15]. Fetal growth and development depends on the unique supply of dietary fatty acids from the mother [16]. Lipids/fats represent a balanced and wholesome diet important to maintain the health and its key components, fatty acids, represent essential nutrients during intrauterine life [6].

Lipids are esters of moderate to long-chain fatty acids, which are carboxylic acids with a long aliphatic hydrocarbon tail, either saturated or unsaturated. Based on the number of double bonds in the hydrocarbon chain, unsaturated fatty acids are further classified as monounsaturated fatty acids (MUFA) (presence of one double bond) and polyunsaturated fatty acids (PUFA) (presence of two or more double bonds).

Long-chain Polyunsaturated Fatty Acids (LCPUFA)

Long-chain polyunsaturated fatty acids (≥20 carbon atoms) are distinguished into two key families; omega-3 and omega-6. Omega-3 fatty acids contain a double bond (C=C) at the third carbon atom from the carboxylic end of the fatty acid chain and omega-6 fatty acids contain a double bond (C=C) at the sixth carbon atom. Among PUFA, linoleic acid (LA; 18:2 omega-6) and alpha-linolenic acid (ALA; 18:3 omega-3) are called 'essential fatty acids' because humans cannot synthesize them in the body and they have to be ingested through the diet [6]. All fatty acids within the omega-3 family are derived from ALA, while all omega-6 fatty acids are derived from LA. Biologically, most active forms of omega-3 PUFA are docosahexaenoic acid (DHA;

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22:6 omega-3) and eicosapentaenoic acid (EPA; 20:5 omega-3). ALA is found in algal oil, flaxseed oil, rapeseed oil (canola), walnuts, and some green leafy vegetables while EPA and DHA are mainly found in marine fish or fish oils. AA is a physiologically important omega-6 fatty acid and is found abundantly in fats, oils, eggs, meat, poultry, cereals, vegetables, nuts, seeds, and human milk.

LCPUFA serve as important constituents of cell membrane phospholipids, play an important role in maintaining the fluidity, permeability, and conformation of cell membranes, perform membrane-associated functions, and act as intracellular mediators of gene expression [17]. AA and DHA are important structural fatty acids in neural tissue. They provide energy, act as the precursors of the metabolically active compounds such as the prostacyclins, prostaglandins, thromboxanes, leukotrienes, and resolvins, and perform functional and structural roles within the body. LCPUFA together with their above-mentioned metabolites are involved in the functioning of transporters, ion channels, and enzymes and in signal transduction pathways [18].

LCPUFA Biosynthesis

Liver is known to play a central role in the fatty acid synthesis and metabolism [19]. Fatty acid desaturases are the enzymes that catalyze the introduction of double bonds at specific positions in a fatty acid chain [20, 21]. Delta 5 (Δ5) and Delta 6(Δ6) desaturases participate in the synthesis of LCPUFA [22, 23]. DHA is endogenously synthesized from its precursor ALA via a series of Δ6 desaturase, Δ5 desaturase, elongase enzymes, and β-oxidation steps [24]. The same series of desaturases and elongases are involved in the conversion of LA into its longer-chain, more unsaturated derivative, AA. These LCPUFA are stored in the adipose tissue in the form of triglycerides.

LCPUFA Metabolism During Pregnancy

There are major changes in the maternal lipid metabolism throughout pregnancy to ensure a continuous supply of fatty acids to the growing fetus [25]. During early pregnancy, LCPUFA consumed through diet are accumulated and stored in the adipose tissue as a result of enhanced lipogenesis. Subsequently in the later stages of gestation, when the fetal growth rate is maximal, there is an increase in the lipolytic activity in the maternal adipose tissue [26, 27]. This increases plasma triacylglycerol concentrations, with smaller rises in phospholipids, cholesterol concentrations [26, 28], and plasma non-esterified fatty acids (a form of free fatty acids present in small proportion) which serve as a source of LCPUFA for the growing fetus. Additionally, there is mobilization of LCPUFA from the maternal adipose tissue depots and selective delivery of maternal circulating LCPUFA to the fetus through placenta [28].

LCPUFA Intake/Status during Pregnancy and Fetal LCPUFA Status

The fatty acid levels in maternal blood lipids serve as indicators of maternal status [29]. Several studies report a decline in the maternal essential fatty acid status from the first trimester of pregnancy until delivery [30]. Additionally, there is depletion in the levels of DHA in maternal total plasma [31], serum [32], plasma phospholipids, and erythrocytes [33, 34]. Report suggests a significant decline in the ratio of DHA to docosapentaenoic acid in maternal plasma phospholipids indicating maternal difficulty during pregnancy to cope up with high demands of DHA [34]. Although these studies provide evidence for compromised LCPUFA status in pregnancy, there is limited information on the maternal factors that influence essential fatty acid metabolism during pregnancy. Presumably, maternal dietary intake/status of LCPUFA is related to the amount of fatty acids delivered to the fetus through the placenta [35, 36]. Since the ability of fetus and placenta to synthesize LCPUFA is low, the fetus primarily depends on placental transfer of LCPUFA [32].

Maternal fatty acid intake can directly influence the plasma and tissue fatty acid profile of the offspring [37]. Infant plasma omega-3 and omega-6 fatty acids and conjugated LA are related to maternal plasma fatty acids [38] while the deficiency of AA and DHA in maternal blood throughout pregnancy results in a suboptimal neonatal DHA status [34]. A study in our department has shown higher levels of DHA and AA in cord blood as compared to maternal blood in terms of pregnancy suggesting that large quantities of maternal LCPUFA are diverted to the fetus [39]. Evidence from several observational studies and randomized control trials (RCT) suggests a positive association between intake of omega-3 fatty acids during pregnancy and birth outcome [40].

Role of LCPUFA in Pregnancy

The different kinds of fatty acids consumed by the mother during gestation are known to influence pregnancy and fetal outcome [6] due to their fundamental roles as structural elements and functional modulators [17]. LCPUFA are required to support the development of the fetus in utero. LCPUFA are required in all stages of pregnancy [1] and play important role in determining length of gestation, initiation of labor, and in placental growth and development [41] (Fig. 35.1).

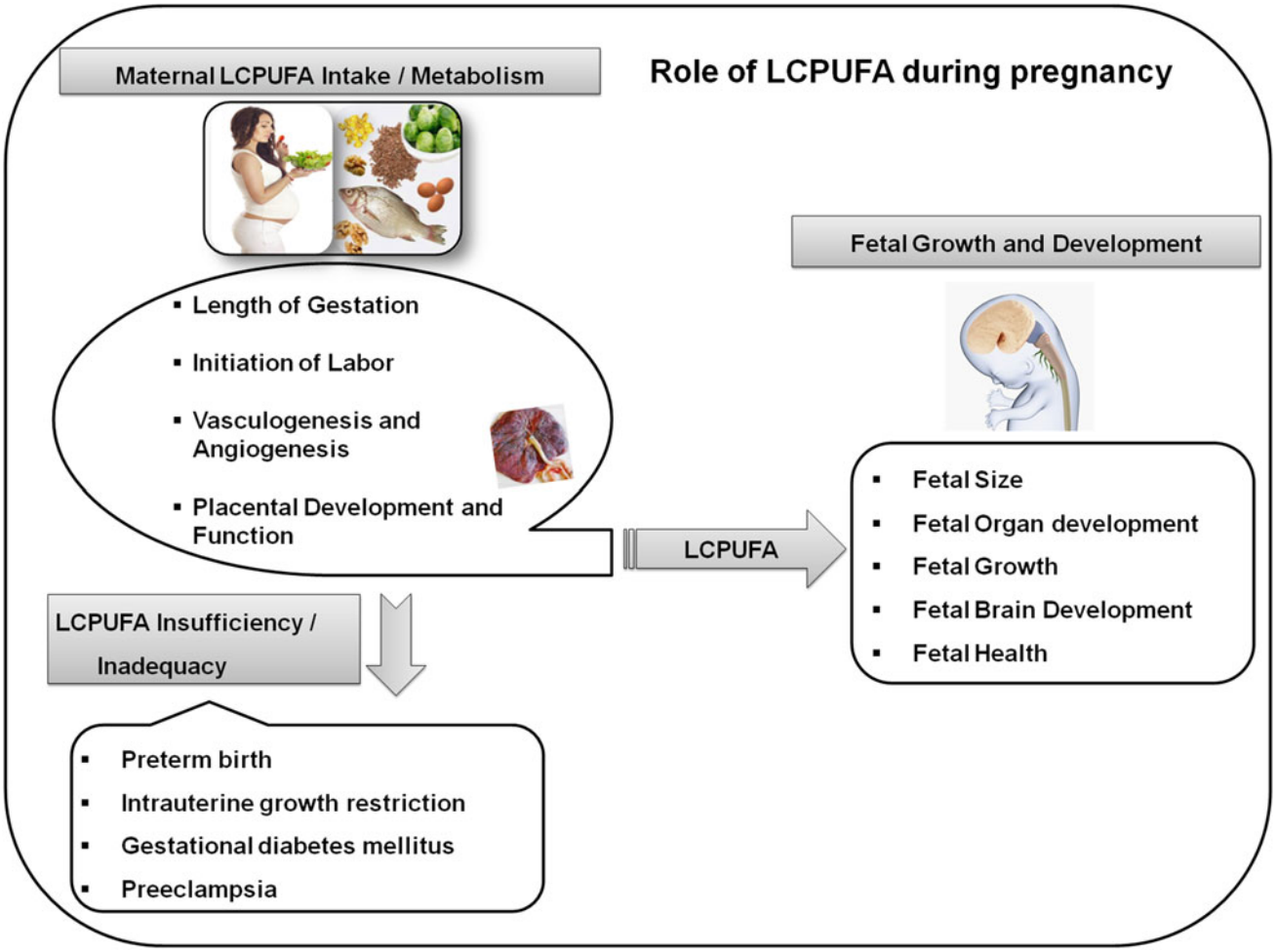

Fig. 35.1 Role of LCPUFA during pregnancy. *LCPUFA* Long-chain polyunsaturated fatty acids. Maternal LCPUFA intake during pregnancy plays an important role in maintaining length of gestation, initiation of labor, placental angiogenesis, and development. However, lower levels of maternal LCPUFA may lead to preterm labor, intrauterine growth retardation, and pregnancy complications such as gestational diabetes mellitus and preeclampsia. Adequate amounts of maternal LCPUFA are essential for fetal growth and organ development, brain development, and overall health

Initiation of Labor

LCPUFA metabolites such as prostaglandins are critical for initiation of labor and parturition process [42]. The rise in prostaglandins is involved in pathway of uterine contractility [43] and their levels increase during labor in the fetal membranes [44].

Among LCPUFA, AA serves as a precursor of the potent 2-series prostaglandins (PGs) E2 and PGF2α, and thromboxane A2 which are required for connective tissue remodeling associated with cervical maturation and rupture of membranes [45, 46]. On the other hand, EPA acts as a precursor for the 3-series of prostaglandins and produces PGE3 and PGI3, which promote myometrium relaxation [42]. These 3-series PGs do not possess any uterotonic activity and inhibit the synthesis of prostaglandins belonging to series 2 [47]. The eicosanoids from omega-3 and omega-6 fatty acids possess opposing modes of action. It is also known that EPA and DHA competitively displace AA in the membrane phospholipids, reduce production of PGE2 and PGF2α, and thereby inhibit the parturition process [48] (Fig. 35.2).

The premature production of PGE-2 and PGF-2α may lead to remodeling of the cervix, ultimately triggering labor by activating matrix metalloproteinases (MMPs) [49]. It has been reported that the concentrations of AA elevate in the amniotic fluid during labor and is accompanied by the elevated levels of PGE2 and PGF2α in the maternal circulation preceding the onset of spontaneous labor [50]. Administration of vaginal PGE2 has been a successful way to induce labor since the 1960s [51, 52].

As the pregnancy progresses, there is a rise in omega-3 fatty acids within the utero-placental unit followed by local production of series-3 prostaglandins, a critical element in cervical

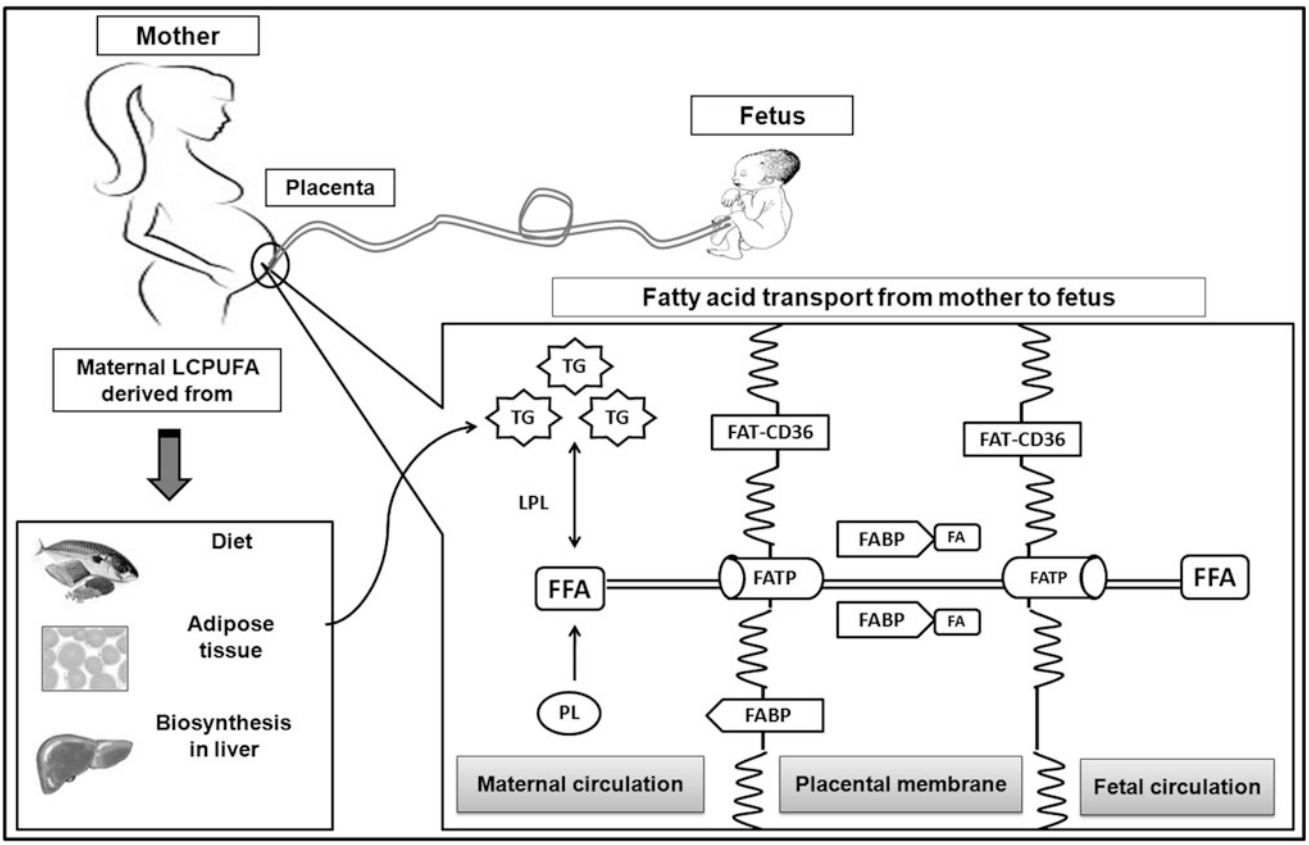

Fig. 35.2 Role of LCPUFA in the maintenance of normal length of gestation and labor. *ALA* Alpha-linolenic acid, *EPA* Eicosapentaenoic acid, *LA* Linoleic acid, and *AA* Arachidonic acid

ripening that plays essential roles in normal initiation of labor [53]. Reports suggest that inclusion of EPA in the diet leads to a reduction in the production of proinflammatory eicosanoids and increased production of prostacyclin (PGI2), promotes myometrial relaxation [54], and prevents preterm labor [55].

Length of Gestation

There is growing evidence that omega-3 fatty acids and their eicosanoid metabolites play vital roles in determining the duration of gestation [54, 56] and parturition process [45]. Improved omega-3 fatty acids status during pregnancy shows promise as series-3 prostaglandins are important in the maintenance of normal length of gestation [57]. Maternal DHA supplementation has shown to increase the length of gestation and infant size [46, 54, 56].

Some RCTs report that 600 mg DHA/d [56] and 800 mg DHA/d [58] significantly increase duration of gestation. On the other hand, 400 mg DHA/d supplementation showed no effect on the gestation duration [59]. No randomized controlled trial has found a reduction in gestation duration or size at birth [56].

LCPUFA in Placental Growth and Development

Placenta is at the interface between mother and fetus and is a key moderator of fetal growth and development [60]. The proper growth, development, and establishment of the placenta with its circulatory system are essential for successful maintenance of mother's health and for the development of the embryo [5].

During pregnancy, vasculogenesis and angiogenesis are critical processes in placental development [61]. Vasculogenesis involves the formation of new blood vessels from angioblast precursor cells, leading to the formation of an initial vascular network. Angiogenesis is the process of development of new vessels from pre-existing blood vessel [62] that plays an important role in the development of capillary network in both maternal and fetal compartments [63]. These processes are regulated by various growth factors, including vascular endothelial growth factor (VEGF) family, placental growth factor (PlGF), transforming growth factor ß (TGF ß) family, and angiopoietins along with proteases such as MMPs as well as their respective receptors [64, 65].

Maternal supply of DHA plays an important role in placental angiogenic processes and vascular remodeling by

increasing the expression of VEGF, angiopoeitin, and tissue inhibitors of metalloproteinases (TIMP) genes [66]. Angiogenic activities of LCPUFA are reported on the first trimester placental trophoblast cell line and have been shown to be highest for DHA followed by EPA and AA [67]. It has been observed that DHA induces maximum tube (capillary-like structures) formation by stimulating cell proliferation in the placenta as compared to other fatty acids [66]. DHA is also known to alter the expression of several genes, such as adipose differentiation-related protein, fatty acid-binding protein-4 (FABP4), FABP3 and cyclooxygenase-2, which are involved in angiogenesis [67]. Studies from our department in women with preeclampsia have shown a negative association between placental DHA levels and maternal anti-angiogenic factor soluble fms-like tyrosine kinase-1 (sFLT-1) levels [68]. A recent review reports that maternal omega-3 fatty acid supplementation during pregnancy is associated with enhanced placental growth and reductions in placental inflammation, oxidative stress in rats [41].

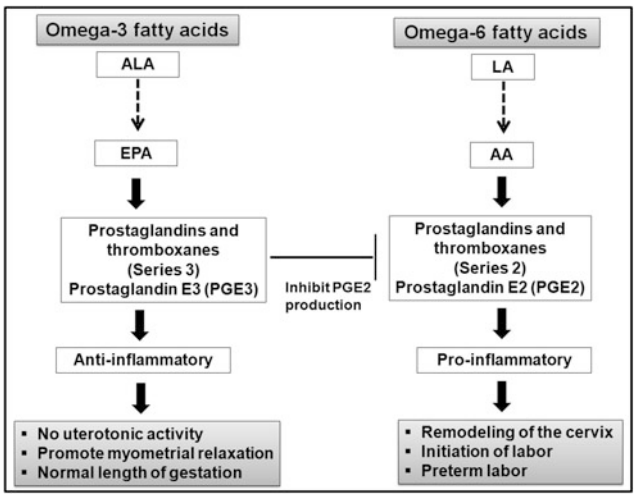

Fig. 35.3 Maternal LCPUFA transport to fetus through placenta. *LCPUFA* Long-chain polyunsaturated fatty acids, *TG* Triglycerides, *LPL* Lipoprotein lipases, *FFA* free fatty acids, *PL* Phospholipids, *FAT* fatty acid translocase, *FATP* fatty acid transport protein, *FABP* fatty acid-binding protein, and *FA* fatty acid

Transport of Maternal LCPUFA to Fetus Through Placenta

The fetus has a limited ability to synthesize LCPUFA because the capacity of the fetal liver for desaturation and chain elongation is not mature in early gestation in humans. Therefore, the fetus is dependent upon the mother for a supply of preformed DHA and AA through placenta. However, maternal lipoproteins which are rich in triglycerides do not directly cross the placental unit and therefore require placental lipoprotein lipases for their hydrolysis to form free fatty acids [26]. These fatty acids are mainly derived from maternal fatty acids bound to albumin, from lipoproteins bound by apoprotein receptors, or from triglyceride-rich lipoproteins released by the triglyceride hydrolases or lipoprotein lipases [69]. These enzymes must be active to facilitate the placental uptake of free fatty acids [70].

The free fatty acids need to get bound with the fatty acid-binding proteins (FABPs; cytosolic and membrane bound FABPs) to get entry into placental cells [46] where they cross the microvillous and basal membranes of placenta by simple diffusion [35]. Additionally, a number of placental fatty acid transport proteins (FATPs) and carrier proteins are present for the transfer of hydrophobic fatty acids from the maternal circulation to the fetal circulation [71] (Fig. 35.3).

Studies from various laboratories have clearly demonstrated the presence of different FATPs both in the cytosol and in the cell membranes and stated their role in the uptake and intracellular transport [72]. Several transport proteins such as plasma membrane fatty acid-binding protein (FATP), fatty acid translocase, or CD36 are located in the placenta to facilitate fatty acid transfer and meet the increased nutrients demand of the fetus during gestation [70, 73]. FATP 1 and FATP 4 have shown to be positively correlated with placental DHA uptake and are important for selective maternal–fetal transfer of DHA [74]. Thus, the placenta plays a critical role in modulating the transport of maternal fatty acids to fetus [41, 75].

Consequences of LCPUFA Inadequacy/Insufficiency

A number of RCTs have demonstrated that the maternal DHA intake during pregnancy can prolong high-risk pregnancies, reduce early preterm delivery, improve birth outcome by increasing birth weight, and head circumference and birth length [1]. However, low concentrations of maternal LCPUFA have shown to be associated with adverse pregnancy outcome, reduced birth weight, and an increased risk of small for gestational age infants [76].

Preterm Labor

Preterm birth is defined as birth before 37 weeks of gestation or fewer than 259 days since the first day of the woman's last menstrual period. As mentioned above, the balance between omega-3 and omega-6 fatty acids plays an important role in the maintenance of normal length of gestation. However, an imbalance between omega-3 and omega-6 fatty acids may lead to disturbances in the production of prostaglandins which are critical in cervical ripening and initiation of labor [57].

Preterm birth is characterized by lower production of prostaglandins by the reproductive tissue [45]. If omega-3 fatty acid accumulation within the feto-placental unit is low and local production of prostaglandins is high, the cervix ripens prematurely with increase in uterine contractions, leading to preterm delivery [57]. Mothers delivering preterm babies are reported to have low levels of omega-3 fatty acids [77]. Additionally, it is reported that the percent total LA, AA, EPA, and omega-6/omega-3 ratio are higher while total omega-3 fatty acids are lower in preterm mothers compared to full-term mothers [77]. An imbalance in the levels of omega-3 and omega-6 fatty acids has been reported in the preterm delivery [47] where a high ratio of omega-6/omega-3 fatty acids results in increased production of PGE2 and PGF2α leading to initiation of labor and preterm labor [54].

Studies from our department have reported reduced erythrocyte DHA levels in mothers of preterm babies as compared to mothers of term babies [78] and reduced levels of placental AA and DHA in preterm deliveries [79]. A study in preterm and full-term human newborns found differences in maternal erythrocyte AA content and hypothesized that high content of maternal erythrocyte AA and AA/EPA ratio may be considered as an early signal of preterm delivery [80]. Similar observations are reported in the fatty acid profile of erythrocyte membrane of Brazilian mothers at delivery [81] and in the fatty acid composition of the colostrum of Iraqi mothers delivering preterm [82].

Supplementation of omega-3 fatty acids during pregnancy has shown to reduce early preterm birth before 34 weeks of gestation by 31 % [83]. Omega-3 fatty acid supplementation has also been reported to reduce the rate of recurrent preterm birth in a randomized trial [84].

Intrauterine Growth Restriction

Normal fetal growth depends on the genetically predetermined growth potential and is modulated by fetal, placental, maternal, and external factors [85]. Intrauterine growth restriction (IUGR) is characterized by the failure of the fetus to reach its genetic growth potential [85, 86] and is associated with increased perinatal mortality and morbidity [86].

LCPUFA status is altered in pregnancies complicated by IUGR [87]. Reports suggest alterations in lipid status in the mother, fetus, and placenta in IUGR pregnancies, i.e., a decrease in the conversion of LA and ALA into AA and DHA, respectively [88, 89]. Abnormal maternal lipoprotein concentrations of cholesterol, low-density lipoprotein (LDL)-cholesterol have been reported in IUGR [90].

A lower proportion of AA and DHA in fetal blood in comparison with maternal blood has been reported in IUGR pregnancies which may be related to inadequate transplacental supply and a fetal lack of desaturases enzymes [88]. Reports suggest that placentas of infants with IUGR have a specific placental phenotype indicating alterations in placental structure and functions [91]. Altered placental lipoprotein lipase activity and placental FABP expression has been reported in IUGR pregnancies indicating disrupted lipid metabolism in these pregnancies [92]. However, only minor changes in passive membrane permeability and composition have been reported in the syncytiotrophoblast membranes in IUGR pregnancies [93]. A case control study reports an increase in AA in the placenta and umbilical artery phospholipids of fetal growth retardation speculating that the differential arterial composition may be responsible for the increased cardiovascular risk of fetal growth-restricted infants in adulthood [33].

Gestational Diabetes Mellitus

Gestational diabetes mellitus (GDM) is characterized by an abnormal glucose tolerance diagnosed for the first time during pregnancy [94]. GDM is associated with adverse obstetric and perinatal outcomes [95] and these mothers are at a risk of developing type 2 diabetes in later life [96]. Intake of specific types of dietary fat has been implicated in GDM risk where diets high in saturated fatty acids increase the risk of developing GDM [97] while PUFA are protective [98].

Reduction in plasma phospholipid of omega-6 fatty acids has been reported in GDM mothers [99]. In GDM, decreased proportion of LCPUFA in fetal plasma has been reported resulting from decreased supply, impaired placental transfer, or altered intrauterine metabolism [100]. Altered maternal metabolism, as a result of maternal hyperglycemia in GDM, affects placental metabolism and leads to an aberrant fetal metabolism [101]. In GDM pregnancies, fetal plasma and red blood cells show an altered lipid pattern compared to controls, with reduced AA and DHA levels [102]. On the other hand, elevated levels of DHA and AA in placental phospholipids are reported in GDM, which subsequently results into impaired LCPUFA transport to the fetus [103]. The composition of placental glycerol phospholipids is known to be altered in GDM and might reflect an aberrant fatty acid transfer across the placenta affecting fetal body composition. Placental mRNA and protein expression of CD36 has shown to be higher while FABP1 mRNA and FABP3 protein expression has shown to be lower in GDM [104].

Disturbance in normal fetal growth and development induced by GDM is associated with long-term adverse effects in the offspring, such as adiposity and type 2 diabetes [105]. GDM is associated with increased fetal weight and the risk for later metabolic and cardiovascular diseases [106]. However, omega-3 fatty acid supplementation in GDM pregnancy is reported to have beneficial effects on maternal high-sensitivity C-reactive protein, malondialdehyde levels, and hyperbilirubinemia of newborn babies [107].

Preeclampsia

Preeclampsia is a pregnancy complication manifested by hypertension, proteinuria, and the varying degrees of ischemic peripheral organ damage, which typically arise in the third trimester of gestation [108]. There are limited studies which have reported lower levels of omega-3 fatty acids from erythrocytes in preeclampsia [109, 110]. It has previously been shown that low erythrocyte levels of omega-3 fatty acids and high levels of omega-6 fatty acids particularly AA are associated with an increased risk of preeclampsia [111] while higher levels of omega-3 fatty acids at mid-gestation are associated with lower maternal blood pressures and pregnancy-associated hypertension [112]. A report suggests that high intakes of energy, sucrose, and PUFA independently increase the risk for preeclampsia [113]. Report suggests that lower concentrations of maternal and fetal LCPUFA in mothers with preeclampsia may be due to the decreased maternal LCPUFA synthesis that further leads to deficiency in the offspring [114]. Some studies report no change in the LCPUFA status [109] while others report higher DHA levels [115]. Further, it is likely that altered membrane lipid fatty acid composition may lead to altered placental development in preeclampsia [116]. Reports suggest a possible role of impaired placental fatty acid oxidation in the pathogenesis of preeclampsia [117]. Recent study from our department also indicates that disturbances in placental fatty acid metabolism exist in preeclampsia [118].

Maternal LCPUFA and Fetal Development

Maternal LCPUFA and its storage in fetal adipose tissue provide an important source of LCPUFA during the critical first months of life for rapid cellular growth and activity [28, 119]. It is known that prior to 25 weeks of gestation, the fetus accumulates only a small amount of lipids and thereafter exponentially accumulates large amount of lipids [28]. It has been reported that the fetus requires approximately 50 mg/kg/day of omega-3 fatty acids and 400 mg/kg/day of omega 6 fatty acids during the early weeks of life [120, 121].

Fetal Size and Weight

If adequate nutrition is available, the fetus can reach its growth potential, resulting in the birth of a healthy newborn of appropriate size. Among LCPUFA, DHA has shown to play important role in determining birth weight [46, 122], fetal growth, and development [1, 58]. Placental phosphatidylethanolamine with AA is known to be associated with fetal growth [123]. Low plasma phospholipid concentrations of EPA, DHA, and dihomo-gamma-linolenic acid (DGLA) and high concentrations of AA during early pregnancy have shown to be associated with reduced birth weight and/or an increased risk of small for gestational length infants [76]. DHA supplementation in large studies has shown slightly higher birth weight by about 50 g at delivery [32]. A meta-analysis of 15 RCTs indicates that maternal omega-3 fatty acid supplementations lightly increase birthweight as compared to placebo but show no differences in birth length and head circumference [40]. A double-blind RCT showed that supplementation with 600 mg/day DHA increases birthweight by 172 g [56]. A recent study reports no association between maternal omega-3 fatty acid compositions in gestational week 24 with fetal weight gain [124]. A study investigating birthweight in a fishing community reports increase in the duration of gestation with increased intake of marine fats but decrease in birthweight [125]. No studies have reported a reduction in infant size at birth by LCPUFA supplementation [56].

Maternal LCPUFA and Brain Development

DHA and AA are found in very high concentrations in cell membranes for fetal neural and retinal development and are known to accrete extensively in these tissues during prenatal period [40, 120, 121]. Brain development is known to accelerate during the second half of pregnancy, lasting until late adolescence [126]. The brain growth spurt that takes place from the third trimester of pregnancy until 18 months after birth also correlates well with DHA accretion in brain phospholipids [127]. During this time, the developing brain is sensitive to acute variations in the supply of DHA. Maternal diet, DHA stores, placental transport, and genetic polymorphisms are reported to influence DHA accretion in the fetal brain.

Several human and animal studies indicate that LCPUFA play a vital role in the development and maintenance of the central nervous system and improved cognitive development and spatial memory [128–131]. However, DHA deficiency has shown to cause retarded visual acuity [132], cognitive impairment, cerebellar dysfunction [133], and various other neurological disorders [134].

High-dosage LCPUFA supplementation at mid-pregnancy has shown to be associated with improved intelligence quotient scores of neurodevelopment [135]. Supplementation studies conducted in pregnancy and/or lactation using DHA suggest that DHA could help in improving cognitive outcome of children in later life [131]. Animal studies indicate that LCPUFA promote early brain development and regulate behavioral aspects, memory, and cognitive functions [136]. These studies have proved that supplementation has many beneficial effects such as increased visual acuity [137], reduced hyperactivity [138], and enhanced cognitive functions,

memory, and attention [136]. Studies based on a rat model of Alzheimer's disease suggest that DHA can be used as a therapeutic agent to improve cognitive decline [139].

Maternal LCPUFA and Fetal Health

Maternal plasma triacylglycerols and non-esterified fatty acids are known to correlate with fetal growth and fat mass [140]. Maternal fasting triglyceride levels are significant predictors of the fatty acid composition of the child's muscle membrane [141]. During gestation and lactation, higher levels of AA, EPA, and DHA are positively associated with child's pre- and postnatal growth [142–144].

A review suggests that maternal intake of omega-3 and omega-6 fatty acids in gestation and lactation can impact the developing infant tissue neuroendocrine and metabolic pathways [145]. Recent studies in humans and animals suggest that inadequate levels of omega-3 fatty acids during the prenatal and postnatal periods influences metabolic diseases [146], lean mass [147], and blood pressure in the offspring [148]. Further, prenatal and early postnatal exposures to low omega-3 fatty acids and high omega-6 fatty acids influence adiposity in children [149]. Children with a lower proportion of LCPUFA in their muscle membrane are at a higher risk for developing insulin resistance [150].

An enhanced maternal–fetal omega-3 PUFA status has shown to be associated with lower childhood adiposity [149]. A review suggests that the provision/supplementation of LCPUFA during critical periods of growth, especially from the 2nd trimester of pregnancy to 5 year, can prevent coronary artery diseases in adult life [151].

Animal studies demonstrate that maternal and post-weaning diet containing omega-3 fatty acids improve the lipid profile in the offspring [152, 153]. The beneficial effects of gestational/prenatal omega-3 fatty acid supplementation in reducing risk for metabolic syndrome markers in the hamster [154] and Wistar rat offspring [155, 156] have also been reported. Maternal supplementation with DHA is reported to decrease blood lipid [157] and improve blood pressure in the adult rat offspring [156].

LCPUFA Recommendations During Pregnancy

Fetal DHA requirement increases exponentially with gestational age due to fetal development. Therefore, a daily intake of DHA during pregnancy is recommended by World Health Organization and the Food and Agriculture Organization of the United Nations [158]. It is advisable that pregnant women should ingest at least 300 mg/day of DHA in order to achieve a better pregnancy outcome [159].

In 2002, the Food and Nutrition Board of the US Institute of Medicine established adequate intake levels (AI) for omega-3 and omega-6 fatty acids[160]. The recommendations suggest that human diets should contain minimum 3 % LA and 0.5 % ALA [161]. The recommended dietary allowances for essential fatty acids are 4.5 % of total energy for pregnant women and 5.7 % of total energy for lactating women. It has been suggested that the intake in Indian pregnant and lactating women should be 300 mg; of which, 200 mg should be in the form of DHA [Indian Council of Medical Research, India [162]. In populations consuming fish, it is recommended that during pregnancy two portions of fish should be consumed per week, with one portion of an oily fish such as mackerel, herring, sardines, or salmon [159].

Conclusion

Dietary intake of LCPUFA before and during pregnancy is critical for maternal health and optimal fetal growth. Accretion of AA and DHA in maternal tissues during pregnancy is the major determinant of length of gestation, parturition, placental growth, and development. Insufficiency/decline in the LCPUFA is associated with adverse pregnancy outcomes such as preterm birth, intrauterine growth retardation, GDM, and preeclampsia. Maternal LCPUFA status positively influences postnatal growth, neuro-cognitive development and helps to improve the health of the offspring.

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Role of Antioxidants in Human Health

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Introduction

The research on use, properties, characteristics, and sources of antioxidants especially phenolic compounds, flavonoids, vitamins, synthetic chemicals, and some micronutrients began in the late eighteenth century. With that initiation, significant attention on antioxidant research has been increasing, with hundred thousand papers published as the evidence on this subject [1]. The French paradox states that low coronary heart disease (CHD) death rates displayed an increased pattern with high intake of dietary cholesterol and saturated fat [2, 3]. French epidemiologists formulated French paradox concept in the 1980s [4]. It is suggested that the endorsement of primary prevention through optimal diet rich in fruit and vegetables with regular physical exercise, and life without smoking, is worthwhile [5]. Keys and his co-workers displayed a much lower incidence of CHD in the period between 1960 and 1975 in men in southern European countries such as Italy, Greece, and Yugoslavia than men in northern Europe, explaining that these differences between countries could be largely because of the differences in ratio of monounsaturated to saturated fatty acids in the diet [6]. At the end of twentieth century, epidemiological studies and associated meta-analyses strongly proposed that long-term consumption of diets rich in plant polyphenols prevented the development of cancer, cardiovascular diseases (CVDs), diabetes, osteoporosis, and neurodegenerative diseases [7]. In the past decade, human trials have suggested that antioxidants might provide mixed results, which we discuss later in this chapter.

Oxidative stress owing to excess free radicals is one of the major factors linking human diseases. An outcome of normal aerobic cellular metabolism is the generation of free radicals, and in-built antioxidant system in human plays a vital role in the prevention of its adverse effect. However, imbalanced defense mechanism of antioxidants together with overproduction or incorporation of free radicals from environment to living system leads to oxidative stress. As explained by Pham-Huy, imbalance between formation and neutralization of reactive oxygen species (ROS)/reactive nitrogen species (RNS) ends up with oxidative stress [8]. Because of ROS imbalance, modulation in the normal cell homeostasis takes place with its extremity to cell death. Accordingly, ROS exercises a wide spectrum of pathogenesis and tends to be the causative factor for number of diseases through its inhibition and activation of proteins, mutagenesis of DNA, activation of gene transcription, and so on [9]. The antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GRx) neutralize the ROS and RNS in the system. The nonenzymatic antioxidants also play a role in maintaining the homeostasis and are metabolic and nutrient antioxidants. Metabolic antioxidants are endogenous, and few of them are lipoic acid, glutathione, L-arginine, coenzyme Q10, and metal-chelating proteins. Nutrient antioxidants are exogenous which cannot be produced in the system and can be acquired through food and as supplements of vitamin E, vitamin C, trace metals, flavonoids, omega-3 and -6 fatty acids [8].

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Natural Antioxidants and Sources

The antioxidants from natural source are the first choice to human health because of its role in prevention and as adjunctive treatment in diseases; in addition, it avoids the adverse responses. It is widely accepted that a plant-based diet intake may reduce the risk of oxidative stress-related diseases, but understanding the complex role of diet in such chronic diseases is challenging because of its complexity with more than 25,000 bioactive food constituents [10]. Fruits and vegetables with numerous micronutrients, including β-carotene (a precursor of vitamin A), vitamin C, vitamin E, and selenium, are termed as essential micronutrients with antioxidant potential [11].

It is hypothesized that antioxidants originating from natural food sources may work as antioxidants with beneficial health effects through mechanisms related to antioxidant defense [10]. Sen et al. recently summarized that common natural antioxidants in foods such as vitamins (vitamins A, C, and E), carotenoids (β-carotene, lycopene, and astaxanthin), polyphenols (tea polyphenols and red wine polyphenols), and flavonoids (isoflavone, xanthones, and anthocyanins) are beneficial against major diseases including cardiovascular disease, neurological diseases, and cancer [12]. Over the past 10 years, researchers and food manufacturers have become increasingly interested in polyphenols. The paramount reason for this interest is the acknowledgment of the antioxidant properties of polyphenols and its great abundance in our diet [13]. Phenolic compounds are plant secondary metabolites which are generally involved in defense against ultraviolet radiation or assault by pathogens, and these are found in herbs and fruits such as berries, apples, citrus fruit, cocoa, grapes, vegetables such as onions, olives, tomatoes, broccoli, lettuce, soybeans, grains and cereals, green and black teas, coffee beans, propolis, and red and white wines [14, 15]. Flavonoids are a class of secondary plant phenolics with significant antioxidant and chelating properties. In the human diet, they are most concentrated in fruits, vegetables, wines, teas, and cocoa [16]. Flavonoids, benzo-γ-pyrone derivatives consisting of phenolic and pyran rings, differ in the arrangements of hydroxyl, methoxy, and glycosidic side groups, and in the conjugation between the A- and B-rings. During flavonoid metabolism, hydroxyl groups are added, methylated, sulfated, or glucuronidated, which determine its function. In food, flavonoids exist primarily as 3-O-glycosides and polymers [17]. The antioxidant properties of flavonoids are attributed through either its reducing capacities or its influences on intracellular redox status which are endorsed by its capacity to transfer electrons, free radicals, and chelate metal catalysts [18], activate antioxidant enzymes [19], reduce α-tocopherol radicals [20], and inhibit oxidases [21]. Figure 36.1 illustrates important antioxidant molecules, tested in clinical trials.

Cardiovascular Disease (CVD)

CVD today is responsible for approximately one-third of deaths worldwide, and this figure likely to increase in both developing and developed countries as risk factors for dyslipidemia, hypertension, obesity, and diabetes [22]. Heart failure (HF) is currently worldwide pandemic with an unacceptable high level of morbidity and mortality in industrialized countries, and there is no curative treatment currently available because of its severe pathology and unknown etiology [23, 24]. Cardiovascular risk factors significantly trigger oxidative stress, which contributes to disruption in the balance between nitric oxide (NO) and ROS resulting in relative decrease in NO bioavailability. This ends up with endothelial dysfunction, which is supposed to be the initial event of atherosclerosis [25]. When focused on hypertension, recently it has been hypothesized that oxidative stress is a key player in its pathogenesis, evidenced with its increase in patients with salt-sensitive hypertension, essential hypertension, malignant hypertension, renovascular hypertension as well as preeclampsia. Because of it, reductions in SOD and GPx activity have been observed in newly diagnosed and untreated hypertensive subjects, and are inversely correlated with blood pressure [26]. Over the past few decades, many studies have shown that low-density lipoprotein (LDL) cholesterol may be rendered more atherogenic by oxidative modification that allows it to accumulate in the artery walls, and antioxidants have been shown to slow down the progression of atherosclerosis [27–29].

Epidemiological studies suggests that increased dietary intake of antioxidants reduces the risk of coronary artery disease (CAD). Earlier clinical trial evidences from a benefit of vitamin E (Cambridge Heart AntiOxidant Study [30], secondary prevention with antioxidants of cardiovascular disease in end-stage renal disease study) [31], vitamin E and slow-release vitamin C (Antioxidant Supplementation in Atherosclerosis Prevention study) [32], and vitamin C plus vitamin E (Intravascular Ultrasonography Study) [33] on cardiovascular endpoints. Studies have shown that diets rich in fruits and vegetables reduce blood pressure in hypertensive patients [34]. Follow-up studies on antihypertensive effect of *Melothria maderaspatana* leaf and tea consumption on blood pressure have shown that it reduced hypertension with its antioxidant efficacy [35, 36]. An early study on hyperlipidemic children also observed that antioxidants vitamins C and E improve endothelial function [37]. Furthermore, in patients with obstructive sleep apnea (OSA), the reduced endothelial-dependent vasodilation was acutely improved by the free radical scavenger vitamin C [38]. Paradoxically, a recent study conducted by Ye et al. on relevant literature and the eligible studies of randomized controlled trials reported on the effects of antioxidant vitamin on cardiovascular outcomes as compared to placebo.

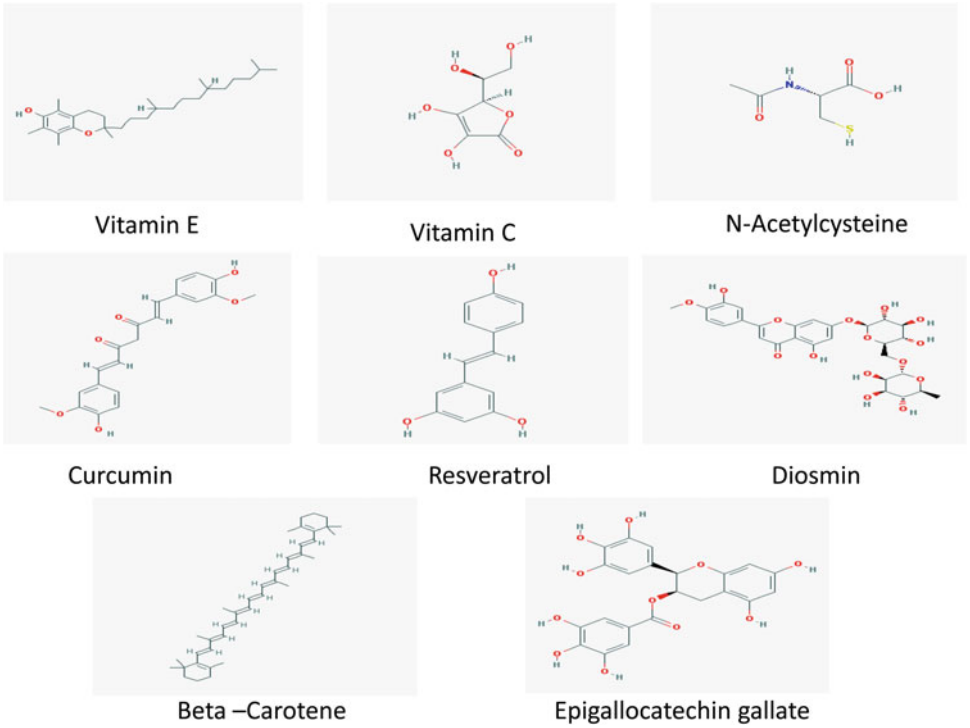

Fig. 36.1 Antioxidant molecules, which underwent clinical trials

The outcome has shown that antioxidant vitamin supplementation has no effect on the incidence of major cardiovascular events, myocardial infarction, stroke, total death, and cardiac death [39]. The reason for this could be that although LDL susceptibility to oxidative damage could lead to atherosclerosis, impact of plasma antioxidant vitamin level on vascular events is largely attributed to plaque formation and rupture.

The in vivo model studies that mimic human disease have postulated the beneficial effect of antioxidant therapy. Many such studies on natural antioxidants proved its protective ability against CVD. When focused on flavonoid, recent literatures have shown that diosmin prevents hypertension and cardiac oxidative stress via its antioxidant role in DOCA-salt-induced hypertensive rats [40] and suppress ischemia/reperfusion (I/R) injury [41]. Additional evidence supports that morin, a bioflavonoid antioxidant, also prevents hypertension and cardiovascular oxidative stress in uninephrectomized DOCA-salt hypertensive rats [42]. In addition, pharmacological model of NO-inhibited rats suggests that phenolic acids such as sinapic acid, veratric acid, syringic acid, and vanillic acid have shown strong antioxidant potential in cardiovascular system and thereby they inhibit hypertension and cardiovascular remodeling [43–46]. With this, antioxidant minerals (copper, manganese, and zinc) also have the potential to prevent or delay the cardiovascular complications of hypertension which has been evidenced with high-salt-diet-induced hypertension in rats [47].

The dietary antioxidants, fish oil, increase antioxidant enzyme activities in macrophages and reduce atherosclerotic lesions in apoE-knockout mice, and the levels of hepatic SOD and CAT activities were remarkably higher in the mice provided with fish oil diet [48]. The protective effectiveness of (blueberries) BB against atherosclerosis in this apoE-deficient mouse model was studied by Wu et al. The result of the study concluded that the potential mechanisms of BB involve the reduction in oxidative stress through its capacity to inhibit lipid peroxidation and enhancement of antioxidant defense [49]. Another study by Robredo et al. accounted that increased oxidative stress with its activity on pro-angiogenic factors could participate in the onset of retinal alterations in apoE-Knockout mice, and treatment with multivitamin plus lutein could effectively prevent these changes [50].

Under the condition of myocardial I/R, cardiac surgery for coronary artery bypass generally involves with cardioplegic arrest and elective global ischemia of the heart. Moreover, the concept of reintroduction of oxygen to the ischemic myocardium causes significant injury, and the mediation of oxidative stress has long been known [51, 52]. Recently, sinapic acid reduced I/R injury in rat heart via attenuation of oxidative stress validated using isolated heart Langendorff method [53]. Further experimental evidence on cardioplegic solution has shown that caffeic acid phenethyl ester prevents lipid peroxidation induced by I/R injury showing its potent antioxidant property [54]. Coronary vascular function after cardioplegic storage may profit by addition of iron chelators (or antioxidants) to cardioplegic solution, confirmed by

Schröder et al. [55]. During cardiopulmonary bypass under myocardial I/R, N-acetylcysteine (antioxidant) significantly reduced the baseline activity of NF-kB, which has been suggested to aggravate myocardial I/R injury [56].

Diabetes Mellitus

Diabetes mellitus (DM) is another most common health problem of the world in the current century. International Diabetes Federation's Diabetes atlas, 2012, reported that the number of people with diabetes is predicted to rise from over 371 million in 2012 to 552 million by 2030 [57]. Diabetes, characterized by a state of insulin deficiency that leads to a rise in glycemia [58], is commonly classified as insulin-dependent diabetes mellitus (type 1, an autoimmune disease where β-cells of the pancreas are affected by the body's defense system) and noninsulin-dependent diabetes mellitus (type 2, a metabolic disorder characterized by insulin resistance and deficiency) [59, 60].

Oxidative stress under hyperglycemic condition mediates excessive generation of free radicals, which in sequence aggravates the development, and progression of diabetes and its complications [61]. Endothelial dysfunction plays a main role in the pathogenesis of diabetic vascular disease such as cardiovascular damage, cataracts and retinopathy, nephropathy, and polyneuropathy [62]. Many experimental studies have shown that the critical point in diabetic patients is shared by diabetic cardiomyopathy, a disorder of the heart muscle, which is one of the major causes of heart failure [63], and diabetic nephropathy strikes one-third of patients with insulin-dependent diabetes as reported by Kedziora-Kornatowska et al. [64]. In addition, neuropathy, hyperglycemia-mediated neural degeneration [65], and retinopathy, retinal capillary cell death [66] are also accompanied by the increased oxidative stress.

Polyphenols from both edible and inedible plants have been reported for its multiple biological effects, including antioxidant activity [67]. Numerous studies have evidenced the role of these compounds in preventing the development of long-term diabetes complications [68]. Many researchers reported the use of antioxidants and its role in reducing the oxidative stress and alleviating diabetic complications [66]. Multiple antioxidants' supplementation reported to attenuate cardiac dysfunction in diabetic rats and plays a role in the prevention and management of diabetic cardiomyopathy [63]. A study by Cinar et al. [69] revealed that vitamin E supplementation significantly lowered liver and lung thiobarbituric acid-reactive substance (TBARS) levels and improved impaired endothelium-dependent vasorelaxation in streptozotocin (STZ) diabetic rat aorta. In addition, vitamins C and E reported to decrease oxidative stress and improve renal function in STZ diabetic rats [70]. Earlier findings by Brands et al. reported the elimination of superoxide and prevention of hypertension and decrease in glomerular filtration precipitated by diabetes [71]. Orally active multifunctional antioxidants reported to delay the cataract formation in STZ diabetic rat models [72]. One such antioxidant, lithospermic acid, evidenced to prevent the development of diabetic retinopathy in spontaneously obese diabetic rats [73].

Many flavonoid compounds such as diosmin [74] and coumarin [75] showed beneficial effect on STZ-nicotinamide-induced type 2 diabetes in rats through its antioxidant effect. Thymoquinone, a flavonoid isolated from *Nigella sativa,* ameliorates chemical-induced oxidative stress and β-cell damage in experimental hyperglycemic rats [76]. Green tea with high level of phenolics reported for its antioxidant nature amends the metabolic syndrome and risks of diabetes and its complications [59]. Improved nerve function was reported in diabetic neuropathy patients with the administration of α-lipoic acid [77, 78]. The combinational effect of vitamins E and C was studied by many researchers. Beckman et al. accounted the positive effect on the administration of vitamins E and C combination for six months on endothelium-dependent vasorelaxation in type 1 diabetic patients [79]. Gaede et al. [80] also reported this combinational effect in improving renal function in type 2 diabetes.

Chronic Kidney Disease (CKD)

Chronic kidney disease (CKD) is one another serious global health problem and is now considered as a key determinant of the poor health outcomes of major noncommunicable diseases [81]. CVD is the cause of death in ~34 % of hemodialysis (HD) patients. Indeed, at all ages, these individuals have a 10- to 20-fold increased risk of death from CVD [82, 83]. Increased oxygen radical formation was found in CKD, in the presence of a reduced antioxidant defense [84]. Dialysis patients are under elevated oxidative stress with increased free and phospholipid-bound F2-isoprostane [85]. Along with the above factors, chronic renal failure (CRF) is associated with impaired endothelium-dependent vasodilation and accelerated atherogenesis [86]. Previous study on patients with CKD showed that impaired renal function and duration of dialysis treatment are associated with increased oxidative stress [87].

The experimental studies with animal model of CRF suggested the beneficial role of natural antioxidants against oxidative stress. Gum arabic (GA) attenuated renal dysfunction in this model of CRF, suggesting the promising potential of GA in protecting renal failure progression. The mechanism(s) of this nephroprotection is uncertain, but may

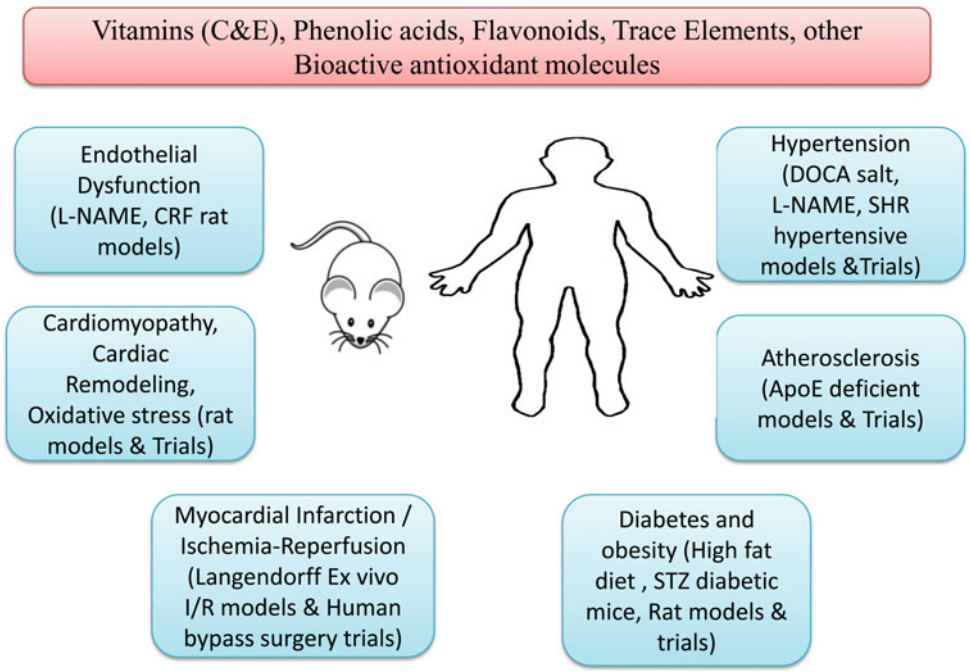

Fig. 36.2 Beneficial role of antioxidants on CVD, CKD, and diabetes in experimental and clinical trials

involve antioxidant and/or anti-inflammatory actions [88]. On extending studies with GA, it was observed that it decreases high levels of several pro-inflammatory cytokines in plasma and kidney of rats with adenine-induced CRF. Further, it could ameliorate the loss of antioxidant defense, decrease adenine-induced superoxide production, and DNA double-strand breaks [89]. Recently, it has been reported that diosgenin, a phytoestrogen, has the potential to attenuate vascular calcification and improve endothelial function in CRF rats with its antioxidant activity [90, 91].

The majority of clinical studies showed a decrease in the biomarkers of oxidative stress with antioxidant therapy. Earlier results from human studies have shown that acute administration of vitamin C reduces the levels of oxidant stress in renal failure and improves NO-mediated vessel dilatation [86]. As a whole, Coombes and Fassett systematically analyzed and reviewed that vitamin E decreases oxidative stress in 20 of the 25 included studies. Of these studies, 19 measured changes in circulating vitamin E after therapy, with 18 studies reporting significant increase in different compartments (serum/plasma, erythrocytes, platelets, and LDL) [92] and also explained that compared with studies using vitamin E, the effects of vitamin C in HD patients were more equivocal. Overall, based on a very small number of events, current evidences suggest that antioxidant therapy in predialysis CKD patients may prevent progression to end stage of kidney disease (ESKD) [93]. Figure 36.2 illustrates beneficial role of antioxidants on CVD, CKD, and diabetes in experimental and clinical trials.

Cancer

Cancer is a proliferative disease, and nearly 12 million incident cases and 8 million deaths due to cancer happened worldwide in 2008 and 65 % of the deaths occurred in less-developed countries [94]. Evidences from last two decades have exposed the mechanism by which oxidative stress can lead to chronic inflammation, which in turn is to be the mediator of chronic diseases such as cancer [95]. In normal cells, free radicals in low concentration play a role in signal transduction before their elimination. However, cancer cells demand high levels of ROS to uphold their proliferation rate and to exhibit an accelerated metabolism [96]. The epidemiological studies in the early 1990s accounted for the significant protective effect of fruits and vegetables against a variety of different cancers [97], signifying that healthy diet could prevent approximately 30 % of all cancers. The beneficial effect of increased consumption of fruits and vegetables was shown to reduce the risk of various cancers such as oral cancer [98] and head and neck cancer [99]. Moreover, flavonoids rich in green tea and black raspberries reported to have promising chemopreventive activity against human oral cancer [100]. It has been shown that several epicatechin derivatives from green tea possess anticarcinogenic activity via enhancing antioxidant and anti-inflammatory activity and by inhibiting lipid peroxidation [101].

The ability of antioxidants and its preventable strategy against cancer has been proved in in vivo *models*. Notably,

an earlier work of Singh and Agarwal supported that the flavonoid silymarin exhibits anticancer effects against skin cancer preventing both photocarcinogenesis and skin tumor promotion in mice, attributed to scavenging free radicals and strengthening the antioxidant system [102]. Another finding suggests that silymarin is effective in preventing N-butyl-N-(4-hydroxybutyl) nitrosamine (OH-BBN)-induced bladder carcinogenesis in mice [103]. Antioxidant from garlic constituent, S-allylcysteine (SAC), acts via decreasing lipid peroxidation and enhancing enzymatic antioxidants such as SOD and catalase, which has been proved in DMBA-induced hamster buccal pouch carcinogenesis model [104]. Few other studies with this model proved the anticancer activity of various compounds through their antioxidant potential, such as ferulic acid [105], rosmarinic acid [106], berberine [107], and andrographolide [108]. Furthermore, Manoharan et al. [109] found that chemopreventive efficacy of curcumin and piperine was elucidated with the anti-lipid peroxidation through the modulating effect on the carcinogen detoxification process.

Moon et al. examined the efficacy of retinol supplementation on the incidence of first new nonmelanoma skin cancer in moderate-risk subjects through randomized, double-blind, controlled trial with 2297 free-living subjects. The results were enrolled and ascertained that daily supplementation with 25,000 IU of retinol was effective in preventing squamous cell carcinoma (SCC), although it did not prevent basal cell carcinoma (BCC) [110]. Clark et al. determined that the selenium supplementation reduces the incidence and mortality of several sites carcinomas [111]. Mooney et al. found a significant reduction in benzo (a) pyreneB(a)P-DNA adducts, a risk factor of lung cancer in women with vitamin treatment, suggesting that antioxidant supplementation may mitigate some of the pro-carcinogenic effects of exposure to B(a)P [112]. Watters et al. [113] found that higher serum vitamin E at baseline was associated with improved overall prostate cancer survival.

However, few cancer prevention trials with vitamin antioxidants fail to show its potential against cancers. Lippman et al. reported that in Selenium and Vitamin E Cancer Prevention Trial (SELECT) with population of relatively healthy men, selenium and vitamin E, alone or in combination, did not prevent prostate cancer [114]. The efficacy of supplemental vitamin E and β-carotene on incident liver cancer and chronic liver disease (CLD) mortality was studied by Lai et al. as a randomized trial in 29,105 Finnish male smokers. The studied groups received the supplementation for 5–8 years and were followed for 16 additional years for outcomes, concluding that long-term supplemental vitamin E or β-carotene had no effect on liver cancer or CLD mortality over 24 years of follow-up [115].

The question on deteriorating role of antioxidant on chemotherapy is still under debate. Some researchers argued that antioxidants scavenge the free radical integral to the activity of certain chemotherapy drugs thereby diminishing treatment efficacy. Others suggest that antioxidants may alleviate toxicity with uninterrupted treatment schedules and a reduced need for lowering chemotherapy doses [116]. The commonly used radio- and chemotherapeutic drugs used for cancer therapy influence tumor outcome through ROS modulation; therefore, ROS regulation in this case is highly significant. ROS are known to promote tumor development and progression through elevated cellular proliferation, evasion of apoptosis, tissue invasion, and angiogenesis [96]. Gorrini et al. [117] clearly mentioned that because of metabolic and signaling aberrations, cancer cells exhibit elevated ROS levels and induce DNA mutations and pro-oncogenic signaling pathways. This can be balanced by an increased antioxidant intake.

Neurodegenerative Diseases

Neurodegenerative diseases including Parkinson's and Alzheimer's diseases occur because of loss in structure or function of neurons, including death of neurons. About 1 % of the population by the age of 65 and 4–5 % of the population by the age of 85 are affected by Parkinson's disease (PD) [118, 119]. It is characterized by the death of dopaminergic neurons in the substantia nigra (SN) with consequential effect on motor function and coordination [120]. Moreover, Alzheimer's disease (AD) is projected to become a public health priority in developing countries with increasing number in the elderly age groups of 65 years and older. The number doubles from 420 million in 2000 to 973 million in 2030 [121, 122]. AD is a progressive neurodegenerative disorder, and the most prevalent form is the dementia characterized by a progressive decline in memory, behavior, and cognitive functions [123]. Abnormal oxidative stress and mitochondrial dysfunction highlight its role as a causative agent in the pathogenesis of neurological diseases including PD and AD [124–127].

Following experimental studies notified the use of antioxidants for the treatment of neurodegenerative diseases. Neuroprotective effects of a citrus fruit flavonoid, hesperidin was reported in human neuroblastoma SK-N-SH cell against rotenone-induced oxidative stress and apoptosis [128]. Chronic antioxidant therapy diminishes the oxidative stress in in vivo mouse model of AD as reported by Siedlak et al. [129]. One such evidence with oral administration of *Withania somnifera,* in vivo PD model, showed the reduction in oxidative damage and improves physiological

abnormalities [130]. The protecting effect of escin against experimental PD was reported to accomplish through its antioxidant and anti-inflammatory properties [120]. Casetta et al. [131] reported the beneficial activity of vitamin E in reducing AD incidence and in delaying the onset of AD. In addition, treatment with antioxidants vitamin E, vitamin C, selegiline, and estrogen reported to exert positive effects against the development of AD [132]. The report of Dai et al. enlightens that the consumption of fruit and vegetable juices with high concentrations of polyphenols, at least three times per week, may play an important role in delaying the onset of AD [133]. The well-known antioxidant, curcumin, with its anti-inflammatory and lipophilic action improves the cognitive functions in patients with AD [134]. The preclinical evidence supported the usage of antioxidants in the prevention of AD [135]. The results of the Alzheimer's Disease Cooperative Study clinical trial in patients with moderate AD suggested that vitamin E delays functional deterioration [135].

Aging

Aging is a biological phenomenon, which could be unavoidable and universal, and it affects all multicellular organisms and common among unicellular organisms, including protozoa, yeast, and bacteria [136, 137]. Aging plays a causative role and is a risk factor for many diseases, including AD and PD. The impact of aging world population continues to grow with 35.6 million living with dementia worldwide in 2010 and a projection to 65.7 million by 2030 and 115.4 million by 2050 [138]. The molecular damage in aging was contributed mainly by free radicals, which lead to pathological changes in the cell and such damages also shared by the metabolites such as sugars and reactive aldehydes and spontaneous errors in biochemical processes [139, 140]. The major mechanism of aging attributes to DNA or the accumulation of cellular and functional damage [141]. Hence, reduction of free radicals by decreasing their rate of production may delay aging.

Some nutritional antioxidants are reported to retard the aging process and prevent disease [142]. Wang et al. [143] reported that combination of S-adenosylmethionine, vitamin E, and vitamin C suppresses the development of age-related metabolic dysfunctions. Chronic melatonin treatment improved mitochondrial function and increased life span in senescent prone mice [144]. The survival of male C57BL/6 mice was increased with the treatment of tetrahydrocurcumin and green tea polyphenol [145]. Flavonoids extended life span in C. Elegan with its antioxidant actions [146], and also antioxidant curcumin and cocoa extended life span in Drosophila melanogaster [147, 148]. Fleenor et al. suggested that gnetin may be a novel therapy for treating arterial aging in humans [149].

Obesity

Together with all the above-explained diseases, obesity also contributes a major part in public health problem in both developed and developing countries [150]. The World Health Organization stated that obesity has been implicated with the genesis of many diseases such as cancer, diabetes, and hypertension [151]. Energy imbalance due to a disorder in lipid metabolism accounts to be the primary cause of obesity [152]. Oxidative damage has been implicated in the pathology of a series of human ailments such as cancers, heart diseases, obesity, and aging [153]. Flavonoid compounds have been accounted to promote health with its antiobesity activity as reported by many researches [154, 155]. Antioxidant-rich saffron compounds are reported to modulate obesity and concurrently its associated metabolic disorders [156]. Exotic fruits with its bioactive components show potential health benefits by acting as antidiabetic, anti-obese, anti-oxidant, and anti-inflammatory. This has been reported the importance of exotic fruits in the prevention of complex pathophysiology of obesity and diabetes [157].

Earlier experimental and human studies attested the anti-obesity activity of many antioxidants. Polyherbal preparations of *Triphala* reported to exhibit hypolipidemic effect in experimentally induced hypercholesteremic rats [158]. The hypolipidemic and antioxidant effects of both fenugreek seeds and triphala as adjuncts to dietary therapy were studied in patients with mild-to-moderate hypercholesterolemia [159]. Vitamin B group supplementation enhances antioxidant capacity and showed anti-inflammatory effect in obese diabetic patients [160]. *Portulacaoleracea* L. showed positive effects on serum lipids' profile of obese adolescent patients attributing to its antioxidant property [161]. Beside these reports, consumption of *Zizyphus jujuba* fruits also showed positive effects on reducing serum TC and LDL-C in obese adolescents, thus preventing the development of atherosclerosis from childhood [162].

Conclusion

Important contribution of oxidative stress in the initiation and progression of diseases brought antioxidants to the human trials in last few decades. The antioxidant therapy has shown beneficial effects in various animal models, some of them were shown to be effective in human trials. Figure 36.3

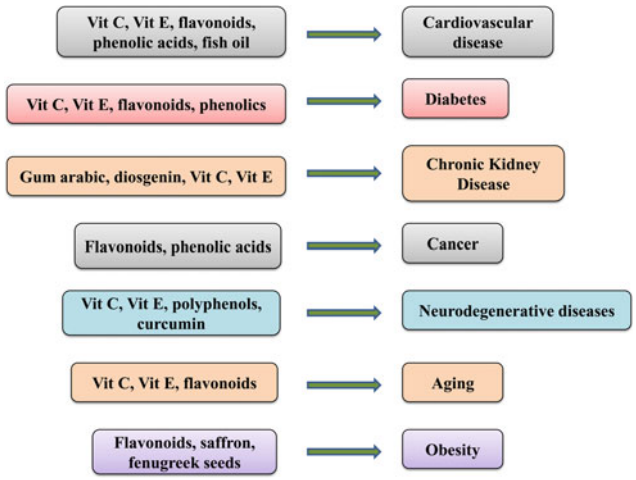

Fig. 36.3 Beneficial effect of antioxidants on various diseases

illustrates the beneficial effect of antioxidants on various diseases. This review concludes that there is a need for large cohort-based clinical trials and follow-ups to confirm the efficacy of antioxidants.

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Role of Omega-3 Fatty Acids in Cardiovascular Disorders

37

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Introduction

A major burden of the increasing morbidity and mortality related to systemic diseases has been attributed to cardiovascular (CV) and cardiometabolic events. Substantial evidence has accumulated in the last decade favouring cardiovascular benefits of fish consumption and their use in the management of different cardiac disorders. This interest in the therapeutic value of fish oils was triggered in the 1970s by the observation of low cardiovascular mortality rate in Greenland Eskimos who are known for their high fish intake [1]. The cross-sectional study conducted on 852 middle-aged men in the Netherlands recorded an inverse relation of coronary heart disease (CHD)-related mortality and the amount of fish consumption [1]. Research in this field over the last 50 years has led to inconsistent results; nevertheless, the evidence from epidemiological, observational, and clinical trials demonstrating the myriad mechanisms of antiarrhythmic, anti-inflammatory, and anti atherogenic potential makes fish oils worth discussion when considering primary and secondary preventions of cardiovascular diseases [2, 3].

Statins have been considered the cornerstone in the management of dyslipidemia, along with adjunct treatment with fibrates, niacin, bile acid sequestrates, or cholesterol absorption inhibitors. The inverse relationship of fish oils or omega-3 fatty acids (omega-3-FA) with low-density lipoprotein (LDL-C) and triglycerides (TG), and the consequent reduction in the risk of cardiovascular events has made the latter, emerge as a pharmacological option in targeting lipid levels, along with statins [4].

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Omega-3-FAs are bioactive compounds consisting of marine-derived eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and the plant derived alpha-linoleic acid (ALA). Food sources of ALA include flaxseed, walnuts, canola oil, and chia seeds that act as a precursor for EPA and DHA synthesis [5]. The tissue levels of omega-3-FA in the non-fish eaters depend on the conversion of ALA to EPA and DHA by a process of elongation and desaturation [6]. EPA and DHA are found in oily fish, namely tuna, mackerel herring, sardines, salmon, trout, and Atlantic cod fish [7]. DHA is also found in algae. EPA and DHA can be considered as conditionally essential fatty acids in view of their various metabolic functions, not duplicated by the other fatty acids [8]. A variety of preparations containing different amounts of omega-3-FA or fish oils are available in the market today owing to the increased awareness about their impacts on health, especially cardiovascular diseases. EPA and DHA are the omega-3-FA of importance. They are provided as food supplements, prescription drugs, and medical foods sourced from fish, krill, algal, and plant oils.

Biochemistry

Fatty acids (FA) are classified on the basis of their carbon–carbon double bonds into saturated, monounsaturated, and polyunsaturated FA. The family of long-chain, highly un-saturated fatty acids consists of the omega-3 and the omega-6 fatty acids. This classification is shown in Fig. 37.1. The omega-3-FA has their carbon–carbon double bond at the third and fourth carbon atoms from the methyl tail end, or "omega" end of the carbon chain [9].

The omega-3-FA consists of α-linoleic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA). Their structures are depicted in Fig. 37.2. ALA can be converted to EPA and

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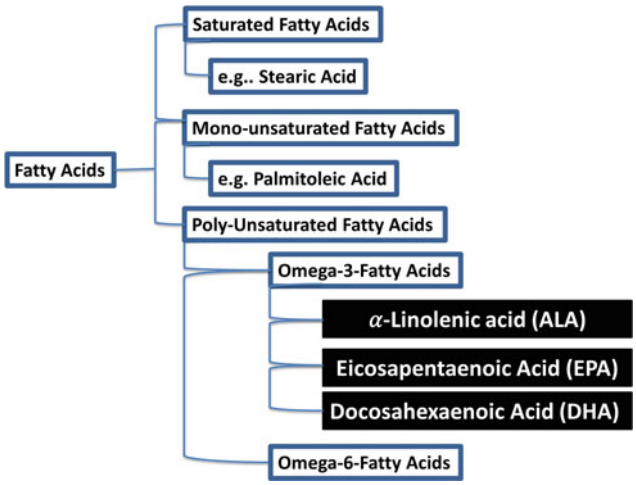

Fig. 37.1 Classification of fatty acids

DHA by the rate-limiting step mediated by the enzymes desaturase and elongase [10]. ALA, EPA, and DHA cannot be synthesized in the human body and are therefore termed essential [11]. Even though fish oils form the main source of omega-3-FA for human consumption, the primary producers are the marine microorganisms such as microalgae [12]. The major dietary omega-6 FA is linoleic acid, found in vegetable oils, seeds, and nuts. Omega-6 FA, mainly derived from meat, can be converted into arachidonic acid (AA) [13].

AA is the main precursor to the eicosanoid group of compounds that metabolizes to form prostaglandins, leukotriene, and thromboxane, by cyclooxygenase, lipoxygenase, or cytochrome P450 AA monooxygenase. These are the main mediators of inflammation, thrombosis and atherosclerosis.

Proposed Mechanisms of Action

The cardio-protective actions of seafood-derived omega-3-FA have been examined in a diverse and continually expanding array of studies. Several hypotheses have been proposed based on various in vitro molecular experiments to large randomized controlled trials.

Various experimental studies document the impact of omega-3-FA on molecular pathways. These include changes in eicosanoid profiles, regulation of gene expressions, tissue metabolism, modulation of cellular and organelle membrane structure and function, and genetic regulation [14]. The cardio-protective benefits are multifaceted and still debated as sufficient human studies are lacking. Omega-3-FA has been found to contribute by anti-inflammatory, blood pressure lowering, antiarrhythmic, and plaque stabilizing effects [15].

Effect on lipids—Fish oils have emerged as a real player in the prevention of coronary events by its anti-atherosclerotic, vascular, and metabolic effects. The anti-atherosclerotic effect of fish oils may be brought about by its influence on endothelial dysfunction, oxidative stress, inflammation, hemostasis, and insulin resistance [16, 17]. A consistent effect of omega-3-FA in lowering plasma TG levels without raising LDL-C levels has been evident from epidemiological, observational, and clinical trial studies. They reduce hepatic TG synthesis and increase the clearance of circulating TG [2, 18]. Omega-3-FA decreases de novo lipogenesis, thus reducing fatty acid availability for TG synthesis. They also reduce the hepatic enzyme activity for TG synthesis and increase fatty acid beta oxidation [19–24]. Patients with hypertriglyceridemia are at about 2 times higher risk for myocardial infarction (MI) and at a higher risk of coronary artery disease (CAD) than those with normal triglyceride

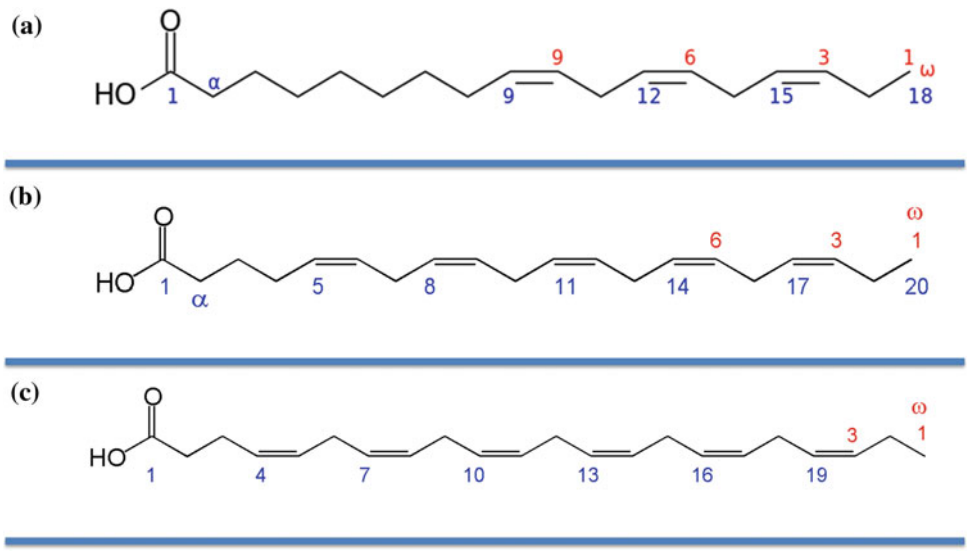

Fig. 37.2 Chemical structure of **a** α-linoleic acid (ALA); **b** eicosapentaenoic acid (EPA); and **c** docosahexaenoic acid (DHA). Note that, from the *n* end (in all three of the above compounds), the first double bond (3 in *red*) appears as the third carbon–carbon bond, hence the name "*omega-3-fatty acid*"

levels. A study by Samuel et al. demonstrated substantial evidence of omega-3-FA reducing triglyceride levels at the dose of 4 g/day. This was a significant breakthrough in implying the cost-effectiveness of omega-3-FA and its additional role in treatment of dyslipidemia caused by elevated triglycerides, despite the efforts made in controlling low-density lipoprotein cholesterol [25].

Antiarrhythmic effect—Ventricular fibrillation caused by triggered activity and re-entry, in addition to other forms of arrhythmias, constitutes the final common pathway for most cardiac deaths. EPA and DHA contribute to the reduction in cardiac dysrhythmias [26–32] by shortening the action potential duration and slowing down the impulse conduction [33, 34]. They stabilize the partially depolarized ischemic myocytes, preventing ventricular fibrillation in post-MI and heart failure patients. They reduce arrhythmias in ischemic heart disease by reducing re-entry [35]. Researchers have recorded the net antiarrhythmic action of omega-3-FA by its effect on the myocardial sodium channels, potassium channels, sodium–calcium exchanger proteins, and its ability to alter membrane fluidity [36]. The landmark diet and reinfarction trial (DART) study demonstrated a 30 percent reduction in coronary artery disease-related mortality after long-term dietary intervention in men who suffered from MI [37]. Similar results were obtained in the GruppoItaliano per lo Studio dellaSopravvivenzanell'InfartoMiocardico (GISSI) Prevenzione study, which showed a substantial reduction in cardiovascular mortality with fish oil supplementation [38]. A study involving the role of omega-3-FA in prevention of sudden cardiac death among patients with dilated cardiomyopathy displayed promising results; however, the patient number was small [39].

Omega-3-FA may be useful in the management of atrial fibrillation (AF), with minimal risk of ventricular arrhythmia [35]. The anti-inflammatory action of omega-3-FA makes them attractive as a potential therapy for AF, as inflammation is thought to play a role in the pathogenesis of AF, and not just an epiphenomenon [40, 41]. This seems to have promising results but needs further research before experimental findings can be translated into clinical benefits.

Anti-inflammatory effect—Omega-3-FA intake reduces the production of eicosanoids, which are arachidonic acid metabolites that promote inflammation and oxidative stress. This effect is evident with an EPA intake of above 2 g per day [42]. The EPA- and DHA-derived E-series and D-series of resolvins (resolution—phase interaction products), neuroprotectins, and maresins are oxygenated metabolites that possess potent anti-inflammatory action and exert immunomodulatory actions on neutrophils, macrophages, dendritic cells, and T cells [43–45]. The eicosanoids that are derived from the omega-6-FA are proinflammatory, while those derived from omega-3-FA are anti-inflammatory. This fundamental difference confers omega-3-FAs and their cardio-protective effects, by inhibiting the development of coronary syndromes through their anti-inflammatory and anti-thrombotic potential [46, 47]. However, this effect in inflammatory diseases such as arthritis, asthma, inflammatory bowel diseases, and psoriasis has shown mixed results and represents areas for further study [48–52].

Effect on endothelium—The cardio-protective effects of omega-3-FA could be mediated by its influence on endothelial function and systemic epoxyeicosatrienoic acids (EETs)/dihydroxyeicosatrienoic acids (DHETs) ratio, especially in patients with hypercholesterolemia [53], peripheral arterial diseases [54], acute MI [55], and type 2 diabetes mellitus [56, 57]. They have been found to target the oxidative stress that causes endothelial dysfunctioning in smokers and hypertensives [58]. Their consumption has been found to lower the levels of circulating E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 through multiple relevant molecular pathways [2, 59, 60]. The main culprit causing endothelial dysfunction is soluble epoxide hydrolase (sEH) that converts (EETs) to (DHETs). Trials have shown omega-3-FA consumption to decrease sEH activity and thus increase EET/DHET ratio [61]. Trials involving fish-based diet, targeting the sEH levels, have shown a beneficial effect on the endothelium sustained for at least 4 weeks in spite of fall in serum omega-3-FA component [61].

Effect on hypertension—Studies have indicated modest reductions in systolic and diastolic blood pressure with omega-3-FA [62]. Mechanisms involve improved autonomic function by augmentation of vagal tone, improvement in left ventricular diastolic filling, and alterations on the cardiac electrophysiological pathways [29, 63–65]. The hypothesis of omega-3-FA reducing heart rate by secondary changes in ionic channels and influencing the parasympathetic stimulation of vagus nerve was evident in an analysis by Mozaffarian et al. [66]. It was shown that fish consumption of 3 or more times per week was associated with a reduction in heart rate of 3 bpm. Another meta-analysis demonstrated that omega-3-FA supplements reduced heart rate by 1.6 bpm independent of the amount consumed [67].

The arachidonic acid-derived prostaglandins, thromboxanes, and leukotrienes mediate vasoconstriction, platelet aggregation, and synthesis of inflammatory mediators [68, 69]. The prostaglandins derived from EPA antagonize the effect of the former. EPA and DHA are the precursors of lipoxins, resolvins, and protectins, which regulate vascular tone and blood pressure [69]. Omega-3-FA appears to suppress the activity of angiotensin-converting enzyme, leading to reduced angiotensin 2 production and leading to vasodilation and inhibition of aldosterone secretion [70]. Omega-3-FA-mediated increase in endothelial nitric oxide production enhances the vasodilatory responses [71–73]. DHA has a modulatory effect on the rennin–angiotensin–

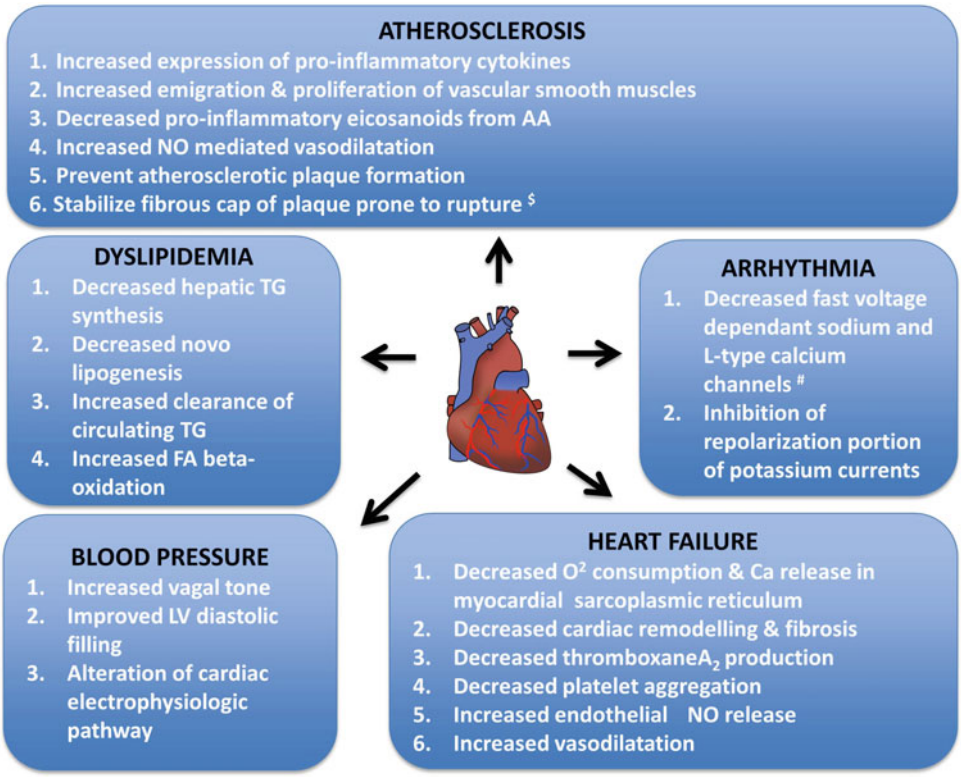

Fig. 37.3 Cardio-protective mechanisms of omega-3-FA (Ref. $[84] and #[85] marked in the figure)

aldosterone system and promotes vascular smooth muscle apoptosis. It thus prevents vascular wall fibrosis and development of secondary hypertension [74].

The supplementation of omega-3-FA in healthy volunteers did not have any effect on their blood pressures [75]. High doses of omega-3-FA (3 g/day) produce small but significant reduction in systolic blood pressure in the older and hypertensive subjects. They may play a role in patients with mild hypertension managed on diet and lifestyle modification before starting antihypertensive [76].

A recent finding in this field is a novel mechanism in which omega-3-FA slows down biological aging by preserving telomere length. In an analysis carried out on 608 patients with stable coronary artery disease, the baseline plasma omega-3-FA levels were found inversely proportional to telomere shortening over 5 years of follow-up. The shortening of telomeres that are tandem repeats of deoxyribonucleic acid (DNA) forming a protective cap at the ends of chromosome has been associated with deterioration of cardiac diseases [77]. Hence, this finding may be a significant breakthrough while determining the significance of omega-3-FA in treatment of CV diseases.

Omega-3-FA and omega-6-FA constitute 30–35 % of total brain fatty acids and are shown to benefit cognitive function. Diets deficient in omega-3-FA may adversely affect learning. However, human studies are lacking [78–80]. The discovery that Alzheimer's disease patients have reduced DHA levels in their brain and peripheral tissues [81, 82], supported by the recent meta-analysis report that dementia is associated with lower blood levels of EPA and DHA, suggests that omega-3-fatty acids may play a significant role in ameliorating neurodegeneration [83].

Figure 37.3 summarizes the various mechanisms of action of omega-3-FA [84, 85].

Effects on Diabetes

A discourse on the CV effects of fish oils would be incomplete if the influence of the latter on insulin sensitivity, insulin secretion, beta cell function, and glucose tolerance is not described. Diabetes has emerged as a major non-communicable disease in both the developing and the developed countries with clinical consequences on atherosclerotic diseases and the CV system. Hence, in addition to the benefits of omega-3-FA on the heart, a description on the association between fish consumption, dietary long-chain omega-3-FA, and the risk of developing type 2 diabetes is warranted.

The trials reviewed below indicate differences between geographical regions in observed associations of fish consumption and dietary intake of long-chain omega-3-FA with risk of type 2 diabetes. Wu et al. performed a systematic review and meta-analysis to understand the relations of

dietary omega-3-FA, dietary fish and/or seafood, and circulating omega-3-FA biomarkers, with incidence of diabetes. There was no significant association established between diabetes and consumption of fish and/or seafood, consumption of EPA and DHA, or with their circulating biomarkers [86].

A clinical trial performed on the effect of omega-3-FA on insulin sensitivity, insulin secretion, beta cell function, and glucose tolerance in healthy individuals demonstrated that fish oils in moderate doses did not affect the above three parameters [87]. Griffin et al. [88] in the study of the effect of dietary ratio of omega-6 to omega-3-FA on metabolic parameters did not find any influence on insulin sensitivity. In a prospective Chinese population-based cohort study by Villegas et al. [89], an inverse relation was found between fish, shellfish, and long-chain omega-3-FA intakes and type 2 diabetes in women. Marine sources of EPA and DHA were not associated with diabetes risk. In a fish-based dietary intervention study in postmenopausal women with type 2 diabetes, an improvement was found in their endothelial function, which had no correlation with serum omega-3-FA concentrations [90]. A similar favorable effect on the endothelial function was found in the normoglycemic offspring of subjects with type 2 diabetes mellitus after fish oil supplementation [91].

A prospective study involving women with mean age of 54.6 years, who were followed up for 16 years, suggested an increased risk of type 2 diabetes with the intake of long-chain omega-3-FA, especially with higher intakes [92]. Van Wouden-bergh et al., in his population-based cohort study on Dutch participants, analyzed a group of non-diabetics ≥55 years. His results showed a positive association between total fish intake and the risk of type 2 diabetes. EPA, DHA, and fatty fish intake was not associated with the development of type 2 diabetes, but lean fish intake was found to be a causative factor [93].

In the analysis of older men and women included in the Cardiovascular Health Study, spanning a period of 15 years, long-chain omega-3-FA was not associated with a higher incidence of diabetes [94]. Akinkuolie et al. reviewed eleven randomized controlled trials on the effect of omega-3-FA on insulin sensitivity. It was concluded that omega-3-FA intervention had no effects on insulin sensitivity compared to placebo [95].

Wallin et al. systematically searched PubMed and ExcerptaMedica (EMBASE) databases through December 2011 to identify prospective studies examining relations of fish consumption, dietary omega-3-FA intake, and risk of type 2 diabetes. This meta-analysis indicated the considerable statistical heterogeneity in the overall summary estimates of the association between fish oil consumption and development of type 2 diabetes, some of which could be partly explained by geographical differences [96].

Table 37.1 Trials on omega-3-FA in type 2 diabetes mellitus (T2DM)

Study (references)	Results
Montori [182]	1. Omega-3 PUFA reduces TG levels in type 2 diabetes mellitus
	2. It causes rise in LDL levels
	3. There is no significant effect on fasting glucose, glycosylated hemoglobin, total cholesterol, and HDL cholesterol
Hartweg [183]	Omega-3 PUFA causes a 25 % reduction in TG levels in patients with T2DM
Hu [184]	Omega-3 PUFA is associated with reduced incidence of coronary heart disease and total all-cause mortality in patients with cardiovascular disease and T2DM
Nettleton [185]	Omega-3 PUFA reduces incidence of conversion to T2DM from impaired glucose tolerance in obese persons

Evidence shows the accurate association between fish and fish oil intake and diabetes remains largely unknown. Noteworthy is the fact that geographical regions play a role in this with studies showing a beneficial effect of fish oils on the prevention of type 2 diabetes in Asian population [97].

The studies on the association of omega-3-FA and diabetes are summarized in Table 37.1. The question of omega-3-FA being a friend or a foe in the context of diabetes remains unanswered. Further basic and clinical studies are essential to confirm the effects of omega-3-FA on diabetes.

Omega-3-FA in the Primary Prevention of Ischemic Heart Disease (IHD)

There has been an increasing interest in the health benefits of omega-3-FA, particularly in their role in the primary prevention of CV diseases. The following studies described below are some of the research work and their results, established in this aspect. Chronic inflammation has been found to be the main causative factor behind cardiovascular diseases [98]. Hence, the recent limelight on the anti-inflammatory potential of omega-3-FA warrants a detailed description. In a recent study, EPA and DHA intake resulted in a decreased expression of genes involved in inflammatory and atherogenic pathways [99]. They also caused a reduction in the circulating levels of C-reactive protein (CRP), tumor necrosis factor (TNFa), and some interleukins, thus reducing the risk of inflammatory events [100, 101]. In addition to lowering the levels of high-sensitivity CRP (hsCRP), EPA and DHA also lowered heat shock protein 27 antibody titers, thereby significantly causing a cardio-protective effect [101].

Omega-3-FA may help prevent cardiovascular events by its beneficial effects on lipids as well. They reduce fasting and post-prandial triglyceride levels and also retard the growth of thrombogenic plaques. Its anti-thrombogenic and mildly hypotensive action makes it worth a try in primary prevention of IHD [102].

Omega-3-FA in the Secondary Prevention of Cardiovascular Disease

Ever since researchers observed the CV benefits of omega-3-FA in the Greenland Eskimos and Okinawa islanders [103, 104], numerous meta-analyses have been performed that show its effect on reduction in CHD mortality, including fatal MI and sudden cardiac death (SCD), in populations with and without established CVD [105–108].

Role in IHD

In a trial by Delgado-Lista et al. [15], there was evidence accumulated that omega-3-FA supplemented for at least 6 months in capsule form or dietary source reduced cardiovascular events by 10 %, cardiac death by 9 %, and coronary events by 18 %, while showing a trend for lower total mortality.

The Japan EPA Lipid Intervention Study (JELIS) in the Japanese patients with hypercholesterolemia demonstrated reduced risk of major coronary events using long-term intake of EPA [109]. These patients with or without history of CHD were randomized to receive 1.8 g/day of EPA with statin or statin alone. Primary endpoints defined as SCD, fatal and nonfatal MI, and nonfatal CHD events including unstable angina pectoris, angioplasty, stenting, or coronary artery bypass graft (CABG) occurred in 2.8 % of patients receiving EPA compared to 3.5 % of the patients receiving control [110]. Patients with a history of CHD in the EPA group showed a significant reduction in major CHD events (19 % reduction compared to the control group), while the patients without history of CHD did not show any significant reduction in major CHD events with EPA treatment. Another finding of the JELIS study was that omega-3-FA found to be beneficial, despite the statin therapy in both the intervention and the control groups. The JELIS study also suggests that omega-3-FA may confer a significant benefit in terms of CV risk reduction in patients with type 2 diabetes [111]. Owing to the high baseline intake of marine fish by the Japanese natives, the incidence of CHD in them was found to be less than half that of the USA [112]. The Japanese have also been found to have an inverse relation between the levels of omega-3-FA and carotid intima media thickness [113], thus providing additional anti-atherosclerotic benefits and reducing the atherosclerotic plaque burden.

The landmark DART trial that focused on secondary prevention of CHD by long-term fish oil intake showed a 30 % reduction in total mortality related to coronary artery disease [37]. In this trial, a group of men, younger than 70 years and with history of angina, were randomly allocated between the following groups: (a) dietary advice to reduce total fat intake and increase intake of omega-3-FA (b) dietary advice to increase intake of fatty fish (c) increased intake of cereal fiber or (d) no specific dietary advice [37, 114]. The subjects were followed up for 2 years and their compliance was monitored by means of dietary questionnaire. The results showed lower mortality due to reduction in CHD deaths in the subjects advised to eat fatty fish. However, the incidence of nonfatal MI was slightly higher in this group [114].

The GISSI-Prevenzione study was another large intervention trial to assess the effect of omega-3-FA supplementation in secondary prevention of mortality in patients with a recent history of MI [115, 116]. A substantial reduction in all-cause and cardiovascular mortality was found in addition to reduction in the incidence of sudden cardiac death within four months of starting therapy with omega-3-FA [38]. In patients treated with omega-3-FA alone, the primary composite endpoints of death, nonfatal MI, and stroke were decreased significantly. There was also a reduction in CV death and SCD that can be attributed to the reduction in life-threatening ventricular arrhythmias, the most common cause of SCD after recent MI. The GISSI-P trial showed a 20 % reduction in death, 30 % reduction in CV death, and a 45 % reduction in SCD by omega-3-FA in patients with recent MI. In another case control study designed to assess the benefits of omega-3-FA in older patients with CHD, the results showed the association of omega-3-FA with a lower risk of fatal CHD but not with nonfatal MI. In this study, patients with fatal MI and other CHD and patients with nonfatal MI were matched to randomly selected controls. After 2 years of randomization, the plasma phospholipid omega-3 concentrations were found to be higher in the subjects who were at a lower risk of fatal CHD [117].

Delgado-Lista et al. [15] conducted a meta-analysis of 21 studies on omega-3-FA and CV events and summarized the following. Omega-3-FA significantly reduced the total mortality, the risk of CV death by 9 %, fatal and nonfatal CHD events by 18 %, and the risk of a CV event of any kind by 10 %.

Role in Heart Failure

The GruppoItaliano per lo Studio dellaSopravvivenzanell'Infartomiocardico—Heart Failure (GISSI-HF) trial was conducted among patients with chronic heart failure. The result showed significantly lower occurrence of death from any cause and CV-related hospitalizations, in the group who received 1 g/day of omega-3-FA compared to those receiving placebo [118].

Investigators have reported a substantial reduction in angiographic vein graft occlusion after 1 year of supplementation with 3.4 g/day of omega-3-FA [119]. A prospective study over 14.2 years concluded that raised serum concentrations of omega-3-FA especially DHA were associated with a lower incidence of heart failure in women [120].

A study on a large prospective cohort of young women indicated that little or no intake of fish and omega-3-FA was associated with an increased risk of cardiovascular disease [121].

Role in Arrhythmia

The Zutphen study showed a lower risk of CHD and SCD in men with increased fish consumption and the lowered risk could have been due to its antiarrhythmic effects. This effect was more apparent in people younger than 65 years; however, no clear dose response of fish intake on the risk reduction was seen [122]. Omega-3-FA may prevent SCD through its antiarrhythmic effect in asymptomatic people developing CHD who are not on statins. The benefits of omega-3-FA may be apparent in the statin users as it has protective mechanisms other than arrhythmia prevention. This has been shown by the JELIS trial and may be useful to prevent SCD in patients who cannot tolerate statins. These findings merit further exploration [123].

Role in Prevention of Secondary Composite Cardiovascular Events

A study by Eritsland et al. was conducted on 610 patients undergoing coronary artery bypass grafting (CABG), to evaluate the benefits of omega-3-FA in these patients. They were randomized to receive either 4 g/day of fish oil or control therapy, in addition to aspirin or warfarin [117]. The supplementation of omega-3-FA was found beneficial as the vein graft occlusion rates per distal anastomoses were found significantly lower in the fish oil group as compared to the control group, one year after the CABG. This study proves that omega-3-FA provides CV benefit in patients undergoing CABG by preventing the rates of vein graft occlusion in addition to increasing the rate and duration of patency of the graft.

The marine-derived omega-3-FA has fallen on hard times based on the findings in recent systematic reviews and meta-analysis that the evidence of omega-3-FA protecting against vascular disease is not clear-cut, and the benefits are not as great as previously believed [124]. Linoleic acid, once thought to reduce cholesterol, is now shown to cause heart disease, instead of preventing it [125].

The study on omega-3 FA and ventricular arrhythmia (SOFA) trial failed to support the antiarrhythmic potential of fish oils. In this study, patients with implantable cardioverter-defibrillators and a history of malignant ventricular tachycardia or ventricular fibrillation were randomized to receive 2 g/day of fish oil or placebo. There was no significant difference seen between the fish oil and the placebo group regarding the incidence of the primary endpoints of the trial, namely implantable cardioverter-defibrillator intervention for ventricular-tachycardia, fibrillation, or all-cause death [126]. However, the antiarrhythmic potential of fish oil cannot be completely abandoned, as this trial had detected a 33 % reduction in the primary endpoint with fish oil supplementation and fish oils may have brought about a benefit smaller than 33 %.

The OMEGA trial was conducted to assess the CV benefits of omega-3-FA. A set of patients with history of recent MI was randomized to receive either 1 g/day of omega-3-FA in addition to the standard guideline treatment or the standard treatment alone [127]. The primary endpoints recorded were the incidence of SCD, total mortality rates, and nonfatal CV events. No statistically significant difference was seen in the primary endpoints between the fish oil and placebo group. It must be pointed out that the use of aggressive pharmacological therapy would have masked any potential beneficial effects of fish oils.

The alpha omega trial [128], which was along similar lines as the SOFA trial, included patients between 60 and 80 years of age with a history of MI who were already receiving their optimal pharmacological therapy (including anti-hypertensive, antithrombotic, and lipid-modifying therapies) supplemented with the following—(a) EPA and DHA (b) alpha-linoleic acid (ALA) (c) EPA, DHA, and ALA, (d) placebo. The primary outcomes assessed were major CVD events, fatal and nonfatal CVD events, and CV interventions. No significant difference was seen in the primary endpoints among the DHA, EPA, and ALA group or the placebo group. But an interesting result of this study was the significant reduction in CVD events in diabetic patients supplemented with EPA and DHA, and also in the non-statin users who were supplemented with omega-3-FA plus ALA. The statin users on the same supplementation failed to show this benefit. The drawback of this study, as in the case of the SOFA trial, was that an optimal pharmacological therapy had been used in the subjects, thus making it difficult to prove the benefit of any new intervention with omega-3-FA.

In a recent meta-analysis conducted by Rizos et al. evaluating the effects of omega-3-FA on all-cause mortality, CV death, SCD, MI, and stroke [129], no benefit was demonstrable. The pitfalls in this trial were the small sample size, inadequate dosage, and the diverse sources of omega-3-FA that would have affected its efficacy [130]. The p value of <0.0063 chosen for this study also caused discrepancy in the results [131].

The effects of omega-3-FA and vitamin B on the incidence of major CVD events in patients with history of CHD or stroke were analyzed in the Supple'mentationen Folates et omega-3 (SU.FOL.OM3) trial [132]. No significant effect on the primary endpoints (major CV events including nonfatal MI, stroke, or CVD death) was seen with supplementation of either. Neither did omega-3-FA or vitamin B reduce the incidence of all-cause mortality or cancer morbidity. Some of the drawbacks of this study included using a low dose of EPA/DHA combination, small sample size, greater use of angiotensin-converting enzyme inhibitors, and angiotensin 2 receptor blockers in the placebo group and short trial duration.

Svensson et al., in their analysis of secondary prevention of CV events by omega-3-FA in patients undergoing chronic hemodialysis, showed that the number of myocardial infarctions was significantly reduced in the patients supplemented by fish oils. However, the primary endpoints of total CV events and death were not lowered by omega-3-FA supplementation. The small sample size, and the large number of withdrawals, was the drawback of the study [133].

The wide discrepancy among the above-listed trials and meta-analysis may be explained by a number of factors. Studies such as the JELIS trial contained a population whose background levels of EPA and DHA were already high due to their high dietary intake of fatty fish and hence had a lower incidence of CVDs as compared to the Western population. Second, most of the studies in this field are the secondary prevention trials, where the effect of fish oils was analyzed on people with pre-existing cardiac diseases. Some of these trials had a short follow-up period and some trials had patients who were already receiving optimal aggressive treatments. These limitations must be considered while interpreting the results from various studies.

Tables 37.2 and 37.3 list the meta-analyses of the association of omega-3-FA on secondary prevention of IHD. Table 37.2 describes the benefits of fish oils on CVS while Table 37.3 shows studies contradicting this.

Table 37.2 Meta-analyses favouring cardiovascular benefits of omega-3-FA

Risk factor	Study (references)	Benefits
Hypertension	Geleijnse et al. [186]	Higher intake (>0.5 g/day) of omega-3 PUFA reduces blood pressure in older hypertensive subjects
Ischemic heart disease	Burr et al. [37]	Fatty fish intake causes 29 % reduction in all-cause mortality in men after recent MI
	GISSI-Prevenzione trial [38]	Omega-3 PUFA causes 20 % reduction in total mortality, 30 % reduction in CV mortality
	Campos et al. [187]	Consumption of ALA reduces risk of MI in patients with MI
	Yokoyama et al. [107]	EPA supplementation caused 19 % reduction in major CV events (primary and secondary prevention) in patients of hypercholesterolemia
Dyslipidemia	Tilander et al. [188]	Fish oil lowers plasma cholesterol (esterified and unesterified), TG and PLs
Arrhythmia	Kumar et al. [189]	EPA and DHA intake associated with significant reduction in recurrence of AF in patients with AF
	Patel et al. [190]	Patients with pulmonary vein antrum isolation treated with omega-3 PUFA lowers incidence of early AF recurrence and procedural failure
Post-PTCA	Bairati et al. [191]	Patients on omega-3 PUFA, who underwent PTCA, had less chances of restenosis
	Marista et al. [192]	EPA and DHA supplementation before PTCA causes small but significant reduction in restenosis rate
Post-CABG	Calo et al. [193]	Omega-3 PUFA administration 5 days before CABG till discharge reduces risk of postoperative AF and duration of hospital stay
	Heidt [194]	Perioperative intravenous infusion of PUFA reduces incidence of AF after CABG and reduces duration of Intensive care unit stay
Heart failure	GISSI-HF study [116]	Omega-3 PUFA reduces mortality and hospital admission for CVS reasons, in HF patients
	Yamagishi et al. [195]	PUFA consumption reduces CV mortality from HF in Japanese population

MI myocardial infarction, *CV* cardiovascular, *AF* atrial fibrillation, *PTCA* percutaneous transcatheteral angioplasty, *CABG* coronary artery bypass grafting

Table 37.3 Meta-analyses contradicting cardiovascular benefits of omega-3-FA

Risk factor	Study (references)	Lack of benefits
IHD	Burr et al. [112]	2 portions of oily fish per week/3 fish oil capsules daily caused higher risk of cardiac death in men <70 years
	Kromhout et al. [196]	Low-dose EPA-DHA/ALA did not reduce major CV events in patients with recent MI on optimal pharmacological treatment
Arrhythmia	Rauch et al. [125]	No significant antiarrhythmic effect/no effect on SCD in patients with recent MI
	Brouwer et al. [197]	No protective effects of PUFA on incidence of recurrent ventricular arrhythmia in patients with ICDs
	Kowey et al. [198]	PUFA treatment does not reduce recurrent AF in patients with paroxysmal AF
Post-PTCA	Reiss et al. [199]	Incidence of angiographic stenosis found higher in fish oil-supplemented group
Post-CABG	Saravanan et al. [200]	PUFA administration starting 5 days before CABG does not reduce risk of postoperative AF
	Farquharson et al. [201]	Fish oil administration for 3 weeks before cardiac surgery causes no significant reduction in incidence of postsurgical AF
Heart failure	Dijkstra et al. [202]	No role of fish intake in prevention of HF
	Belin et al. [203]	No significant association between EPA and DHA, ALA or trans-fatty acid intake on incidence of HF

MI myocardial infarction, *CV* cardiovascular, *AF* atrial fibrillation, *PTCA* percutaneous transcatheteral angioplasty, *CABG* coronary artery bypass grafting

Omega-3-FA in Preventing Cardiac Events in Patients with Chronic Kidney Disease (CKD)

Ever since the cardio-protective benefits of omega-3-FA have been brought to light, several researches have been conducted to find out the effects of omega-3-FA supplementation on CVD risk reduction in patients with chronic kidney disease. CVD is a major cause of mortality and frequent cause of hospitalization in patients with CKD [134, 135] and is likely due to the chronic inflammation, dyslipidemia, malnutrition, atherosclerosis, and vascular calcification [136]. Among the mechanisms involved in the cardio-protective action of fish oils, studies have laid an emphasis on modifications of erythrocyte membrane fatty acid content, including an increased omega-3-index and decreased oleic acid.

It is well known that raised dietary intake of saturated FA augments the amount of the cell membrane total saturated fatty acid content that promotes occurrence of CV events [137, 138]. Omega-3-FA supplementation has been shown to decrease the total saturated fatty acid content in CKD patients as well as general population [139–141]. CKD has been found to be an inflammatory state with elevated inflammatory biomarkers such as C-reactive protein, serum homocysteine, and plasma fibrinogen [142, 143]. These inflammatory markers further increase the risk of CVD in patients of renal dysfunction. Omega-3-FA-derived resolvins and protectins have been found to attenuate the levels of these mediators of inflammation [144–146], thus reducing the oxidative stress and accumulation of advanced glycosylation end products [147]. Renal fibrosis has been found to be decreased with intake of omega-3-FA [147].

In some of the recent researches, the erythrocyte membrane oleic acid levels were found to be associated with a higher CV risk. Its levels were found significantly elevated in patients with acute coronary syndrome [148, 149] and also in CKD patients on hemodialysis who were at a higher risk of CVD [139, 150] Oleic acid plays the culprit in causing adverse CV outcomes by stimulating vascular smooth muscle migration and proliferation via the direct activation of extracellular signal-regulated kinase (studies in vitro). Studies also showed amplification of angiotensin 2-induced protein kinase C activation and reactive oxygen species generation in vitro [151, 152] omega-3-FA supplementation has been found to decrease erythrocyte membrane oleic acid levels in CKD patients on dialysis [139, 140].

The triglyceride lowering potential of omega-3-FA may be useful in controlling dyslipidemia occurring in patients of CKD, thereby lowering the CVD risk. In CKD patients undergoing dialysis, elevated triglycerides constitute the primary dyslipidemia. On the contrary, the levels of total cholesterol, triglyceride, and LDL cholesterol are normal or low in malnourished patients with CKD [153]. The supplementation of omega-3-FA may raise the omega-3-index favoring a cardio-protective effect in CKD patients. The exact mechanism of action of omega-3-FA on the structure and functions of cell membrane receptors, which increases the omega-3-index, remains to be elucidated.

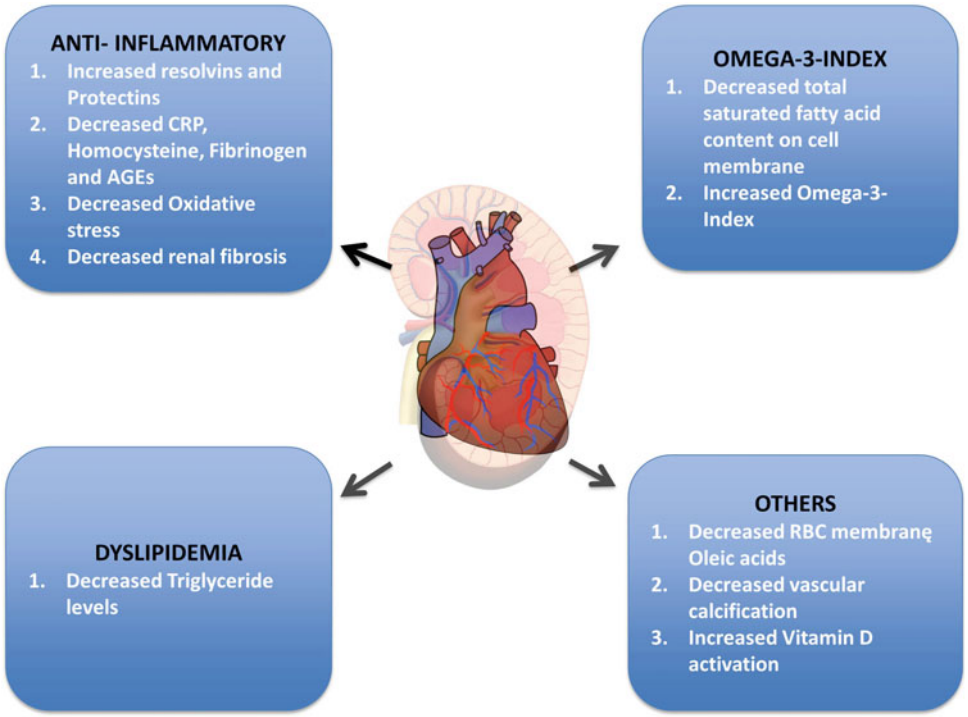

Fig. 37.4 Cardio-protective mechanisms of omega-3-FA in CKD

Among the studies being done to evaluate the beneficial effects of omega-3-FA in CKD, the propensity of omega-3-FA in inhibiting vascular calcification is worth a mention. Vascular calcification, prevalent in patients with CKD, is an independent predictor of CV mortality [154]. Vascular calcification is evident in plain radiographs of patients on dialysis. Omega-3-FA is found to have a positive effect on vascular system by reducing pulse wave velocity [155], and increasing the levels of Fetuin-A that antagonizes the vascular calcifying process [156]. Vitamin D deficiency is more common in patients with renal dysfunction than in the general population and this has been shown to aggravate CVD risk [157, 158].

In summary, omega-3-FA minimizes cardiovascular disease (CVD) risks by reducing inflammation, decreasing oxidative stress, exerting antiarrhythmic effect, and by its anti-atherosclerotic potential. In addition to these, the CVD risks in patients of CKD may be reduced by modification of erythrocyte membrane fatty acid content thereby raising the omega-3-index, decreasing total saturated fatty acids, oleic acids, and by alterations in the cell signalling systems. Noteworthy are the ongoing studies on the active involvement of omega-3-FA in prevention of vascular calcification and vitamin D activation in patients of CKD. The anti-inflammatory effect of vitamin D is exerted by its biologically active metabolite 1,25 (OH) 2D, which has anti-proliferative and anti-inflammatory effects on the endothelial cells of the vascular wall [159]. Omega-3-FA supplementation has shown to increase the concentration of 1,25 (OH) 2D, in dialysis patients [140]. Hence, fish oils may have a cardio-protective effect in CKD patients through activation of vitamin D. Figure 37.4 depicts the cardio-protective effects of omega-3-FA in CKD.

Omega-3-Index and CV Health

The omega-3-index is defined as the percentage of EPA + DHA content in the red blood cell membranes. This index has emerged as a new risk factor for death caused by CVDs [160]. An omega-3-index of less than 4 % is associated with low cardio-protection, while an omega-3-index of 8 % or higher is associated with increased cardio-protection [161]. The erythrocyte membrane FA content reflects the FA content of the myocardium [161]; hence, its modification can alter the risk of CV events. The first evidence in support of the above relation was brought to light when studies on heart transplant patients, before and after fish oil supplementation, demonstrated that arachnoid acid declined in both myocardial and red blood cell (RBC) membrane phospholipids, in patients receiving fish oils [161, 162]. A low omega-3-index was associated with an increased risk of ventricular fibrillation during acute ischemic phase of MI and SCD [163, 164].

Omega-3-FA intake augments the fatty acid composition in the myocardial phospholipids and the omega-3-index may be a reasonable approach to assess the cardiac omega-3-FA content and predict future CVD events [165, 166]. The

Table 37.4 Dosage recommendations of omega-3-FA

Indication	Doses recommended
CVD—primary prevention [166]	EPA and DHA intake of 500 mg/day or ≥two 3.5-oz servings of fatty fish at least twice a week
CVD—secondary prevention [2]	EPA and DHA of 1 g/day
Hypertriglyceridemia [8]	2–4 g/day of EPA and DHA as capsules under physicians' observation in addition to other lipid-lowering treatment
Pregnancy and lactation [168]	≥300 mg/day of DHA
Type 2 diabetes [169]	2–3 oz. servings of fish weekly

relevance of the association of omega-3-index with the incidence of SCD index is evident in the following data: In the Western countries where the average omega-3-index is <5 %, the incidence of SCD is 150/100,000 person years, whereas in Japan where the consumption of fish is higher, the omega-3-index is >9 %, and the incidence of SCD is 7.8/100,000 person years [167].

Pharmacological Preparations and Dosages

With the escalation of interest in the role of omega-3-FA in CV health, a new era of research has spawned in this potentially latent natural supplement teeming with health benefits. Authoritative bodies have defined the recommendations of fish oil, both in the pharmacological and dietary forms, depending on the various clinical scenarios. The American Heart Association (AHA) recommends EPA and DHA intake of 500 mg/day [168] or ≥two 3.5-oz servings of fatty fish at least twice a week [169] for primary prevention of CVD. For secondary prevention, 1 g/day of seafood sourced EPA and DHA is advised, in consultation with a physician [2]. In hypertriglyceridemia, it is recommended to take 2–4 g/day of EPA and DHA as capsules under physicians' observation in addition to other lipid-lowering treatment [8]. Pregnant and lactating women are advised to consume ≥300 mg/day of DHA [170], at the same time avoiding fish with higher levels of mercury. Given the current evidence, supplementation of omega-3-FA appears to be beneficial in the management of T2D. Currently, the American Diabetes Association recommends that T2D patients consume 2- to 3-oz. servings of fish weekly [171].

Among the pharmacological preparations available in the market, a 1 g capsule of Lovaza™ (GlaxoSmithKline) containing 465 mg of EPA and 375 mg of DHA as fatty acid ethyl esters [172] may be given to patients with hypertriglyceridemia at doses of 4 g/day. Amr101™ is another preparation of EPA as an ethyl ester. It was recently developed by Amarin Pharma and is well tolerated at the dosage of 4 g/day that reduces non-high-density lipoprotein (HDL-C), apolipoprotein B, lipo-protein-associated phospholipase A2, very low-density lipoprotein (VLDL-C), and total cholesterol in patients with hypertriglyceridemia [173].

Although many international authorities have issued dietary recommendations for omega-3-FA, there is a pressing need to establish a dietary reference intake (DRI) for these fatty acids [8]. This will ensure reduced uncertainty among consumers and health professionals regarding target omega-3-FA intake. The DRI, as issued by the institute of medicine (IOM) of the National Academies, consists of four nutrient-based reference values—estimated average requirements (EAR), recommended daily allowance (RDA), adequate intake (AI), and tolerable upper intake level (UL). Table 37.4 gives the various dosage recommendations of omega-3-FA.

Adverse Effects

The only adverse effect of clinical significance is nausea that occurs at doses 4 g/day or higher [174]. Some complain of fishy taste while belching, which can be overcome by freezing the capsule or taking it along with meals [175]. The consumption of fish oils through diet was doubted to cause an increased mercury intake, especially through big fishes such as king mackerel, blue fin tuna, shark, or swordfish [176]. This factor was questioned while considering fish intake in females of reproductive age group, pregnant females, lactating mothers, and young children. However, it was found that inadequate intake of DHA in pregnancy may impair the neural development in the fetus [177, 178]. Omega-3-FA has been found to play a significant role in brain growth and cognitive development during late gestation and leading into childhood [179]. Omega-6-FA, in addition to omega-3-FA, has also been shown to be essential for fetal and infant development and deficiencies in these FA may lead to neural and vascular complications of preterm babies [180].

Seafood, unlike fish oil supplements, conferred additional cardio-protective benefits as it contains high-quality proteins

and vitamin D, along with omega-3-FA and hence, the recommendations in the above-mentioned population subset have been laid down.

The potential risk of omega-3-FA-induced bleeding events in patients on anti-platelets and anticoagulants has sought much attention. But, recent meta-analyses have proven them to be safe unto 4 g/day [174].

Conclusion

Studies on the effects of omega-3-FA in CV and other systems have demonstrated mixed results and have provided us with multiple options for further research. Although these bioactive compounds demonstrated modest reduction in cardiovascular events in earlier randomized clinical trials, recent studies have challenged the benefits of omega-3-FA in the current era of optimal medical therapy. On careful analysis of the current body of evidence, certain pitfalls in the results of some trials can be attributed to faulty study design, inadequate dosage of omega-3-FA, and differences in study populations [181]. CVD is a major economic burden and hence, the role of omega-3-FA in the primary and secondary prevention of CVD, along with its beneficial effects in a vast array of various medical conditions, needs to be better exploited.

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Beneficial Effect of Long-Chain Omega-3 Fatty Acids in Psoriasis

38

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Introduction

Psoriasis is the autoimmune skin disorder characterize by inflammation, redness, and itching. The genetic and environmental causes leading to abnormalities in the skin lipid metabolism and higher production of CD+, CD8+ T cell and pro-inflammatory cytokines also tend to aggravate the disease. Psoriatic lesion has enriched epidermal keratinocytes and leukocytes cells. Keratinocytes actively play an important role in recruitment and activation of leukocyte and others cytokines [1, 2]. Omega-3 (ω) fatty acids are polyunsaturated fatty acids, which have double bond at third carbon atoms from methyl end of the molecule. Instances of these fatty acids include alpha-linolenic acid (ALA, 18:3), eicosapentaenoic acid (EPA, 20:5), docosapentaenoic acid (C20:5n-3:DPA), and docosahexaenoic acid (DHA, 22:6). Among these, EPA and DHA are most widely used diverse therapeutic benefits including psoriasis [3]. Consumption of these molecules clinically tends to provide multiple benefits in the prevention of several dreadful disorders like cardiovascular diseases, type 2 diabetes, inflammatory bowel diseases (IBD), psoriasis, rheumatoid arthritis, and mental disorders [4]. Inside the body, EPA and DHA are formed from linolenic acid, but their conversion is quantitatively too low. Therefore, these are considered as essential fatty acids and it mainly derived from diet sources and necessary for human health [5]. Nutrition experts suggested that ω-6:ω-3 fatty acids ratio of 5:1 is desired for normal function of cell [6]. Nowadays, food habits in the world characterized by major consumption of saturated fatty acids and poor proportion of ω-3 fatty acids [6]. The higher ratio of ω-6/ω-3 in diet promotes the pathogenesis of several disorders such as cardiovascular, inflammatory, and autoimmune diseases, whereas higher level of ω-3 fatty acids founds great potential to suppression of these dreadful disorders [7]. There are several studies reported on prevention of diseases using ω-3 fatty acids as a major component of diet. It has been observed that administration of 1.7 g/day of ω-3 fatty acids helps in suppressing cardiovascular diseases [8]. At the dose of 3–4 g/day, ω-3 fatty acids reduce plasma triglycerides level in hypertriglyceridemia patient [9]. Another study has reported that administration of ω-3 PUFA at 1–8 g/day produces beneficial effects in the treatment of psoriasis, IBD, eczema, and rheumatoid arthritis [10]. Besides, the ratio of 2.5/1, 5/1, 10/1 ω-3 PUFA/ω-6 PUFA is effective in the suppression of colorectal carcinoma and asthma, whereas ratio <2:1 is useful to prevent recurrence and metastases in mammary cancer [11]. In the last few years, transformation in nutritional habits with high dietary intake of ω-3 fatty acids enriched food products has been developed such as bakery products, milk egg derivatives, and juices. However, the most vital natural resource of ω-3 fatty acids is fish oil [12, 13]. Several literature reports revealed that enhanced level of prostaglandins and leukotrienes in psoriasis patient. Another study revealed that i.v. infusion of EPA and DHA loaded lipid emulsion can result for the improvement in the psoriatic condition via inhibition of PGs and leukotriene's formation [13, 14]. Another study has reported that administration of 3.6 and 14 g/day of EPA and DHA up to 6 month results into improve severity in psoriasis patients with minimal side effects [15]. Another literature report revealed that daily administration of 1.8 g EPA in 28

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patients of psoriasis for 8 weeks can result in significant inhibition of psoriasis area and severity index (PASI) [16].

Grimminger et al. observed that i.v. administration of 2.1 g of EPA and 21 g of DHA in 20 psoriasis patient reduces 45–76 % chances of PASI [17]. Similarly, another study demonstrated that administration of 10 fish oil capsules (each contain 1.5 g of EPA) in 32 psoriasis patients with enhanced benefits for itching and erythema [18]. Mayser et al., demonstrated that administration of 200 mL/day of ω-3 fatty acids containing 4.2 g of EPA and DHA results in 11.2 % decrease in PASI score [19]. Escobar et al, reported that application of 15.8 % EPA and 10.1 % DHA through topical routes on 25 psoriasis patients with greater inhibition of erythema, itching, and significant reduction in thickness of psoriatic plaques [20]. Overall, EPA and DHA also metabolize to potent anti-inflammatory molecules such as RvE1 and RvE2 [21]. However, there are two major limitations which hindered their clinical applications such as lipid peroxidation and restricted permeation across the biological membranes. This can be overcome by co-administration with antioxidants and nanomedicines for enhancing the therapeutic benefits of ω-3 fatty acids.

Pathophysiology of Psoriasis

Proinflammatory cytokines play an important role in the pathogenesis of psoriasis; they are recruited and activated by keratinocytes. Chemically they are glycoprotein; it includes lymphokines, monokines, interferon's, interleukins, and growth factors. Moreover, these attracts the neutrophils to the cluster differentiation (CD11) and along with it produces various interleukins such as IL-23, IL-15, IL-20, interferon-α, inducible nitric oxide syntheses (iNOS), and tumor necrosis factor-α. These all biomarkers have lead to keratinocyte hyperproliferation and epidermal hyperplasia in psoriasis [22, 23]. Every cell contains arachidonic acid (AA; 20:4n-6), which is a major substrate for eicosanoid synthesis [24]. Eicosanoid includes prostaglandins (PGs), thromboxanes, and leukotrienes (LTs) [25]. These are key elements for inflammatory response and also induce other pro-inflammatory cytokines which are involved in pathogenesis of psoriasis. Thus, AA metabolism plays an important role in psoriasis cascade [26]. Protein kinase (PK) is a serine threonine kinase which is sensitive to reactive oxygen species (ROS). Their activation results to induce human leukocyte antigen-1 (HLA-class-I), TNF-α, and CDId. Further their combination with keratinocyte natural killer T cell mediates cytokines production [27]. Phosphorylation of protein kinase results in translocation to nuclear membrane and produces nuclear factor (NF-K) by phosphorylation of RelA P65 protein. Nuclear factor responsible for DNA binding and it result to elicit various biomarkers involved in psoriasis [28, 29]. Reactive oxygen species (ROS) includes superoxide, hydrogen peroxide, hypochlorous acid, peroxynitrite, hydroxyl radical, and superoxide anion [30]. Their overproduction or inadequate removal of ROS creates oxidative stress, which causes umpteen abnormalities such as lipid peroxidation, damage to DNA, and production of various inflammatory cytokines [31], and they strongly involved in the pathogenesis of psoriasis. Furthermore, many cytokines activating the other pathways such as tyrosine kinase and gene expression produce inflammatory response in psoriasis [32]. In last, overall we conclude that alteration in dermal vascularization, abnormal eicosanoid metabolism, lymphokines production, and oxidative stress, and these all are considered as strong predisposing factors in psoriasis cascade.

Omega-3 Fatty Acids and Their Potent Metabolites as Nutritional Value in Psoriasis Pharmacotherapy

Phospholipids are a major composition of membranes; by acting of PLA2, it liberates AA and further action of COX and LOX produces prostaglandins (PGI2, PGE1, PGE2, etc.) and leukotrienes (LTB4, LTC4, etc.) [33, 34], and the pictorial depiction of AA metabolism involved in psoriasis is shown in Fig. 38.1. Literature supports that fish oil combination with ultraviolet B (UVB) successfully reduces the toxicity associated with UVB and prolongs the duration of action with better efficacy [35]. Cyclosporine is widely

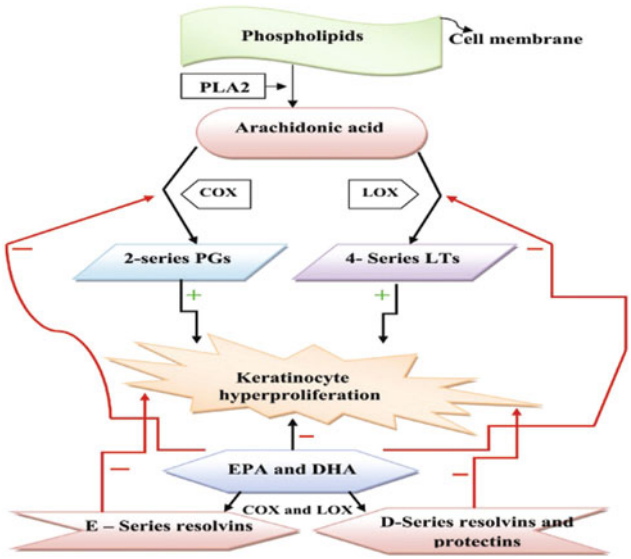

Fig. 38.1 Involvement of membrane phospholipids in arachidonic acid and their products formation and key role of EPA-DHA and its metabolites exerting inhibitory action on PGs and LTs to resolve psoriasis

reported in psoriasis treatment, but they have major limitation due to nephrotoxicity, adding of fish oil with cyclosporine reduces its toxicity [36]. There are few products of ω-3 fatty acids such as Omacor, which containing 84 % EPA + DHA or EPA/DHA ratio 1.2:1.0 and MaxEPA® containing 30 % EPA + DHA or EPA/DHA ratio 1.5/1.0 is widely employed in psoriasis treatment by blocking of LTB4 and 12-HETE formation [37]. Another different study revealed that i.v. infusion of lipid-based emulsion contains EPA and DHA found overall improvement in the severity of psoriasis via blocking of AA metabolism. Other than these beneficial effects, ω-3 fatty acids also suppress ROS formation by virtue of upregulation of gene regulation of antioxidant enzymes [38, 39]. There are some patents also describes the use of purified ω-3 fatty acids which have more than 85 wt% to produces beneficial effect in autoimmune disorders. Moreover, 50 mg DHA and 300 mg EPA loaded into unit dosage form and has been employed for the treatment of autoimmune-based disorders [40]. These are summarized in Table 38.1.

US patent no, US 2010/0331415 A1, describes the administration of EPA and DHA with the dose of 0.5–1 g/day up to 6 months results to found better efficacy in psoriasis patient [41]. Another US patent demonstrated the EPA, DHA, and stearidonic acid (SDA, 18:4n-3) produces beneficial effect in psoriasis patient [42]. Another US patent no, 7,919,685, describes the positive effects of EPA, DHA, and SDA in psoriasis by the inhibition of TNF-α, IL-1, and IL-6 [43]. US patent no. 2007/0219271A revealed the combination of ω-3 fatty acid with cyclosporine or MTX, and it administered in psoriatic patient found synergistic antipsoriatic action over alone use of said drug [44]. Another US patent describes resolvin and DHA metabolites such as resolvins D1 (RvD1), resolvins E1 (RvE1), and 10, 17S, docosatrienes of DHA metabolite, and these are significant inhibitors of TNF, PGs, and LTs [45].

DHA-Derived Resolvins and Protectins

DHA is a potent molecule; it constitutes D series resolvin and protectin D1. It formed by subjecting of 15-lipoxygenase (15-LOX), and this catalyzes hydroxylation of DHA at C17 position and produces 17S-H (P) DHA [46]. Another hydroxylation result produces resolvins D1, D2, D3, D4 (RvD1-RvD4), and dihydroxyl compound known as protectins D1. Thus, 17S configuration is progenitor of all these mediators and commonly called as 17S-resolvin-D class; 10R, 17S-dihydroxydocosahexaenoic acids are known as 17S-protectins D1. These all metabolites [47] have to establish as potent anti-inflammatory molecules to resolve psoriasis (enlisted in Table 38.2).

Table 38.1 Role of EPA and DHA (n-3 PUFA) in psoriasis

S. No.	n-3 PUFA	Outcomes	References
1	Administration of 1.8 g EPA on 28 psoriasis patient for up to 8 weeks	It receive reduction in PASI	[98]
2	Administration of EPA with etretinate in psoriasis patient at the dose of 20 mg up to 40 days	It significantly reduces keratinocytes hyperproliferation and improvement in to resolving of psoriatic plaque	[35]
3	Oral administration of 5.4 g EPA and 3.6 g DHA in combination with UVB on 18 patient for up to 15 weeks duration	It receive significant reduction in PASI as compared to alone use of UVB	[99]
4	Intravenous application of EPA and DHA lipid-based emulsion in 83 plaque types psoriatic patients for up to 14 days	It found reduction in PASI by more than 15	[19]
5	Intravenous administration of EPA and DHA on acute guttate type of psoriasis patient up to 10 days	Psoriatic patient receives moderate clinical improvement in resolving of psoriasis	[17]
6	Topical administration of 15.8 % EPA + 10.1 % DHA in 25 plaque psoriasis patient for 4 weeks	Significant reduction in PASI	[20]
7	Given combination of cyclosporine with 6 g of EPA and DHA on 20 psoriasis patient for up to 3 month	It found greater reduction in PASI as compared to alone use of cyclosporine	[100]
8	Administration of betamethasone dipropionate with fish oil	Betamethasone has to reduce hyperproliferation and further addition of fish oil enhanced inhibition of rat edema from 43.15 to 70.35 %	[101]
9	Application of fish oil topically for 4 week	It reduces itching and erythema in psoriasis patient	[20]
10	Individual administration of ω-3 fatty acid with petrolatum base and 3 % salicylic acid with emollient base in psoriasis patient	PASI score reduces up to 41.7 and 28.3 %, respectively	[19]

Table 38.2 Impact of DHA and EPA metabolites in psoriasis

Mechanism of action	Benefits	References
It act by inhibition of IL-12 and further to reduce IL-6, IL-23, and IL-15	It reduces thickness of keratinocyte proliferation in psoriasis	[102]
Inhibition of IL-23, IL-6, and TNF-α	It significantly reduces keratinocyte hyperproliferation and differentiation	[103]
It enhances phagocytosis and apoptosis of PMNs	Chemokines production inhibited	[103]
It reduces Chemotaxis	Prominently decrease cytokines production	[104]
It inhibit superoxide formation	Significantly reduces ROS formation, oxidative stress, and cytokines production	[104]]
It enhances L-secretin and downregulates the CD18 expression	Resulted to inhibit pro-inflammatory cytokines and further reduces keratinocytes hyperproliferation	[105]
It block IL-8 secretion	It reduces cytokines and keratinocytes hyperproliferation	[104]
It inhibit LTB4 and PGs formation	Results to decrease psoriatic plaque by to decrease keratinocyte hyperproliferation	[106]

EPA-Derived Resolvins

EPA has two end metabolites named as E-series resolvin E1 (RvE1) and resolvin E2 (RvE2). The intermediate compound between two is 18-hydroperoxy eicosapentaenoic acid (18-H (P) EPA). The action of 5-LOX produces 18R-RvE1, 18S-RvE1 [48, 49], and 18R-RvE2 or 18S-RvE2. Further, these molecules suppress the dendritic cell migration [50] and inhibit IL-12, PGs, NF, and LTB4 production (shown in Table 38.2).

Omega-3 Fatty Acids: Immunosuppression in Psoriasis by Various Mechanisms

The administration of fish oil containing ω-3 fatty acids in psoriatic patient results to suppress natural killer cell and to inhibit the formation of IL-1, IL-6, and TNF-α. Administration of fish oil in psoriatic patient lowers the number of peripheral blood CD4+ T cells and inhibits the T cell expression [51, 52]. T cell signaling is transduced by T cell receptor (TCR) and antigen-presenting cell (APC). Their activation completed in two steps, early and late phase. Early phase involves mobilization of intracellular Ca^{2+}; protein kinase C activation; expression of immediate early genes like C-fos (is a protein encoded by FOS gene), C-jun (is a protein encoded by a gene) [53]; and activation of mitogen-activated protein kinase (MAPK). Other is plasma membrane which also mediates the early phase of T cell-inducing cascade. They includes Src family, protein tyrosine kinase, Lck, Fyn, G-protein, growth factor receptors, Plcy1, LAT, and MAPK. Late phase involves transition of cell from S phase to G/M phase and prolongs PKC activation [54, 55], whereas ω-3 fatty acid administration inhibits the various signaling cascade like as PKC, MAPK, and Ca+2 ions channel. Furthermore, they also alter IL-2-induced Janus kinase signal transducer, activator of transcription signaling pathways including PLA, PLC/PLD, and downstream cell signaling. Rising in Ca^{2+} concentration makes higher nuclear factor T cells (NF-AT) activity and gene expression; it results to induce T cell expression and leads to keratinocyte hyperproliferation [56]. Thus, EPA and DHA targeting on these biomarkers reduces keratinocyte hyperproliferation up to significant level (Fig. 38.2).

PKC Activation

Protein kinase is available as PKC, PKC1, and PKCII. The presence of phorbol esters in the body is largely responsible for the activation of PKC. There are various studies reporting the influence of ω-3 fatty acids on PKC activation [57, 58]. Speizer et al. reported that activation of PKC in S49 lymphoma cells, purified bovine and rat, and application of EPA and DHA shows binding of radioactive phorbol esters under the absence of phospholipids [59]. With the presence of phospholipids, EPA and DHA reduce the catalytic action of PKC and phorbol ester binding. Administration of fish oil into mice for two weeks, it results to inhibit suppress signals and effector function in T cells [16], whereas in human it shows immunosuppressive effects. The membranes which have PKC-α and PKC-ε cause MAPK activation and upstream of ERK1/2 results to inhibit the translocation of NF-κβ (Fig. 38.2). However, administration of EPA and DHA revealed that they act on these molecules and to inhibit the IL-2 gene expression and ultimately significantly reduced the keratinocyte proliferation [60, 61].

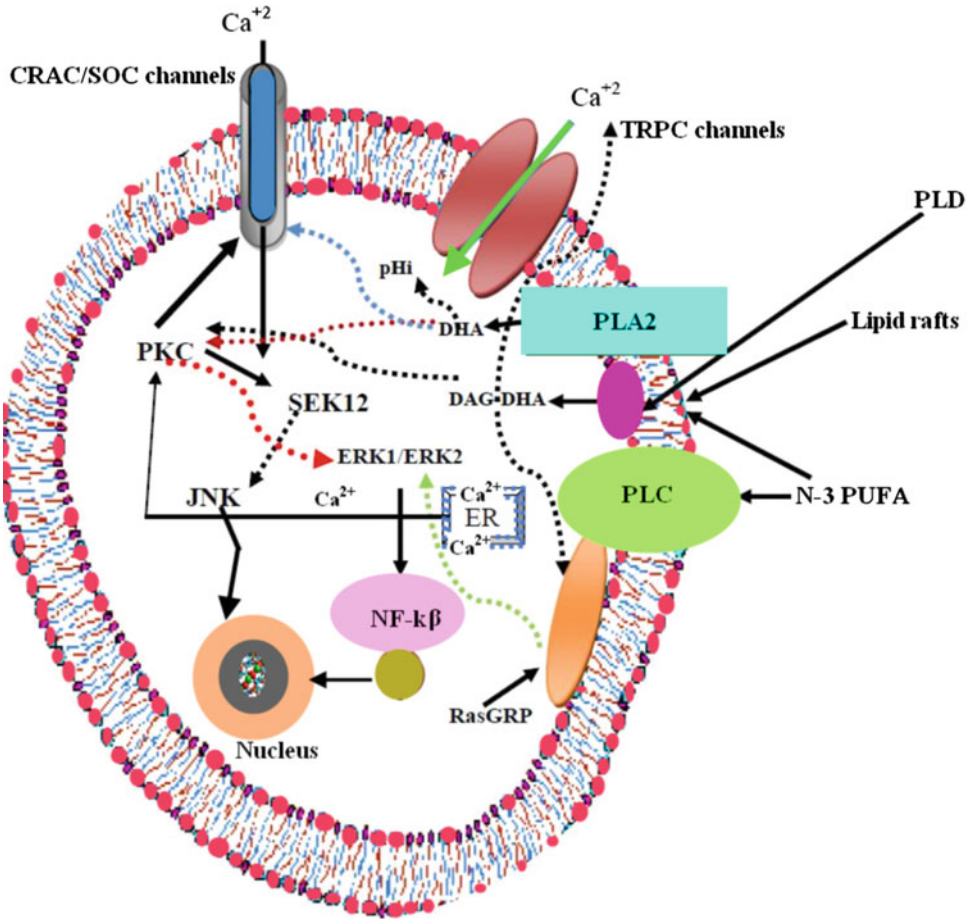

Fig. 38.2 Role of n-3 PUFA in T cell signaling, they act on PLD to produce DAG-DHA/EP, and further it induces Ca^{2+} intracellular transition through various channels; it activates RasGRP and exerting action on PKC; these all lead to inhibit gene transcription and show immunosuppression to resolve psoriasis via inhibition of JNK and ERK_1/ERK_2

MAPK Cascade

Transcription of genes involved in T cell progression, and they are mediated through Ras/Raf-1/MEK1/2/MAPK pathway. This has been similar to Jurkat T cell, and it involves translocation of NF-κβ, which may responsible for transcription of IL-2 gene into the nucleus [62].

Thus concerning to phosphorylation, AA may activate to MAPK phosphorylation with a PKC-dependent manner [63]. Gorjao et al., reported that uptake of EPA and DHA significantly suppresses the phosphorylation of ERK1/2 in T cell expression and further also inhibits the PKC activation coupled MAPK phosphorylation [64]. Moreover, it also found that EPA and DHA administrations result to suppress IL-2-induced phosphorylation of ERK1/2 [65]. Transient receptor potential (TRP) is a family of ion channel, and it classified into three main groups, one is canonical TRP family (TRPC), which showed as TRPC1-7 (several protein products of the mammalian TRPC). This ion channel further activated under the presence of AA, linolenic acid, and oleic acid. Whereas other member is TRPC3, TRPC6, and TRPC7, they are activated by diacylglycerol (DAG). There are numerous literature reported the mRNA encoding TRPC3/6 channels in Jurkat T cell and their maximum expression founds during G1 phase of cell cycle [66]. Moreover, 1-stearoyl-2-arachidonyl-sn-glycerol (SAG) and 1-stearoyl-2-docosa hexaenoyl snglycerol (SDG) have been reported in the modulation of TRPC6 ion channel. Another study has reported on the Src kinase, which also involved in the activation of TRPC6 channel by DAG molecular species including SAG, DOG, and SDG [67]. Moreover, PKC is activated at different degree of efficiency by SAG and SDG. Another different study revealed that EPA and DHA inhibit the IL-22 production and DAG formation in mitogen-stimulated T cell of mice, and further, they exert inhibitory effects and produce antipsoriatic action. After S phase of cell cycle completion in keratinocytes proliferation, another co-stimulatory signal passes through B 7.1 and B7.2 antigen, which available on APC or B cell. Furthermore, activation of PLC-MAPK, C-Jun terminal kinase, P38/MAPK, and protein kinase is resulted by the combination of T cells expressed CD28 antigen with B7.1 and B7.2. Moreover, this signal induction also induces to JNK pathway, which is regulated by an enzyme such as MEK

analogue to ERK1/ERK2 [68, 69]. Recently reported an administration of ω-3 fatty acids causes JNK inhibition, and it may attribute due to non-phosphorylation of JNK [70]. Many researchers well documented the use of ω-fatty acids significantly inhibits the formation of prostaglandins like PGE2 and PGD2 and leukotrienes including LT4 in psoriatic cells. Furthermore, some EPA-derived products may significantly inhibit the macrophages and monocytes secretary cytokines like TNF-α and ILs [71]. James et al., reported EPA and DHA have to antagonize the AA metabolism and to produce less active eicosanoid [72]. Gil found that administration of fish oil metabolizes to produce 15-hydroxy eicosatetraenoic acid, which shows antiproliferative effect [10]. Furthermore, EPA and DHA derived two other novel anti-inflammatory mediators named as resolvins and protectins (Fig. 38.1), and they inhibit inflammatory biomarkers, which involved in psoriasis [73].

Nuclear Factor κ (NF-κ)

NF-κ activation predominantly by bacterial endotoxin called as bacterial lipopolysachharides (LPS). It cascade the dissociation of NF-κ and move into nucleus and upregulates the various cytokines, adhesion molecules, and cascade the COX-2 gene, which leads to keratinocyte hyperproliferation. Taking of diet which enriched with ω-3 fatty acid, it results to inhibit principal transcription factor (NF-κ) and to suppress the formation of TNF, IL, and many growth factors via to inhibit LPS. Another study revealed the use of ω-3 fatty acids in psoriasis patient shows signification suppression of NF-κ-induced inflammation [74, 75].

Challenges Face Toward Effective Delivery of Omega-3 Fatty Acids in Psoriasis Pharmacotherapy

As we know from above discussion, ω-3 fatty acids (EPA and DHA) have an important for good health. Lipid peroxidation of ω-3 fatty acids is serious issue, and it leads to destroy its therapeutic function [76]. Another challenge is their inappropriate absorption and metabolization after oral administration. The oxidation of ω-3 fatty acid depends on the fatty acid composition, physical forms, and colloidal states of the lipid [77]. The presence of pro-oxidants is enough to initiate oxidation such as singlet oxygen, transition metals, and lipoxygenase enzyme [78]. Antioxidant approaches have been applied to inhibit the oxidation of ω-3 fatty acids. Antioxidants may classify into natural and synthetic. Natural antioxidant application is wide over synthetic due to lesser toxicity associated with natural antioxidants. There are some examples of natural antioxidant including vitamin E, carotenoids, flavonoids, anthocyanins, and phenolic compounds; they act via blocking the free radical generation [79, 80]. Recently, Quarcetin have to found good oxygen radical absorbance capacity and inhibit lipid oxidation in marine oils enriched ω-3 fatty acids. Another study revealed that polyphenolic enriched apple skin extract has good potential to protect EPA and DHA against oxidation [81].

Scope of Nanomedicine in Effective Delivery of Omega-3-Fatty Acids

Oral metabolization and restricted permeation through skin are a great challenge in ω-3 fatty acids' drug delivery, and it may attribute due to availability of metabolic enzymes and barriers which prevents the availability of required amount of drugs to desired site [82, 83]. To overcome the limitations, emergence of nanomedicines has been investigated to improving the absorption of ω-3 fatty acids' drug delivery. Nanomedicines have size range of 1–1000 nm; they include nanoencapsulation, liposomes, microsphere, microemulsion, and nanoemulsion [84]. They provide the controlled drug delivery, better stability, and high drug payload with spectacular systemic and topical application. Moreover, their higher surface area to volume ratio results to improve the biopharmaceutical aspects of drugs. Furthermore, the smaller size of nanomedicine provides higher skin permeation and extends circulation time and delivers the active moieties at targeted sites via passive and active targeting [85]. The high degrees of unsaturation cause increase susceptibility of oxidation to obtain toxic hyperoxides which alters the lipophilicity and oral bioavailability of ω-3 fatty acids. Microencapsulation is an approach to prevent oxidation via encapsulation [86–88]. They enhance the characteristics of ω-3 fatty acid in terms of solubility, thermal stability, targeted drug delivery, and higher cellular uptake.

From the last decade, researchers have developed various lipid-based nanocarriers such as nanoemulsions, microemulsions, and self-emulsifying formulations to improving bioavailability of drugs [89]. Nanoemulsion is a nanosized lipophilic-based transparent emulsion, which composed of oil, water, surfactant, and co-surfactant. They have a droplet size less than 100 nm. Their higher surface area would responsible for higher solubility and better transport of drug [90]. Sharma et al. developed ω-3 fatty acids' nanoemulsion gel with the help of fish oil, which gives ω-3 fatty acids, unitop 100 as surfactant, PEG 400 as co-surfactant, and carbopol 971 as gelling agent. This formulation produces 48.76 % anti-inflammatory activity and adding of betamethasone dipropionate in fish oil and results

to synergize the anti-inflammatory action by 87.64 % [91]. Similarly, microemulsion also gained as potential nanocarriers in drug delivery via oral, topical, and nasal route. It composition is similar like nanoemulsion [92]. They are easy and low cost in preparation and improve the bioavailability of several drugs by various routes administration [93]. Baboota et al., developed ω-3 fatty acids, microemulsion gel by using of linseed oil as oil phase, and it shows higher skin permeation and can be better tool for the management of psoriasis [94]. Microspheres are nanocarrier with particle size of 1–1000 μm. It gains a novelty in drug delivery by safe and efficient targeted action [95]. Fish oil has been utilize for ω-3 fatty acid and has been loaded into microspheres with the help of wheat gluten as polymer by microencapsulation techniques employing double emulsification (O/W/O) method and subsequent heat polymerization. Further different ratio of fish oil loaded wheat gluten microsphere has been prepared and further optimized with the help of zeta potential, confocal microscopy, and scanning electron microscopy. Therefore, entrapment efficiency was found to 81.8 % at the ratio of 3:3 fish oil–wheat gluten microsphere [96]. However, surface characteristics of microsphere such as electrostatic property and porosity make it as better oral controlled release profile of FO-loaded microsphere and delayed oxidation in FO-loaded microsphere [96]. Marinosomes is a marine lipid-based liposome preparation and widely applicable in the prevention and the treatment of skin diseases. Marine lipid composed of 68 % w/w phosphatidylcholine (PC) and 23 % w/w of phosphatidyl ethanolamine, whereas PC contains 30–59 % EPA and DHA. Moreover, they characterized by transmission electron microscopy (TEM), and they found oligolamellar and multilamellar vesicle. Topical application of marinosomes shows optimum penetration of EPA and DHA and skin tolerance [97].

Conclusion

Omega-3 fatty acids exert beneficial effects prominently by altering the lipid composition, frequency of phosphorylation of PTK, downregulation of the signaling pathways like MAPK, ERK1/2, DAG, and T cell inhibition, which plays a key role in the pathogenesis of psoriasis. Targeting on various biomarkers by EPA-DHA and use of nanopharmaceuticals have provided improved efficacy of such novel treatment strategies against oxidation and facilitating improved absorption. Recent studies have revealed that EPA and DHA delivered through nanocarriers like nanoemulsion, microemulsion, and microsphere have helped in multifold augmentation in treatment efficacy. However, the need for increasing depth of research on nanomedicines is still needed on their safety and efficacy. In future, nanomedicines may become first-line approach in effective delivery of ω-3 fatty acids for the treatment of psoriasis.

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Pharmacokinetics and Safety Profile of Omega-3 Polyunsaturated Fatty Acids

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Introduction

Omega-3 long-chain polyunsaturated fatty acids (n-3 PUFA, with ≥ 20 carbon atoms) are a family of fatty acids (FA) that contain two or more double bonds, one of which is located three carbon positions from the methyl terminus ("omega-3" or "n-3"). The main n-3 PUFA in the diet are 5,8,11,14,17-eicosapentaenoic acid [EPA, 18:5(n-3)], 7,10,13,16,19-docosapentaenoic acid [DPA, 22:5(n-3)], and 4,7,10,13,16,19-docosahexaenoic acid [DHA; 22:6(n-3)]. Over the last few decades both epidemiological studies and randomized controlled trials (RCT) have shown that diets rich in EPA and DHA, obtained either from dietary sources or from pharmacological supplements, exhibit hypotriglyceridemic, anti-inflammatory, antithrombotic, vasodilatory, and antiarrhythmic properties and these pleiotropic effects are associated with a lower risk of cardiovascular diseases [1–6].

Fish is the main dietary source of n-3 PUFA. EPA, DPA, and DHA are abundantly present in the flesh of cold-water oily fish (mackerel, tuna, salmon, sturgeon, mullet, bluefish, anchovy, sardines, herring, trout, and menhaden), in shellfish-rich diets, and in fish oil supplements. Lean fish, such as cod, store lipid in their liver [7]. The various marine sources of n-3 PUFA differ in terms of absolute amounts and chemical binding type [8]. Fish and derived unrefined raw fish oils contained n-3 PUFA mainly esterified as either triglycerides (TG) or phospholipids (PL) and, to a lesser extent, due to a partial hydrolysis, as free fatty acids (FFA). In fish oil, EPA and DHA content comprise $\sim$30 % (18 and 12 %, respectively) of the FA present, which means that a 1 g fish oil capsule can provide $\sim$0.3 g of EPA + DHA [7]. Fish roe from herring, salmon, pollock, and flying fish contain between 38 and 75 % of their lipids in the form of PL and phosphatidylcholine (PC) as the predominant lipid class, whereas phosphatidylethanolamine (PE) is the second most abundant [8–10]. As expected, the content of n-3 PUFA in fish oils is highly variable depending on the fish (tuna oil is richer in DHA than EPA, while cod liver oil is richer in EPA than DHA) and seasonal conditions. Indeed, an analysis of eight commercially available capsules of fish oil-derived products found that the content of EPA and DHA ranged between 8.7 and 26.4 % and 8.9 and 17.4 % (wt%), respectively [11].

The initial preparations derived from unrefined raw oils extracted from oily fish or lean fish liver contained a mixture of TG with various FA. However, since fish oil contains a mixture of TG with various FA, the concentration of n-3 PUFA is relatively low, i.e., below the large amounts of fish oils required to achieve the therapeutic benefits derived from EPA and DHA. Higher concentrations of EPA and DHA can be reached using a supplement of unesterified FFA. However, FFA are highly susceptible to oxidation and their oral ingestion causes "fishy" taste, gastrointestinal complaints and can be potentially toxic due to the contaminants present (heavy metals-mercury, dioxin-like compounds and polychlorinated biphenyls) found in particular in fatty fish [12, 13]. This led to the development of formulations of n-3 PUFA containing high concentrations of purified EPA and DHA with low levels of contaminants in a fixed, predefined ratio, with higher oral bioavailability and reasonable patient compliance to be used for dietary supplementation and in controlled clinical studies [14]. The production process, involving purification, transesterification and low pressure distillation, allows greater dosing of n-3 PUFA than dietary fish oil supplements (up to 0.9–1 g per capsule), with undetectable levels of contaminants and a reduced "fishy" taste [13, 14]. Thus, these formulations are an effective way to increase n-3 PUFA intake without changing dietary habits [15, 16].

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These high concentrations of EPA and DHA are available in different formulations, such as natural (typically derived from fish oil) and reconstituted triglycerides (rTG), FFA (OM-3 FFA) or ethyl esters (OM-3 EE), the two latter forms being derived from natural sources of fish oil TG. Natural fish oil contain 100 % TG, whereas chemically reconstituted triglycerides (rTG) are a mixture containing TG as the main component (~50–60 %), diacylglycerides (DAG, 38–42 %), and monoacylglycerides (MAG, 1–3 %) [17]. The OM-3 FFA formulation is encapsulated and coated with a polyacrylate material that facilitates a delayed release in the gut, leading to a significant reduction in the incidence of gastrointestinal side effects [18]. The OM-3 EE and OM-3 FFA formulations are well tolerated and have clearly improved the safety profile of the initial fish oil formulations. In nTG, DHA is mainly located in the position sn-2, while EPA is in position sn-1 and sn-3 [19]. The re-esterification process adds, on average, one extra n-3 FA to each TG molecule, and in rTG, EPA and DHA are randomly distributed in the sn-2 and sn-1/2 positions of the glycerol backbone [19, 20]. This contrasts with mammalian marine oils (also rich in n-3 PUFA), where EPA, DPA, and DHA are preferentially located in the sn-1/3 positions in the TG molecule [21]. Krill oil (KO) is a novel source of n-3 PUFA extracted from the Antarctic crustacean krill (*Euphausia superba* and *Euphausia pacifica*). In krill, almost half (30-65 %) of the n-3 PUFA (including EPA and DHA) are incorporated into PL (35 % as PC and 16 % as PE in *E. superba* and 29 % and 26 % in *E. pacifica*, respectively) and FFA, whereas DPA was present in smaller amounts [8, 10, 22, 23]. The highest EPA and DHA content occurred in *E. superba* (EPA 15–21 %, DHA 9–14 %), although the proportion of PL in the total lipids of KO has been reported to vary between 30 and 60 %, depending on krill species, age, season, and harvest time [23]. A specific analysis of the composition of krill oil (Neptune Krill Oil™) used in the study of Schuchardt et al. [24] showed that, contrary to the manufacturers' indications, a large percentage of n-3 PUFA was present as FFA (22 % of the EPA content and 21 % of the DHA content). These differences in the composition and positions of n-3 PUFA have an important role in their absorption and bioavailability as discussed later in the chapter.

Synthesis Pathways of the n-3 PUFA

n-3 PUFA are essential dietary components for mammalian cells as they lack the enzyme delta-15 desaturase required to introduce the n-3 double bond into linolenic acid (LA, 18:2n-6) and synthesize de novo the precursor α-linolenic acid [ALA (9,12,15-octadecatrienoic acid; 18:3(n-3))]. Furthermore, mammalian cells can only partially convert ALA to EPA and DPA and to a very small extent to DHA. Thus, mammalian cells must obtain ALA which must be supplied from the diet. Plants, however, possess delta-15 desaturase and are able to synthesize ALA from LA. ALA can be found in green leafy vegetables and legumes; some plant oils (canola, echium, perilla, chia, soybean, linseed, flaxseed, and rapeseed), nuts (walnuts, hazelnuts, pecans), and seeds (flaxseed, linseed) are the main sources of ALA in the Western diet [25].

The metabolic conversion of ALA to longer, more unsaturated, n-3 PUFA (EPA, DPA, and DHA) involves a series of desaturation (introduction of a double bond) and elongation (addition of 2-carbon units) reactions occurring primarily in the hepatic endoplasmic reticulum [7, 25, 26] (Fig. 39.1). At this level, dietary ALA is first metabolized to stearidonic acid (SDA; 18:4n-3) by delta 6-desaturase. Then, SDA can be elongated to form eicosatetraenoic acid (ETA, 20:4n-3; 8,11,14,17-eicosatetraenoic acid) that can be desaturated by *delta* 5-desaturase to form EPA which can be further elongated to form DPA. Then, DPA, via elongase and delta-6-desaturase, is converted into tetracosahexaenoic acid (24:6n-3), which is translocated from the endoplasmic reticulum to the peroxisome where two carbons are removed by β-oxidation to generate DHA. The intermediates of the n-3 PUFA generated during these enzymatic steps can either be incorporated into PL or become the substrate for a further elongation/desaturation reaction. DHA can also be a substrate for metabolic retroconversion to EPA and DPA by peroxisomal acyl-CoA oxidase and one cycle of β-oxidation [25–27]. In young rats, DPA is predominantly retroconverted to EPA and is incorporated into PL pools [28], and short-term supplementation with pure DPA significantly increases the hepatic concentration of DHA and the EPA concentration in the liver, heart, kidney, adipose tissue, and skeletal muscle, presumably by the process of retroconversion [29]. In humans, the calculated retroconversion rate of DHA to EPA and DPA following the ingestion of ^{13}C-DHA is relatively low (1.4 %), i.e., insufficient to provide the preventive and therapeutic benefits associated with EPA and DHA [30]. However, human retroconversion rates increase up to 12 % following high chronic DHA consumption [31] and in an essential FA-deficient cell line (EPC-EFAD) [32]. Retroconversion of DHA to EPA is also hormonally regulated, decreasing in women receiving hormone replacement therapy [33]. DHA biosynthesis may be impaired in diseases such as retinitis pigmentosa [34] and is modified in smokers [35].

Short-term studies and intervention studies evaluating the FA composition of circulating lipids in healthy volunteers supplemented with high doses of ALA in fish oils, supplements or foods found that the enzymatic conversion

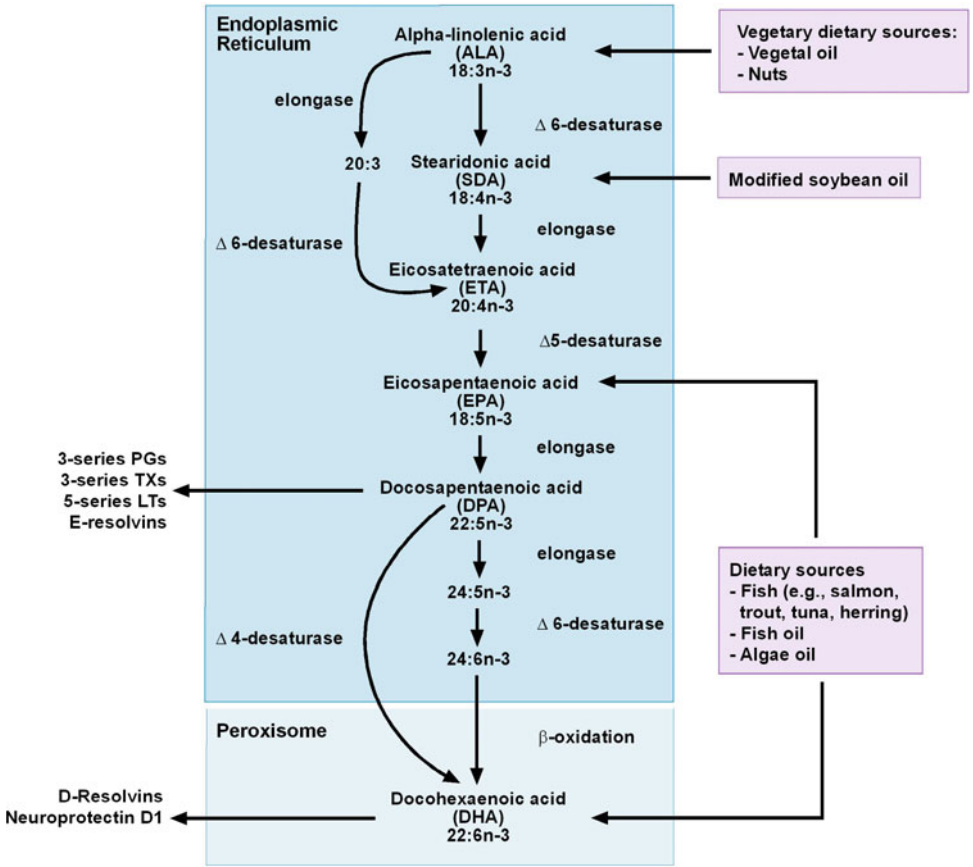

Fig. 39.1 Biochemical pathways for the interconversion of n-3 long-chain polyunsaturated fatty acids (n-3 PUFA). Mammals convert ALA to n-3 PUFA (EPA and DHA) via a series of desaturation and elongation reactions in the liver endoplasmic reticulum (ER). However, the synthesis of DHA from 24:6n-3 requires the synthesis of 24:6n-3 in the ER and its passage into the peroxisome where they undergo one cycle of beta-oxidation to produce DHA

efficiencies of ALA to EPA, DPA, and DHA vary considerably among species and appear to be relatively inefficient in individuals who consume a typical Western diet, particularly in men [14, 27, 36–41]. Several studies have analyzed the conversion of ALA to EPA, DPA, and DHA in humans using labeled [^{13}C]- or [^{2}H]-ALA as a tracer [41–44]. These studies showed that ALA present the highest rate of β-oxidation among all n-3 PUFA, so that $\geq$15–35 % of dietary ALA is rapidly catabolized to CO_2 for energy [36–38, 45, 46], and only a small proportion (<1 %) is converted to DHA [40, 47]. Thus, it has been calculated that the overall amount of DPA and DHA converted from ALA is about 0.13 and 0.05 % of the starting ALA, respectively. Furthermore, diets high in ALA increase the rate of its β-oxidation, reduce its accumulation in plasma, and decrease its conversion to EPA and DHA [38, 45]. Thus, ALA cannot reliably replace EPA and, particularly, DHA in the diet, and it is unlikely that increased consumption of ALA might produce a significant benefit in improving the overall n-3 fatty acid status and health outcomes in humans [26]. Thus, the most effective strategy to attain higher plasma concentrations of EPA and DHA is to optimize their oral absorption and bioavailability. However, in n-3-deficient patients an increased ingestion of ALA markedly increases the plasma concentrations of both EPA and DHA [48].

Although in mammals n-3 and n-6 fatty acids cannot be interconverted, the same series of desaturases and elongases involved in the conversion of ALA to EPA are also implicated in the conversion of LA into its more unsaturated derivatives [e.g., arachidonic acid or AA; (5*Z*,8*Z*,11*Z*,14*Z*)-5,8,11,14-eicosatetraenoic acid; (20:4n-6)]. Therefore, the conversion of ALA to EPA competes with the conversion of LA to AA, so that background diet can influence the conversion of ALA into long-term n-3 PUFA. In fact, in young adult males, the conversion of deuterated ALA into its long-chain derivatives was reduced by 40–54 % when dietary intake of LA increases from 15 to 30 g/day [49].

Factors that modulate the interconversion of n-3 PUFA. Several factors can influence the activities of delta 6- and delta 5-desaturases, including age, diet (dietary n-3/n-6 PUFA content), hormones (insulin, estrogens), oxidative stress, liver disease, and feedback inhibition by end products [7, 35–39, 45, 47, 50, 51]. Thus, the conversion of ALA to n-3 PUFA may be more efficient in infants than in adults. A recent study in 22 preterm infants showed that an average of 42 % of DHA was biosynthesized from ALA in 1-month-old infants

consuming formulas with 0.64 % w/w DHA. This drops to 11 and 7 % at 3 and 7 months of age [52]. Another two studies found an increase in plasma PL DHA contents [53] and in both PL and red blood cell (RBC) DHA content [54] when ALA was added to infant formulas that had no n-3 PUFA. Moreover, dietary supplementation with EPA and DHA reduced the conversion of ALA to DHA by 70 % [38, 40, 45].

Males and females differ in the extent to which ALA is converted into EPA and DHA [55]. Tracer studies using [^{13}C]- or [^{2}H]-ALA and subsequently collecting blood and breath samples over a variable period of time (24 h to 3 weeks) found that young women (mean age 28 years) convert a greater proportion of ALA into EPA and DHA compared with men (mean age 36 years) [36, 37]. In men, these studies showed that $\geq$15–35 % of dietary ALA is rapidly catabolized to carbon dioxide for energy [56], so that the fractional conversion of ALA to EPA ranges between 0.3 and 8 % and the conversion of ALA to DHA is even lower and often is undetectable (<1 %) [27, 36–40, 42, 44, 45, 47, 49–51]. This explains why increased consumption of ALA is largely ineffective to raise the plasma levels of DHA, to decrease plasma TG levels [57], and to provide the cardiovascular benefits associated with EPA and DHA [26, 56]. Conversely, conversion of ALA to n-3 PUFA is more efficient in women. In young women, the conversion of ALA to DPA increases up to 21 % and up to 9 % is converted to DHA, whereas the rate of ALA oxidation is significantly reduced ($\sim$22 vs. $\sim$33 % in men) [36–38]. Thus, women have lower circulation concentrations of EPA and DPA but higher circulating DHA levels than men, and this difference is independent of dietary intake [55, 58, 59]. These differences can be related to gender differences in β-oxidation, subcutaneous adipose tissue composition and mobilization, and/or greater activity of desaturase and elongase enzymes involved in the synthesis of n-3 PUFA by estrogens [36, 44, 50, 55]. In fact, women using oral contraceptives have higher rates of DHA synthesis and higher concentrations of DHA in plasma CE than those who did not, whereas parenteral testosterone decreases DHA levels in female-to-male transsexual subjects [37, 58, 59]. Moreover, estrogen-based hormone replacement therapy in postmenopausal women increases the concentrations of ETA and DHA in plasma CE [55]. The greater capacity of women for ALA conversion may be of interest in pregnant women to meet the demands of the fetus for DHA ($\sim$400 mg/week during the third trimester). Since desaturase activities in developing human liver are lower than in adults, assimilation of DHA by the fetus came primarily by supply of DHA by the mother [26, 60]. Of note, during pregnancy circulating estrogen plasma levels increased due to the synthesis and secretion by the placenta and plasma PC DHA concentrations increased by $\sim$33 % between 16 and 40 weeks of gestation [61]. Thus, it is possible that the increase in estrogen circulating concentrations may up-regulate ALA conversion to DHA during pregnancy.

Pharmacokinetic Properties

As with other nutrients, the digestion and absorption, distribution in the circulation and body tissues, biotransformation, and excretion can differ among the different n-3 PUFA contained in different chemical macromolecules, including FFA, natural and reconstituted TG and EE. In general, tissue distribution of n-3 PUFA remains unknown for ethical reasons, but caution should be exercised before extrapolating preclinical data to humans. Additionally, the pharmacokinetic information is scarce in patients with hepatic or renal disease and in pregnant women and is not available for children.

Digestion of n-3 PUFA

The absorption of PUFA is a complex process that is subject to considerable interindividual variability [8]. Approximately 90–95 % of dietary lipids ingested from foods consist of TG, where three fatty acids (FFA) are esterified to a glycerol backbone. TG undergoes lipolysis by several lipases in the gastrointestinal tract prior to absorption (Fig. 39.2). Lipid digestion is initiated in the oral cavity and continues in the stomach, where lipids are hydrolyzed by both lingual and gastric lipases [62]. The stomach is also the major site for the emulsification of dietary fats. The mechanical effects of peristalsis produce small partially emulsified fat droplets with hydrophobic TG cores surrounded by polar molecules, including PL, cholesterol (CL), FA, and ionized proteins [63]. Emulsification continues in the upper small intestine where peristaltic movements further reduce the fat droplets and TG are mixed with bile salts and pancreatic juice containing lipid digestive enzymes [pancreatic lipase and its cofactor colipase, carboxyl ester lipase, and phospholipase A2 (PLA2)] to form an aqueous suspension of small fatty droplets to maximize exposure to pancreatic lipases for lipid hydrolysis [63]. Pancreatic lipase hydrolyzes TG ester bonds in the small intestine presenting a high specificity for the sn-1,3 positions of the glycerol backbone as compared with the sn-2 position [64] leading to the release of non-esterified FFA and 1- and 2-monoacylglycerols (MAG) and small amounts of diacylglycerols (DAG) which are incorporated into mixed micelles before passive diffusion from the intestinal lumen across the apical membrane of the enterocytes via various FA-transport proteins, including FA-binding protein, CD36, and FA-transport protein-4 (FATP4) [20, 65–67]. The reduced expression of FATP4 in the apical membrane of small

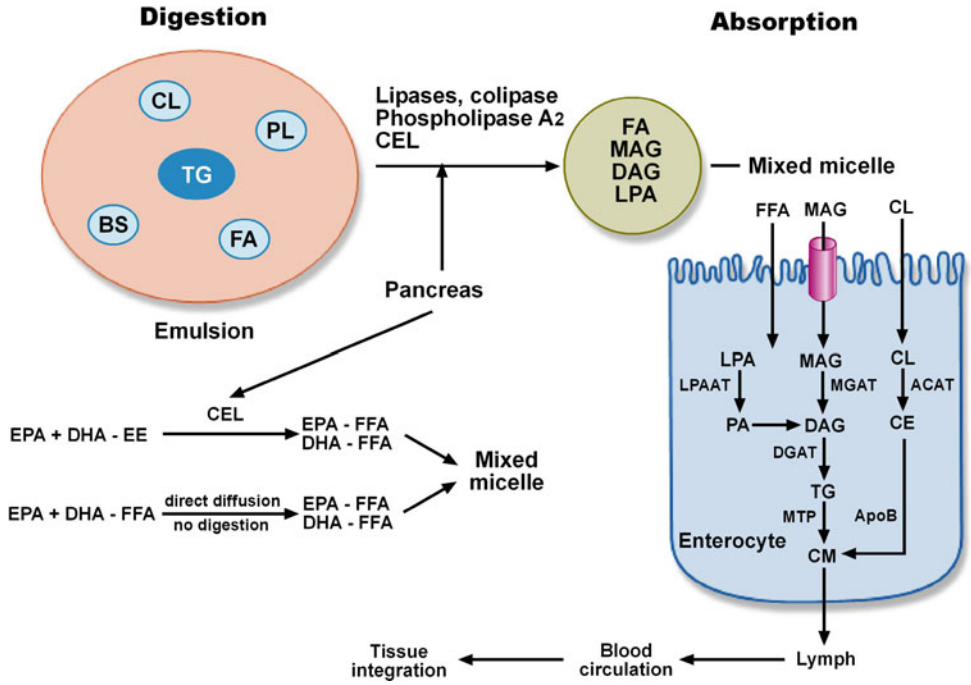

Fig. 39.2 Digestion and absorption of EPA and DHA from n-3 PUFA as ethyl esters (EE) or free fatty acid (OM-3 FFA) formulations (modified from Refs [63, 67]). Abbreviations: *ACAT*, acyl-CoA:cholesterol acyltransferase; *apoB*, apolipoprotein B; *BS*, bile salts; *CE*, cholesteryl esters; *CEL*, carboxyl ester lipase, also known as bile salt-dependent lipase (or BSDL); *CL*, free cholesterol; *CM*, chylomicrons; *DAG*, diacylglycerol; *DGAT*, diacylglycerol acyltransferases; *EE*, ethyl esters; *FA*, fatty acids; *FFA*, free fatty acids; *GPAT*, glycerol-3-phosphate acyltransferase; *LPA*, lysophosphatidic acid; *LPAAT*, LPA acyltransferase; *MAG*, monoacylglycerol; *MGAT*, monoacylglycerol acyltransferases; *MTP*, microsomal triglyceride transfer protein; *PA*, phosphatidic acid; *PAP*, phosphatidic acid phosphorylase; *PL*, phospholipids; *TG*, triacylglycerol

intestine mucosal enterocytes decreases the uptake of PUFA by 50 % [68]. Dietary EPA and DHA, either as sn-2-MAG or as FFA released from the sn-1 and sn-3 positions by pancreatic lipase, depending on the molecular structure of dietary TG, are taken up by enterocytes in the form of mixed micelles via passive diffusion [20]. In contrast to TG which are highly hydrophobic, PL are hydrophilic due to the polar headgroup and they are not hydrolyzed by lingual or gastric lipases and are not dependent on bile salts to form micelles. They are mainly hydrolyzed in the upper segment of the jejunum by pancreatic PLA2 which releases the FAA from the *sn*-2 position that can also be taken up into the enterocytes.

The n-3 PUFA EE formulations (OM-3 EE) are up to 50 times more resistant than the natural TG form to pancreatic lipase hydrolysis and to be absorbed require an additional hydrolysis by the carboxyl ester lipase, to be converted into EPA and DHA free fatty acids (EPA-FFA and DHA-FFA) before they can be incorporated into mixed micelles and transported into the enterocyte [63, 67, 69–72]. Therefore, digestion and absorption of OM-3 EE are considerably slower than TG and PL, and both processes are highly dependent on the fat meal content, which stimulates pancreatic enzyme activity and, thereby, enhances absorption [72–75]. In contrast, the FFA forms of EPA and DHA (EPA-FFA and DHA-FFA) are not dependent on pancreatic lipase activity and do not require an additional hydrolytic step to be directly incorporated into the micelle for absorption and, thus, present an improved bioavailability that is less dependent on meal fat content [71–74, 76].

Once inside the enterocyte and before free and MAG-bound FAA can be transferred to the lymph and the blood, up to 75 % of TG are re-esterified in the endoplasmic reticulum by the monoacylglycerol acyltransferase (MGAT) into DAG (monoacylglycerol pathway). PL from the diet and bile, mainly lysophosphatidic acid (LPA), are acylated by the 1-acyl-glycerol-3-phosphate acyltransferase (AGPAT) to form phosphatidic acid, which is also converted into DAG (glycerol phosphate pathway). DAG is then converted by the diacylglycerol acyltransferase (DGAT) into TG [77]. Dietary cholesterol is acylated by acyl-CoA:cholesterol acyltransferase (ACAT) to cholesteryl esters (CE). Then, and facilitated by microsomal triglyceride transfer protein (MTP), the TG are incorporated together with dietary cholesterol and a small amount of CE into chylomicrons particles with a surface layer of PL to which is

attached a single molecule of the truncated form of apoB (apoB48) which are released into the lymph through the basolateral membrane and, finally, enter the bloodstream via the thoracic duct. Chylomicron TG arising via the monoacylglycerol and the phosphatidic acid pathways differ mainly in the composition of the FA in the sn-2 position [78]. The similarity in the FA of the sn-1 and sn-3 positions of the chylomicron TG from rats fed oil or ester is consistent with a hydrolysis of the acylglycerol products of the phosphatidic acid pathway to 2-MAG prior to reconversion to TG via the monoacylglycerol pathway and secretion as chylomicrons. Ikeda et al. [75] proposed that inefficiency in the lipolysis of EPA and DHA can delay or lower the transfer of MAG and FFA into the enterocyte, limiting the supply/availability of 2-MAG necessary for the re-synthesis of TG for incorporation into chylomicrons. Once in the circulation, chylomicrons are catabolized by the endothelial-bound lipoprotein lipase (LPL), an enzyme which hydrolyzes between 70 and 90 % of total TG content, allowing the delivery of FFA into the skeletal muscle for energy production and adipocytes for storage [79]. The resultant chylomicron remnant particles are finally cleared from the circulation by the low-density lipoprotein (LDL) receptors in the liver where fatty acids, including n-3 PUFA, are either stored or released to the circulation as very low-density lipoproteins (VLDL) to be stored in extrahepatic tissues (skeletal muscle, adipose tissue, RBC, and so forth).

Oral Bioavailability

One strategy to raise plasma concentrations of n-3 PUFA is to optimize their absorption and bioavailability. The term bioavailability refers to the rate and extent to which a nutrient is absorbed from a drug product and becomes available at the site of action. It can be determined by measuring the peak plasma concentration (C_{max}) and the area under the concentration curve over time (AUC), respectively.

Christensen et al. [21] compared the absorption of EPA and DHA in mesenteric lymph duct-cannulated rats following intragastric administration of two oils with different intramolecular TG structures. In one oil presented a specific TG structure with EPA and DHA located in the sn-2 position (specific M-n3-M), whereas other oil had a random FA distribution (random M-n3-M). The lymphatic transport of EPA and DHA increased following intragastric administration of specific M-n3-M compared with random M-n3-M, suggesting that specific M-n3-M with EPA and DHA predominantly in the sn-2 position of the TG was a more readily absorbed source of EPA and DHA. Similarly, Sadou et al. [19] found that after consumption of fish oil, DHA incorporates faster than EPA in plasma TG because DHA was mostly on sn-2 and EPA on sn-1,3 positions. Furthermore, measurement of the cumulative concentration of labeled ALA in stool collected over 5 days following ingestion of 750 mg [^{13}C]-ALA showed that in healthy volunteers and in patients with hypertriglyceridemia, non-esterified n-3 PUFA present in the diet were rapidly and efficiently absorbed (>96 %) [26]. This percentage was comparable to the uptake of ALA (98 %) in patients with ileostomies who were fed with six breakfasts of 5–100 g linseed oil [80].

However, there is evidence that oral availability of n-3 PUFA (EPA and DHA) can be very different in foods and in different chemical formulations (TG, rTG, EE, and FFA) and, additionally, is subject to considerable interindividual variability [8]. Köller et al. [81] evaluated the effect of a convenience drink enriched with 200 mg EPA and 300 mg DHA in 190 subjects with atherosclerotic disease with a n-3 index <5 %. After 8 weeks they showed that the mean n-3 index increased from 4.37 to 6.80 but the interindividual variability in response was high (between 4.3 and 11.8 %), although the study population was rather homogeneous.

The n-3 index measures the content of EPA + DHA in RBC membranes because they are composed almost exclusively of PL, so that purification steps are unnecessary. It has been demonstrated that there is a strong correlation between the combined concentrations of DHA and EPA in RBC membranes (n-3 index) and in plasma as well as in cell membranes of other tissues, like heart, liver, and kidney [82, 83]. Moreover, Carver et al. [84] found that among subjects $\leq$18 years of age, there was no significant relationship between cerebral cortex and RBC FA levels, while in those >18 years there was a significant relationship between brain and RBC levels for several FA. Indeed, the uptake of PUFA by RBC may be considered as a marker for their accretion into brain [85] and retina [86]. Thus, the n-3 index is a biomarker of long-term EPA and DHA status providing useful information on whether the body is receiving an adequate supply of n-3 PUFA [87].

Clinical Trials

Two different types of studies have been performed in humans to assess the oral bioavailability of n-3 PUFA (Table 39.1): short-term (postprandial, i.e., 24–72 h) and longer-term (from 4 weeks to 6 months) interventions (for a review, see 8,88).

Short Clinical (Postprandial) Trials

In most of these studies, EPA or EPA plus DHA were administered at daily doses ranging from 1 to 3.1 g to healthy [24, 72–74, 76, 89, 90] or to overweight subjects [67], doses which are clearly above the normally recommended intake levels. Oral bioavailability of n-3 PUFA was determined by measuring the content of n-3 PUFA in plasma

Table 39.1 Short-term (posprandial) and long-term human studies on the availability of several formulations of n-3 PUFA (modified from Ref. [8])

Authors (Reference)	Study	n	Duration	Test formulation	Doses of EPA + DHA (g/d)	Blood sampling	Result
Short-term (postprandial) studies							
El Boustani et al. [76]	–	8	24 h	FO: TG vs. FFA vs. EE	1 EPA	Plasma-TG	FFA > TG > EE
Lawson and Hughes [73]	CO	8	8 h	FO: TG vs. FFA vs. EE	1 EPA, 0.67 DHA	Plasma-TG	FFA > TG > EE
Lawson and Hughes [74]	R, PG	16	12 h	FO: TG vs. EE	1 EPA, 0.65 DHA	Plasma-TG	TG > EE
Beckermann et al. [72]	CO	8	32 h	FO: TG vs. EE	TG: 1.68 EPA, 0.72 DHA FFA: 1.35 EPA, 1.05 DHA EE: 1.86 EPA, 1.27 DHA	Plasma-TG	FFA > TG > EE
Nordoy et al. [90]	PC, CO	5	5	FO: rTG vs. EE	28g TG: 14EPA, 8 DHA 28 g EE: 13.7 EPA, 10.4 DHA 28 g EE + 12 g olive oil	EPA+DPA Peak in chylomicrons	TG = EE No difference
Wakil et al. [89]	DB, CO	7	24 h	FO: nTG vs. rTG vs. eTG vs. eDG vs. eMG	0.9 EPA, 0.35 DHA	Plasma	rTG > nTG > eTG, eDG, eMG
Schuchardt et al. [24]	DB, CO	12	72 h	FO-rTG vs. FP-EE vs. KO	rTG: 1 EPA, 0.672 DHA EE: 1 EPA, 0.672 DHA KO: 1.05 EPA, 0.63 DHA	Serum-PL	KO > rTG > EE
Davidson et al. [67]	R, OL, CO	54	24 h	OM-3 FFA OM-3 EE	FFA. 2.2 EPA, 0.8 DHA EE: 1.7 EPA, 1.38 DHA	Plasma	FFA > EE
Long-term studies							
Reis et al. [95]	R, DB, PC, PG	89	6 mo	FO: TG vs. EE	EE: 3.7 EPA, 2.5 DHA TG: 3.4 EPA, 1.4 DHA 12 g olive oil	Plasma-PL	No difference
Hansen et al. [71]	R, DB, PC	31	7 wk	FO: TG vs. EE	EE: 2.2 EPA, 1.2 DHA TG: 2.2 EPA, 1.4 DHA	Plasma-PL Plasma-CE	TG > EE
Krokan et al. [93]	PG	40	2 wk	FO: rTG vs. EE	EE: 2.2–7.7, EPA, 1.2–4.2 DHA TG: 2.2–4.4 EPA, 1.4–2.9 DHA	Total serum lipids Serum-PL	No difference
Visioli et al. [98]	–	16	6 wk	Fish vs. FO-EE	Fish: 0.15 EOA, 0.54 DHA EPA: 1.29 and 2.58 DHA: 0.96 and 1.92	Plasma	Fish > FO-EE
Cao et al. [102]	R, SB, G	20	2 mo	FO vs. LO	FO: 1.296 EPA, 0.86 DHA LO: 3.51 AL, 0.9 LA	n-3 index Plasma PL	FO > LO
Elvevoll et al. [99]	–	71	8 wk	FO: smoked salmon vs. cooked salmon vs. cooked cod vs. cod liver	Salmon or cod liver: 400 g/wk Cod liver oil: 15 ml/day	Plasma	Fish > FO

(continued)

Table 39.1 (continued)

Authors (Reference)	Study	n	Duration	Test formulation	Doses of EPA + DHA (g/d)	Blood sampling	Result
Harris et al. [100]	–	23	4 mo	Fish vs. FO	0.45 EPA + DHA	n-3 index	Fish = FO
Maki et al. [103]	R, DB, PG	76 obese	4 wk	KO vs. FO	KO: 0.216 EPA, 0.09 DHA FO: 0.212 EPA, 0.18 DHA	Plasma FA	No difference
Dyerberg et al. [17]	R, DB, PG, PC	72	14 d	FO: FBO vs. rTG vs. FFA vs. EE vs. CLO	rTG: 1.85 EPA, 1.29 DHA FFA: 2.18 EPA, 1.4 DHA EE: 1.87 EPA, 1.39 DHA FO: 2.04 EPA, 1.48 DHA CLO: 1.38 EPA, 1.87 DHA	Serum-TG, CE and PL	rTG > FDO = DLO = FFA > EE
Neubronner et al. [104]	R, DB, PC	129	6 mo	FO: rTG vs. EE	rTG: 1.01 EPA, 0.67 DHA EE: 1.01 EPA, 0.67 DHA	n-3 index	rTG > EE
Ulven et al. [105]	O, R, PG	113	7 wk	KO vs. FO	KO: 0.543 EPA + DHA FO: 0.864 EPA + DHA	Plasma	KO > FO
Ramprasath et al. [96]	R, DB, PC, CO	24	8 wk	KO vs. FO	KO: 0.6; FO: 0.6	Plasma and RBC FA	KO > LO
Offman et al. [101]	OL, PD	52	2 wk	OM-3 FFA vs. OM-3 EE	OM-3 EE: 0.465 EPA, 0.375 DHA OM-3 FFA: 0.55 EPA, 0.2 DHA	Plasma	FFA > EE
Laidlaw et al. [97]	R, OL, CO	35	4 wk	FO	rTG: 0.65 EPA, 0.45 DHA EE: 0.756 EPA, 0.228 DHA KO: 0.15 EPA, 0.09 DHA TG: 0.18 EPA, 0.22 DHA	Plasma	rTG FO > EE FO > TG salmon > PL KO

*: given as natural triglyceride, reconstituted triglyceride, enzymatically synthesized triglyceride, monoglyceride and diglyceride
AL alpha-linolenic acid, *CLO* cod liver oil, *CO* cross-over, *d* day, *DB* double-blind, *h* hour, *OL* open label, *PG* parallel groups, *R* randomized, *wk* week, *FA* fatty acid, *FFA* free fatty acids, *FO* fish oil, *EE* ethyl esters, *LA* linoleic acid, *LO* linseed oil, *nTG* natural triglycerides, *PD* parallel design, *PL* phospholipids, *REM* emulsion formulation, *RF* reference formulation, *TG* triglycerides, *rTG* reesterified triglycerides

lipid fractions (TG, PL, FFA, CE) or RBC lipids, after administration of a single (or multiple) doses of n-3 PUFA in different chemical formulations. In general, the bioavailability of EPA and DHA as FFA formulations was higher as compared with TG or EE formulations, and higher for the TG and rTG form compared with the EE form. Thus, the bioavailability of EPA + DHA in rTG > FFA > TG > EE [8, 17, 24, 67, 72–74, 76, 88–90].

El Boustani et al. [76] administered EPA (1 g) in four different chemical forms and analyzed the kinetics of EPA incorporation into plasma TG. They found that incorporation into plasma TG was markedly smaller and later when EPA was administered as EE rather than as FFA. Lawson and Hughes [73] used the transient rise in plasma TG fatty acids after single-dose ingestion of fish oil as TG, FFA, or EE with linseed oil to determine the relative absorption of fish oil fatty acids in 8 volunteers. As free acids, the fish oil FA were well absorbed ($\geq$95 %). As TG, EPA (1 g) and DPA (0.67 g) were absorbed only 68 and 57 % as well as the FA. The EE were absorbed only 20 and 21 % as well as the FA. The incomplete absorption of EPA and DPA from fish oil TG correlates well with known in vitro pancreatic lipase activity. A double-blind study compared the bioavailability of 20 % EPA in 4.5 g of natural fish oil TG (nTG), rTG, enzymatically synthesized triglyceride (eTG), monoglyceride (MAG), and diglyceride (DAG) [89]. Seven healthy volunteers were given the supplements on 5 occasions and the area under the curve for the next 24 h ($AUC_{0–24h}$) was calculated. Over a 24-h period, there was a significant difference between the mean of $AUC_{0–24h}$ of EPA from rTG (30.2) and that of the eTG and MAG (11.9 and 13.4, respectively, $P < 0.05$) (Table 39.1). Thus, EPA bioavailability from rTG was higher than that obtained from eTG and MAG alone, but was not significantly different from that of DAG and natural TG. The higher bioavailability of rTG compared to eTG may be due to the rTG components (DAG and MAG) facilitating the intestinal phase digestion and acting as emulsifying agents in the stomach and thereby increasing the absorption and bioavailability from rTG [91]. In the case of nTG versus eTG, since both forms are almost 100 % TG and the only difference is that EPA is mainly located in positions sn-1 and 3 in nTG whereas it is randomly distributed in all three glycerol positions in the eTG, this implies that short-term bioavailability of ePA in the positions sn-1 and 3 of the glycerol backbone is higher than in sn-2. This is possibly related to the accessibility of sn-1 and 3 FA to pancreatic lipase compared to the inaccessibility of sn-2 FA, although other explanations are plausible.

Dyerberg et al. [17] compared three concentrated preparations (EE, FFA and rTG) with placebo oil in a double-blind design, and with fish body oil and cod liver oil in single-blind arms. Seventy-two volunteers were given 3.3 g of EPA plus DHA daily for 2 weeks. Bioavailability of EPA + DHA from rTG was superior (124 %) compared with natural fish oil, whereas the bioavailability from EE was inferior (73 %). FFA bioavailability (91 %) did not differ significantly from nTG and the stereochemistry of FA in acylglycerols did not influence the bioavailability of EPA and DHA. A tentative explanation for an even higher index of bioavailability of rTG than of natural TG could be that rTG contains both DAG and MAG molecules, which may facilitate micellar formation and thus enhance the LC n-3 FA absorption into the enterocyte.

In healthy volunteers and in patients with hypertriglyceridemia following a single dose of OM-3 EE (Lovaza, containing ~465 mg of EPA and ~375 mg of DHA), the maximal incorporation of EPA into TG occurred after 4–6 h [73, 74, 76, 90]. However, Epanova (containing 1 g of fish oil-derived OM-3 FFA, EPA and DHA being the most abundant) is directly absorbed in the small intestine. Following repeat dosing with Epanova (4 g/day) under low-fat meal conditions for 2 weeks, the C_{max} were achieved between 5 and 8 h after dosing for total EPA and between 5 and 9 h after dosing for total DHA and steady state plasma concentrations of EPA and DHA within 2 weeks [92]. The ECLIPSE (*Epanova® compared to Lovaza® in a pharmacokinetic single-dose evaluation*) trial, a randomized, open-label, single-dose, 4-way crossover study, compared the bioavailability of Epanova and Lovaza administered during periods of low-fat and high-fat (20 g) consumption to 54 overweight adults [67]. Bioavailability was determined by the ln-transformed $AUC_{0–t}$ during a 24-h interval for EPA and DHA (baseline-adjusted). The $AUC_{0–t}$ for total EPA + DHA during the low-fat period was 4.0-fold greater with OM-3 FFA compared with OM-3 EE, while during the high-fat period, $AUC_{0–t}$ for OM-3 FFA was approximately 1.3-fold greater than OM-3 EE. The individual EPA and DHA plasma concentration–time profiles showed 9.5-fold and 2.0-fold improved bioavailability, respectively, with OM-3 FFA during the low-fat period compared with OM-3 EE (Tables 39.1 and 39.2). During the low-fat period, 59 % of subjects dosed with OM-3 FFA maintained an $AUC_{0–t}$ that was $\geq$50 % of the respective high-fat $AUC_{0–t}$ (vs. 6.0 % of subjects dosed with OM-3 EE). Moreover, individual subject responses demonstrated the superior predictability of OM-3 FFA over OM-3 EE when switching between the low-fat and high-fat consumption periods.

Conversely, other studies showed an equal absorption of n-3 PUFA in the form of either TG or EE [90, 93, 94]. Luley et al. [94] compared the absorption of EPA and DHA from natural fish oil TG with that from EE at two different levels of EPA and DHA (54 and 35 %) and found no difference in

Table 39.2 Pharmacokinetic parameters in several human studies

Authors (Reference)	n/duration	Test formulation	Dose/day	Parameter		
				T_{max} (h)	C_{max}	AUC_{0-t}
Davidson et al. [67]	51/24 h	OM-3 FFA, low-fat	4 g, single dose of Epanova or Lovaza	8.1 (22.6**)	225.8 nmol/mL*	2650.2 nmol h/mL*
		OM-3 EE, low-fat		10.2 (11.6**)	61.1 nmol/mL*	661.9 nmol h/mL*
		OM-3 FFA, high fat		6.0 (20.9**)	543.2 nmol/mL*	4604 nmol h/mL*
		OM-3 EE, high fat		6.2 (19.0**)	401.9 nmol/mL*	3589 nmol h/mL*
		EPA-FFA, low-fat			39.1 μg/mL	465.4 μg h/mL
		EPA-EE, low-fat			4.7 μg/mL	48.8 μg h/mL
		DHA-FFA, low-fat			30.1 μg/mL	337.0 μg h/mL
		DHA-EE, low-fat			15.1 μg/mL	163.0 μg h/mL
Offman et al. [101]	52/14 days	EPA + DHA EE	4 g	6.0	206.7 nmol/mL	3320 (75.8) L
		EPA + DHA FFA		6.0	1350 nmol/mL	19100 (34.2) nmol h/mL
		EPA-EE EPA-FFA		6.5 6.0	34.2 μg/ml 295.0 μg/mL	576 (65.7) μg h/mL 4230 (33.4) μg h/mL
		DPA-EE		6.0	30.6 (68.3) μg/mL	537 (90.5) μg h/mL
		DPA-FFA		6.0	124.1 μg/mL	1660 (41) μg h/mL
Wakil et al. [89]	7/24 h	EPA from rTG EPA from nTG EPA from eTG EPA from MG	4.6 g containing 20 % EPA	8 8 8 6		30.2 (6.2)[a] 25.3 (11.2)[a] 11.9 (12.8)[a] 13.4 (12.5)[a]
Wakil et al. [119]	6/ 24 h	EPA EE EPA EE with exines	4.6 g from fish oil containing 20 % EPA	6.8 7.6		19.7 (4.3)[a] 2 (1.4)[a]
Rusca et al. [106]	49/28 days	EPA-EE from Seacor and IBSA	1 g/day Seacor: 45.5 %, EPA 40.7 % DHA IBSA: 43.4 % EPA, 45.5 % DHA	5.3 (3.6)[a] 4.6 (3.5)[a] 7.2 (0.9)[a] 5.9 (2.2)[a]	98.1 μg/mL 102.5 μg/mL 66.0 μg/mL 67.4 μg/mL	2090.0 μg day/mL 2295.7 μg day/mL 1103.3 μg day/mL 1092.4 μg day/mL
Galli et al. [107]	40/24 h	EPA-EE from Seacor or IBSA	12 /day Seacor: EPA 485 mg, DHA 348 mg IBSA: EPA 434 mg, DHA 434 mg	10.5 (7.8)[a] 11.1 (7.36)[a] 11.5 (9.4)[a] 11.8 (9.3)[a]	7.0 mg/L 5.70 mg/L 11.0 mg/L 9.2 mg/L	98.8 mg/L x h 92.8 mg/L x h 128.0 mg/L x h 136.4 mg/L x h

* Geometrical means. ** $t½$: apparent first-order terminal elimination half-life calculated as 0.693/Kel

[a]Mean (standard deviation)

AUC area under the plasma drug concentration–time curve, C_{max} peak plasma concentrations, *H* hours, t_{max} time to reach C_{max}

absorption. Similar results were reported by Krokan et al. [93] who found that the absorption of EPA and DHA from synthetic EE rich in EPA and DHA were fully comparable to that of natural TG containing smaller amounts of these FA. Nordoy et al. [90] compared the content of n-3 FA in chylomicron TG and the increase in chylomicron TG after a test meal including a large dose of n-3 FA provided either as a rTG (14 g EPA + 8 g DHA) or as an EE formulation (13.7 g EPA + 10.4 g DHA in EE form). They found an equally similar absorption of n-3 PUFA from EE and rTG, despite a lower rate of hydrolyses by intestinal lipase of FA from EE than from TG in vitro. However, the results of this study can be explained because the n-3 FA were given as part of a lipid-rich meal.

Various factors can explain the differences in oral bioavailability of the EE and TG formulations [8]. (1) The finding of a lower bioavailability of EPA and DHA from OM-3 EE than from FFA or TG is in accord with the finding that in vitro pancreatic lipases hydrolyze OM-3 EE 10–50 times more slowly than the corresponding glyceryl esters in TG [70], and require additional digestion with bile salt-dependent lipase and the EE bond must undergo hydrolysis by pancreatic lipases, which appear to be less active than lipases, to be converted into FFA for intestinal absorption [63, 67, 69, 71–74, 76, 93]. Therefore, absorption of the EE formulation is not only lower than that of TG and PL forms but increased during consumption of high-fat meals which increased pancreatic enzyme activity [73, 74]. In contrast, OM-3 FFA formulations are expected to be more readily absorbed than OM-3 EE formulations since a cleavage from a bound form is unnecessary and their bioavailability had little or no dependence on meal fat content [67, 72, 73, 76]. Thus, the OM-3 FFA formulation has the potential to effectively decrease plasma TG levels while the patient follows a low-fat diet. (2) The higher availability of EPA and DHA from rTG compared with natural TG is probably due to the steric position of the FA in the TG molecule. In natural TG, n-3 PUFA are primarily bound at the sn-2 position, which makes it difficult for lipases to cleave them by hydrolysis, while rTG contain higher amounts of n-3 PUFA at the sn-1/3 position, which lipases prefer to hydrolyze. However, other explanations are possible, including that DAG and MAG, which are also present in rTG may facilitate micellar formation and, thus, enhance the n-3 PUFA absorption into the enterocyte [17]. (3) Differences in post-absorptive re-esterification of FFA to TG in the enterocyte, a process which requires glycerol and 2-MAG molecules. With TG formulations, both glycerol and 2-MAG are supplied as a substrate. The slower absorption of the fatty acids from the EE feeding was attributed to a lower efficiency of the phosphatidic acid pathway, which is required in the absence of dietary 2-MAG, although other explanations cannot be excluded. Thus, during peak absorption the cellular and lymphatic appearance of fatty acids from the digestion and absorption of the EE was nearly 50 % lower than that from the corresponding TG [70].

Long-Term Clinical Trials

Several longer-term studies analyzed the bioavailability of EPA and DHA in various n-3 PUFA formulations in healthy [17, 71, 93, 95–102], overweight subjects [103] and in subjects with normal or slightly elevated total blood cholesterol and/or triglyceride levels [104, 105] and in patients with coronary artery disease [95] with contradictory results (Table 39.1). The duration of these studies varied from 4 weeks to 24 months, total numbers of subjects participated in studies ranged from 24 to 129, and the daily doses of EPA + DHA ranged from 0.24 g [97] up to 7.3 g [93]. Additionally, the end points varied among studies, being the FA composition of serum TG and/or plasma FA [17, 71, 93, 95, 103], the percentage of EPA plus DHA (relative to total FA) in RBC [97, 100], the FA composition of both plasma and RBC [96], and the whole blood n-3 PUFA [97].

The bioavailability of EPA and DHA in FFA formulations was compared with the EE and TG formulations with conflicting results. As previously reported in short-term studies, in two trials EPA + DHA in TG or rTG formulations were reported to be significantly more bioavailable compared with the EE formulations [71, 104], while another two studies found no difference in bioavailability for these two forms of n-3 PUFA [95, 105]. Neubronner et al. [104] randomized 150 volunteers to receive for 6 months: (1) fish oil concentrate with EPA + DHA (1.01 g + 0.67 g) given as rTG group; (2) corn oil (placebo group); or (3) fish oil concentrate with EPA + DHA (1.01 g + 0.67 g) given as EE. The omega-3 index increased faster and significantly in both groups treated with n-3 PUFA, but the increase was greater in the rTG group than in the EE group. Another randomized double-blind study compared the effects of EE and TG on plasma fatty acids in 31 healthy normolipidemic men allocated to receive fish oil concentrate as either EE or TG with equal amounts of EPA (2.2 and 2.2 g, respectively) and DHA (1.2 and 1.4 g, respectively) or placebo daily for 7 weeks and found equal incorporations of EPA and DHA into plasma PL from the two formulations [71]. Another study compared the long-term effects of 2 different fish oil preparations (EE and TG) versus olive oil in patients with coronary artery disease. Eighty-nine subjects were randomly assigned to receive capsules containing 6 g/day (TG group) or 7 g/day (EE group) of n-3 PUFA, or capsules containing 12 g/day of olive oil for 6 months. Plasma PL fatty acid analysis showed a 5-fold increase in EPA levels in both fish oil groups [95]. However, this study investigated high unphysiological doses of n-3 PUFA and the doses of

EPA + DHA were different in the EE group (6 g/day) and in the TG group (5 g/day).

An open-label, randomized, parallel-group study compared in 48 healthy subjects two oral formulations of OM-3 EE, one provided by IBSA Institut Biochimique SA (434 mg EPA + 434 mg DHA) and a prescription formulation provided by SPA Società Prodotti Antibiotici S.P.A. (485 mg EPA + 348 mg DHA) [106]. Both formulations were given t.i.d. at a dose of 3 g/day for 28 days. The two formulations increased plasma and RBC concentrations of both DHA and EPA to a similar extent (Table 39.2). Overall, bioequivalence criteria were fully met for both $AUCss_{0–28d}$ and Css_{max} when analysis was performed using the baseline as covariate. However, when the assessment was conducted after substraction of baseline values, the 90 % CIs for Cssmax ratios were within the bioequivalence range, whereas the intervals for AUCss 0–8 ratio were borderline for bioequivalence. On the contrary, in another randomized double-blind crossover study recruiting 50 volunteers with the lowest blood concentrations of n-3 PUFA and treated with a single high dose (12 g) of both OM-3 EE formulations the pharmacokinetic analysis revealed that they were bioequivalent [107]. Indeed, a satisfactory bioequivalence for the $AUC_{0,24h}$ 90 % confidence interval of the ratio between the two formulations were in the range for bioequivalence (for EPA 0.98–1.04 and for DHA 0.99–1.04) and the same was true for the C_{max} and time to C_{max} (t_{max}) (90 % CIs were 0.95–1.14 and 1.10–1.25 for EPA and 0.88–1.02 and 0.84–1.24 for DHA) (Table 39.2). The authors suggested that differences in OM-3 EE bioavailability in healthy volunteers may at least in part reflect differences in the baseline plasma or tissue concentrations of these n-3 PUFA. Thus, they proposed that in order to obtain reliable bioequivalence data of products present in the daily diet, the participating healthy volunteers must be selected among individuals with low and homogeneous baseline concentrations of the variables to be determined and should not be exposed during the study to food items containing the product under evaluation, e.g., fish.

In a phase 2b, open-label, clinical study in which patients were taking OM-3 FFA (Epanova, 4 g/day for 52 weeks), trough plasma levels of EPA reached steady state levels by week 16 at which EPA levels increased 351 % from baseline [92]. Furthermore, the findings of the ECLIPSE trial demonstrated that the short-term administration of Epanova markedly increased bioavailability of n-3 PUFA in overweight subjects during low-fat consumption periods which represent a therapeutic advantage over the OM-3 EE in patients with severe hypertriglyceridemia. Therefore, the ECLIPSE II study compared the pharmacokinetics of Epanova and Lovaza (4 g/day for 14 days) in 52 healthy subjects who were equally allocated in two open-label, parallel-group cohorts in conjunction with a low-fat diet [101]. Systemic bioavailability (C_{max} and $AUC_{0–24h}$) during the 0- to 24-h dosing interval ($C_{max,ss}$) of unadjusted total plasma EPA + DHA were approximately 3- and 3.9-fold higher, respectively, for Epanova relative to Lovaza (5.8-fold and 6.5-fold higher after baseline adjustment) (Table 39.2). Furthermore, after 14 days of repeat dosing, subjects receiving Epanova had substantially higher and less variable trough levels of unadjusted total EPA + DHA compared with Lovaza which supported that after repeated administration in conjunction with a low-fat diet, the absorption of OM-3 EE was significantly lower than that of OM-3 FFA. This is likely attributable to the reduced amount of pancreatic lipase released in response to a low-fat diet. Interestingly, the greater bioavailability of Epanova was associated with a significantly greater decrease in serum triglycerides relative to Lovaza (21 vs. 8 %; $P = 0.013$).

Krill oil. A few studies have compared the bioavailability of n-3 PUFA found in fish oil and KO, in which a large percentage of the n-3 PUFA are bound to PL (Table 39.1) [8, 96, 97, 103, 105]. It was initially proposed that the bioavailability and tissue incorporation of n-3 PUFA from KO was higher in krill oil fed animals than fish oil fed animals, but recent data demonstrated that the bioavailability of EPA and DHA from KO is less than previously suggested. This can be explained because these trials used different designs (some not suitable for absorption studies), duration, selection criteria and concentrations of n-3 PUFA. Furthermore, KO composition is variable in terms of proportions of PL (34–58 %), FFA (6–22 %), and TG (23–36 %) between batches in the various KO preparations used in clinical studies, as affected by processing technologies and by seasonal variations, so that comparisons of the results obtained using different sources of KO is difficult [8, 23, 108]. Thus, further well-designed studies are needed to demonstrate possible differences in oral bioavailability of n-3 PUFA between fish oil and KO.

In a randomized, double-blind parallel arm trial, overweight and obese men and women were randomly assigned to receive capsules containing 2 g/day of menhaden oil ($n = 26$, 0.212 g EPA + 0.178 g DHA per day), KO ($n = 25$, 0.216 g EPA + 0.09 g DHA per day), or olive oil ($n = 21$) for 4 weeks [103]. Results showed that plasma EPA and DHA concentrations increased significantly more in the KO and menhaden oil groups than in the control group, but there were no differences between fish oil and KO groups. Ulven et al. [105] conducted an open single-center, randomized parallel study in 113 healthy volunteers with normal or slightly elevated total blood cholesterol and/or triglyceride levels that were treated with KO (3 g/day, EPA + DHA = 543 mg) or fish oil (1.8 g/day, EPA + DHA = 864 mg) for 7 weeks. KO significantly increased

the plasma level of EPA, DPA, and DHA to the same extent as the dose of fish oil, which suggested KO was more bioavailable than fish oil by a factor of 1.59. However, in these two studies the daily doses of n-3 PUFA were not matched in the two treatment groups.

A double-blind crossover trial compared the uptake of three EPA + DHA formulations (1680 mg EPA + DHA) given as either rTG, EE, or KO (80 % PL-bound EPA and DHA and 20 % free EPA and DHA) in 12 healthy young men [24]. The highest incorporation of EPA + DHA into plasma PL (a proxy for bioavailability) was produced by KO (mean $AUC_{0–72h}$: 80.03 ± 34.71 % × h), followed by fish oil rTAG (59.78 ± 36.75 % × h) and EE (47.53 ± 38.42 % × h). Although a trend for greater bioavailability of total plasma EPA + DHA from KO was noted, likely attributable to the FFA content, the plasma PL $AUC_{0–72h}$ of EPA + DHA among all three treatments was not significantly different. Interestingly, FA analysis of the supplements showed that, contrary to the manufacturers' indications, the KO sample (Neptune KO™) contained 22 % of the total EPA amount as free EPA and 21 % of the total DHA amount as free DHA, while the two fish oil samples did not contain any free FA. The high content of free EPA and DHA in KO might have a significant influence on the availability of both n-3 PUFA. Another randomized double-blind crossover trial compared the apparent bioavailability of PL and TG forms from KO and fish oil [96]. In this study, 3 g of KO (0.777 g of EPA + DHA), fish oil (0.663 g EPA + DHA) or corn oil were daily given to 24 healthy subjects. Each treatment lasted 4 weeks and was separated by 8 weeks washout phases. KO increased plasma and RBC n-3 PUFA concentrations, including EPA and DHA, and reduced n-6:n-3 PUFA ratios compared with fish oil group. Moreover, the n-3 index significantly increased following KO supplementation compared with fish oil and control. However, the doses of EPA plus DHA were different, and the fish oil contained a very high level of linoleic acid (32 vs. 2 % in the krill oil).

In another open-label, randomized, crossover study, 35 healthy subjects were treated with either fish oil in concentrated TG form (rTG: EPA 650 mg, DHA 450 mg), EE fish oil (EPA 756 mg, DHA 228 mg), KO in PL form (EPA 150 mg, DHA 90 mg), or salmon oil in TG form (EPA 180 mg, DHA 220 mg) for 4 weeks [97]. At the prescribed dosage, the statistical ranking of the four products in terms of increase in whole blood n-3 PUFA levels was concentrated rTG fish oil > EE fish oil > TG salmon oil > PL KO. Whole blood EPA percentage increase in subjects consuming concentrated rTG fish oil was more than 4 times that of KO and salmon oil. Risk reduction in several elements of cardiovascular disease was achieved to a greater extent by the concentrated rTG fish oil than by any other supplement.

Possible differences in the availability of n-3 PUFA in KO and fish oil are probably due to different types of chemical bonds. Most of the n-3 PUFA in KO are bound as PL, which indicates that intestinal digestion and absorption of PL-bound n-3 PUFA might be more efficient than that of n-3 PUFA as TG or EE. PL are amphiphilic and exert emulsifying properties in such a way that the pancreatic lipases and phospholipases (primarily PLA2) responsible for cleaving can easily access the emulsified lipid substrate in oil droplets.

Limitation of the Short and Long-Term Studies

The short-term (postprandial) bioavailability studies are useful to assess directly the rate and extent of EPA and DHA absorption and the role of influencing factors, such as age, gender, dietary composition, matrix and galenic formulation, but they do not provide information on their tissular distribution, concentration at the site of physiological activity and biotransformation [8]. In these studies, oral bioavailability was estimated by measuring the levels or proportions of n-3 PUFA (EPA and DHA) in total lipids of plasma, serum, chylomicrons or in plasma lipid fractions such as TG, PL, FFA, and CE [8, 88]. Long-term studies analyzed not only the absorption of n-3 PUFA, but also their tissular distribution and biotransformation. However, due to ethical problems n-3 PUFA distribution is limited to certain peripheral tissues, while the distribution and retention in the brain and heart are almost unknown.

Unfortunately, all these studies present important methodological differences in design, number of participants, age and gender of participants, length of treatment, doses of n-3 PUFA administered (doses were commonly higher in the long-term studies) and content in EPA and DHA, parameters assessed to estimate bioavailability, compliance of study product intake, fat content in the diet, the blood compartment where n-3 PUFA were analyzed, galenic formulation and/or dietary background (intake of fish/other n-3 PUFA containing products) [88]. Because of all these differences, the results achieved were somewhat inconsistent between studies. Of note, the small number of patients (mean 8, between 5 and 54 subjects of mixed gender) indicated that they were underpowered from a statistical point of view to establish the differences between treatments. The same criticism can be applied to the long-term studies (mean 74, ranging from 24 to 129 subjects). Because of all these differences, it is difficult, if not impossible, to compare the outcomes of the studies leading sometimes to contradictory results. Furthermore, patients are treated with fixed doses, ignoring the large interindividual variability in uptake of EPA + DHA and the differences related to age, gender, and body weight. Thus, it would be necessary to calculate the individual doses according to the n-3 index, instead of using

fixed doses of EPA + DHA in the intervention group. Furthermore, most studies did not report sufficient details of the analytical and quality control matters related to the measurement levels/concentrations in plasma or RBC [88]. Finally, most of the studies are performed in young healthy volunteers, while the bioavailability in older patients, with comorbidities and receiving polypharmacy, i.e., those that can benefit most from n-3 PUFA supplementation, remains uncertain.

There is only limited information about the rate of incorporation and the half-life of EPA and DHA in blood and the information came mainly from single-dose supplementation studies and it is very likely that results from short-term studies cannot be extrapolated to what can occur after chronic supplementation. Indeed, following prolonged supplementation of n-3 PUFA (EE or TG), EPA reached its maximum level within 1 week, while the rise in DHA continued for several weeks [71, 109]. Moreover, Sadou et al. [19] analyzed the incorporation of n-3 PUFA into plasma lipids over a short-term (8 h) and a longer-term (30 days) following supplementation with 2 g EPA and 1.3 g DHA daily. They found a marked difference in incorporation at 8 h and 30 days. Thus, at 8 h for EPA the ratio of incorporation into TG (TG value = 1), CE, and PL was 1:0.04:0.67, whereas the equivalent values at 30 days were 1:1.65:3.29; similar differences between 8 h and 30 days existed for DHA. These findings highlight the difficulties in comparing the results from short- and longer-term studies and suggested that the intramolecular structure of n-3 fatty acid TG affects incorporation of EPA and DHA (see below *Influence of chemical binding form*).

Factors Regulating the Absorption

Oral absorption and bioavailability of n-3 PUFA is a complex process that can be markedly influenced by a number of factors, including age, fat content of the meal, position of fatty acids in the glycerol backbone of TG, the position of double bond from the carboxyl-end, the galenic formulation, and the actual matrix in which the n-3 PUFA were provided [8, 88].

Age and Gender

Graf et al. [110] compared the incorporation of ^{14}C-DHA in different tissues from rats of 3 different ages (2, 4, and 10 weeks). Interestingly, only in the oldest animals did they observe significant differences in the uptake of ^{14}C-DHA in PC form compared with ^{14}C-DHA in TG form (2-fold). In a clinical study, healthy elderly (74 ± 4 years) and younger (24 ± 2 years) volunteers were treated with fish oil capsules (680 mg/day of DHA + 320 mg/day of EPA) for 3 weeks, followed by a 2-week washout period [111]. At the end of the treatment, DHA incorporated into plasma lipids increased in both groups, but 42 % more in the elderly group than the young group, while EPA in plasma lipids rose similarly in both groups. The explanation for this age-related difference is uncertain. During the washout, plasma DHA declined to a similar value in both groups.

Carver et al. [84] studied the human cerebral cortex and RBC FA composition in subjects aged from 2 to 88 years and used linear regression models to describe the relationship between age and FFA composition in both tissues. They found that changes occurred after the approximate age of 18 years. Among subjects $\leq$18 years of age, polyunsaturated FA generally decreased with age, with the exception of DHA, which demonstrated a significant increase, while the level of monounsaturated FA generally increased to the age of 18 years. Moreover, in these subjects there was no significant relationship between cerebral cortex and RBC FA levels. However, in subjects >18 years of age there was a significant relationship between brain and RBC levels for several FA, particularly 16:0. These data demonstrated that the levels of cerebral cortex FA change from early childhood through late adulthood and that the levels of several RBC FA might be useful in predicting brain FA levels in adults. A systematic review of 51 publications showed significantly lower values of DHA in total plasma lipids (32 and 33 studies) and in plasma PL (21 and 23 studies) in men than in women [112]. Indeed, women had a significantly higher proportion of DHA in plasma/serum, RBC, and adipose tissue lipids compared with men. Thus, gender distribution should be regarded as a significant potential confounding factor in every study assessing data on FA composition.

Fat Content in Diet

Generally, it is recommended to take n-3 PUFA capsules with a meal because they are thus better tolerated than when ingested on an empty stomach. However, there are no studies which compared the bioavailability of n-3 PUFA when the capsules are ingested on an empty stomach compared to a simultaneous ingestion with a meal. Furthermore, even though the fat content composition of the meal can markedly modify the availability of n-3 PUFA, most of the short- and long-term studies did not report the dietary fat intake during the intervention.

In healthy volunteers, the absorption of n-3 PUFA from both the EE form and TG form increased 3 times when fish oil capsules were ingested with a high-fat meal (44 g of fat) than when taken with a low-fat meal (8 g of fat) [73, 74]. This effect was confirmed in the ECLIPSE trial, which demonstrated that the bioavailability of OM-3 FFA was markedly greater by a factor of 4 as compared with the EE form when the capsules were ingested with a very low-fat breakfast and lunch even though the OM-3 FFA formulation had 42 % less DHA compared with the OM-3 EE

formulation [67]. This marked difference can be explained probably because the EE form requires pancreatic lipase for digestion prior to absorption and the fat diet stimulated the release of pancreatic lipases. Thus, the OM-3 FFA formulation has the potential to effectively decrease severe triglyceride levels while the patient follows a low-fat diet.

Interestingly, in some recent RCT with a neutral result of n-3 PUFA on cardiovascular disease prevention the least bioavailable formulations of EPA + DHA were used, and capsule ingestion was poorly timed, which might result in a poor oral bioavailability [6]. Moreover, study participants were recruited irrespective of their baseline levels in EPA + DHA, and treated with fixed doses, ignoring the large interindividual variability in uptake of EPA + DHA. Thus, study participants should be advised to ingest EPA + DHA with the main meal of the day (which varies in different countries), usually containing sufficient fat to increase their oral bioavailability.

Influence of Chemical Binding Form

The bioavailability of different formulations of n-3 PUFA also depends on the position of the FA in the glycerol backbone of TG and the position of double bond from the carboxyl-end [8, 88]. The higher availability of EPA and DHA from rTG compared with natural TG observed in some kinetic studies is probably related to the steric position of the FA in the TG molecule. In natural TG, n-3 PUFA are primarily bound at the sn-2 (internal) position, which makes it difficult to be hydrolyzed by lipases, while rTG contain higher amounts of n-3 PUFA at the sn-1/3 (external) position, which lipases prefer to hydrolyze [20]. In processed fish oils supplements, however, DHA is predominantly localized to the sn-2 position compared to EPA which is more randomly esterified to all three positions of the TG backbone.

Sadou et al. [19] investigated how the distribution of EPA and DHA in the sn-2 and sn-1/3 positions of fish oil TG influenced their respective incorporation into TG, CE and PL of low- and very low-density lipoprotein (VL/LDL) and high-density lipoprotein (HDL). Nine healthy volunteers were studied over both a short-term (0–8 h) and a long-term (30 days) period of daily supplementation with 2 g EPA and 1.3 g DHA given as 11 g fish oil TG in which DHA was predominantly situated in the sn-2 position. They found a marked difference in incorporation at 8 h and 30 days. Thus, at 8 h for EPA the ratio of incorporation into TG (TG value = 1), CE, and PL was 1:0.04:0.67, whereas the equivalent values at 30 days were 1:1.65:3.29; similar differences between 8 h and 30 days existed for DHA. These findings highlight the difficulties in comparing the results from short- and longer-term studies and suggested that the intramolecular structure of n-3 fatty acid TG affects incorporation of EPA and DHA. The authors proposed that DHA (predominantly situated at the sn-2 position) was mainly incorporated into plasma TG, while EPA (predominantly situated at sn-1/3) was mainly incorporated into plasma PL, thus emphasizing the important role of the TG structure and its potential manipulation for modulating availability of either or both FA.

Subbaiah et al. [113] analyzed the incorporation of EPA and DHA into PC and CE in 6 normolipidemic males after feeding 12 g marine lipid concentrate/day for 28 days. The time course of incorporation of EPA into plasma PC and CE showed a precursor–product relationship, while the DHA concentration of CE was markedly lower than that in PC, and the EPA-DHA ratio was 2-fold to 6-fold higher in CE than in PC at all time intervals. The authors suggested that these differences were related to the fact that EPA and DHA are competitive inhibitors for the enzymatic transfer of FA from PL to CE. But because the affinity of the enzyme lecithin-cholesterol acyltransferase (LCAT) is higher for EPA than for DHA, the transfer of EPA from PL to CE was higher compared with DHA. This would also explain why incorporation of EPA into CE was high, while the incorporation of DHA into CE was negligible and why the DHA incorporation into PL was more rapid than into CE in several studies [19, 30, 113, 114].

Differences in availability of n-3 PUFA between KO and fish oil are also probably related to different types of chemical bonds. Most of the n-3 PUFA in KO are bound as PL, which are amphiphilic and exhibit emulsifying properties which facilitate the formation of mixed micelles and facilitate intestinal digestion and makes the absorption of PL-bound n-3 PUFA more efficient than that of n-3 PUFA bound to TG or EE.

Effect of the Galenic Formulation

Changes in the galenic formulation of n-3 PUFA supplements are aimed to increase availability and/or improve tolerance [67]. A few studies have examined the influence of the galenic formulation of fish oil supplements on the bioavailability of n-3 PUFA (Table 39.3) [91, 115–123].

Emulsions are widely used as carriers for lipophilic micronutrients of bioactive molecules and emulsification is an important step in the digestion and absorption of fats. Thus, emulsifying fish oil may allow the production of more pleasant-tasting supplements and enhance digestion and absorption of n-3 PUFA. Two kinetic studies showed that consumption of n-3 PUFA in an emulsified form was more effective than the consumption of n-3 PUFA oils to increase in EPA and DHA in plasma PL [91, 118]. A randomized, crossover-designed, open-label trial was performed in which 10 healthy volunteers received emulsified fish oil and capsular TG fish oil orally. Compared to standard fish oil, consumption of a single dose of the emulsified fish oil supplement increased the rate and extent of absorption of

Table 39.3 Role of the galenic formulations on the oral availability of n-3 PUFA in humans

Authors (Reference)	Study	n	Duration	Test formulation	Doses (EPA + DHA, g/d)	Sampling	Result
Harris et al. [115]	R	16	1 mo	FO: oil vs. emulsion	2.2	Chylomicrons	No difference
Wallace et al. [116]	OL	25	1 mo	FO: food enriched with microencapsulated oil vs. normal gelatin capsule	0.9	Platelet FA composition	No difference
Kurowska et al. [117]	R, CO	12	24 h	PO: gastric acid resistant vs. normal gelatin capsules containing perilla seed oil	Enteric coated: 11 g containing 6.039 g ALA Uncoated: 11.5 g containing 6.003 g ALA	Plasma	No difference
Garaiova et al. [91]	R, CO	24	9 h	n-3-rich oil mixture: vs. oil emulsion	NS	Plasma, plasma-TG	Emulsion > oil
Raatz et al. [118]	R, OL, CO	10	48 h	FO-TG: emulsified FO vs. capsular FO oil	FO: 4	Plasma PL	Emulsion > oil
Wakil et al. [119]	OL, CO	6	24 h	FO (EPA-EE) oil vs. microencapsulated oil	FO: 4.6 g (20 % EPA)	Plasma	Microencapsulated oil > oil
Haug et al. [120]	R	17	26 h	TG FO: gelled emulsion vs. soft gel capsules	Gelled emulsion: 3.08 EPA + 2.34 DHA Gel capsules:3.03 EPA + 2.3 DHA	Plasma	Emulsion > capsules
Schneider et al. [121]	DB, CO	12	72 h	FO-rTG: gastric acid resistant vs. uncoated gelatin capsule	1.008 EPA + 0.672 DHA	Plasma PL	No difference
Hussey et al. [122]	OL, R, PG	88	2 wk	OM-3 EE vs. emulsion formulation	0.363 EPA + 0.294 DHA	Plasma	LEM > RF
Sanguansri et al. [123]	CO	6	24 h	FO gelatine capsules vs. different food products fortified with microencapsulated FO	FO capsule: 0.266 n-3 PUFA Fortified food: 0.284 (0.05 EPA + 0.234 DHA) per serving of food product	Ileal effluent	No difference

CO cross-over, *d* day, *DB* double-blind, *h* hour, *OL* open label, *PG* parallel groups, *R* randomized, *wk* week, *FA* fatty acid, *FFA* free fatty acids, *FO* fish oil, *EE* ethyl esters, *nTG* natural triglycerides, *PL* phospholipids, *PO* perilla seed oil, *REM* emulsion formulation, *RF* reference formulation, *TG* triglycerides, *rTG* reesterified triglycerides

EPA and DHA and reduced the n-6/n-3 FA ratio in plasma PL over 48 h [118]. The authors hypothesized that emulsification favoured the action of digestive lipases by simplifying the emulsification occurring in the stomach. However, it is possible that differences in absorption could be related, in part, to the vehicles of fat supplements as the emulsified fish oil was supplied in a semiliquid form while capsular fish oil was a gelatin encapsulated liquid oil. It is possible that the gelatin capsule breakdown affected the initial rate of absorption of the FA from the capsular formulation. In another randomized crossover study, 13 volunteers received an oil mixture (concentrated fish oil 43 %, borage oil 31 % and flaxseed oil 26 %) and 11 received the oil emulsion as part of an otherwise fat free meal [91]. The postprandial plasma TG (postprandial $AUC_{0–9h}$) and the ALA, EPA, and DHA levels for the emulsified oil group were significantly higher compared with the non-emulsified oil group; however, pre-emulsification did not affect the absorption of shorter chain less saturated FA. The authors proposed that pre-emulsification of fish oils prior to ingestion reduced droplet size which enable pancreatic lipases to cleave TG-bound n-3 PUFA more easily. Another study compared the bioavailability of EPA + DHA following the administration of an emulsion formulation (LEM) of Lovaza as compared to the reference formulation (RF) in healthy volunteers when dosed for 2 weeks [122]. Following single doses, the dose-normalized EPA plasma-corrected AUCs were 14-fold (total) and 12-fold (free) higher and DHA plasma-corrected AUCs were 10-fold (total) and 13-fold (free) higher for LEM compared to RF. EPA and DHA incorporation into PL increased dose-dependently with both treatments. However, an 8-fold increase over baseline was

observed in EPA incorporation for LEM compared to a 4-fold increase for RF. DHA incorporation increased to a lesser degree, and RBC incorporation also increased. All these data showed that LEM improves the oral bioavailability of EPA and DHA.

Dietary supplements often use capsule coatings that are resistant to gastric acid to mask unpleasant taste, reduce the gastrointestinal side effects, such as reflux or heartburn, and increase patient compliance. Haug et al. [120] compared the bioavailability of DHA and EPA delivered by two different formulations: TG fish oil in traditional soft gel capsules and TG oil as droplets trapped inside a gelatin matrix (gelled emulsions). The $AUC_{0–26h}$ of EPA and EPA + DHA in blood plasma from the gelled emulsions was significantly increased (44.9 and 43.3 %, respectively), and the maximum incremental concentration of EPA and EPA + DHA increased (100.4 and 105.6 %, respectively), compared to soft gel capsules. Thus, bioavailability of EPA and DHA can be improved by incorporating emulsified TG fish oil in a gel matrix prior to oral ingestion. This vehicle is soft and chewable with the possibility of adding flavors, sweeteners and color, being ideal for delivery of PUFA to consumers having problems swallowing large capsules or cod liver oil.

Microencapsulation has been used to mask unpleasant taste in food science, to protect against light and airborne oxidation and to reduce the gastrointestinal side effects but can also enhance the availability of FA. Wakil et al. [119] investigated whether encapsulating the EE form of fish oil with exine microcapsules extracted from readily available and renewable *Lycopodium clavatum* spores can enhance the bioavailability of EPA-EE. Six healthy volunteers ingested 4.6 g of fish oil containing 20 % EPA in EE form, first alone and then after encapsulation within plant spore exines as microcapsules (Table 39.3). Mean $AUC_{0–24h}$ of EPA from EE encapsulated with exines increased by a factor of 10 as compared to EE without exines, probably due to an easier breakdown of finely dispersed oil droplets by lipases, so that the oil is transported through the mucosal lining into the blood stream more efficiently.

Another study compared in 6 patients with a permanent ileostomy the differences in bioavailability between different food products (orange juice, yoghurt and cereal bar) fortified with microencapsulated fish oil [123]. Patients received alternately 284 mg n-3 PUFA (50 mg EPA and 234 mg DHA) via different food products or 266 mg n-3 PUFA (48 mg EPA and 218 mg DHA) via fish oil capsules in a crossover manner. The amount of undigested or unabsorbed n-3 PUFA was measured in the ileal effluent during the next 24 h in order to calculate the amount released, digested, and absorbed. The bioavailability between different food products fortified with microencapsulated fish oil and fish oil gelatin capsules was equal and only 0.58–0.73 % of the total n-3 PUFA dose was recovered in the ileal effluent over 24 h post-ingestion. The n-3 PUFA content in the ileal effluent peaked at 2–8 h and declined after 10 h after consumption of fish oil capsules and fortified orange juice; however, two peaks in n-3 PUFA content were observed, first at 2–8 h and again at 14–16 h, after consumption of fortified yoghurt and cereal bar. This study showed that the delivery of fish oil through food products fortified with microencapsulated fish oil does not compromise the bioavailability of the n-3 PUFA, but the food matrix in which the microencapsulated oil was delivered may alter the transit kinetics through the small intestine. Surprisingly, in this study the authors did not collect blood samples to analyze the amount of LC n-3 FA absorbed. In Crohn's disease patients, after 6 months of treatment the incorporation of EPA and DHA into plasma and RBC PL was enhanced from enteric-coated fish oil capsules compared to uncoated capsules [18]. Thus, the dose needed to achieve the EPA + DHA incorporation rate into RBC PL membranes was one-third of that from uncoated capsules.

Another two studies compared the effect of EPA- and DHA-rich fish oil capsules and ALA-rich perilla seed oil capsules with enteric coatings (which are resistant to gastric acid) with regular soft gelatin capsules containing the same oil [117, 121]. In 12 healthy subjects randomly assigned to a single dose of coated (6.039 g ALA) and uncoated (6.003 g ALA) capsules, no pharmacokinetic differences were found between the two formulations [117], but significantly greater increases in plasma ALA levels from baseline to 24 h were observed after ingestion of the coated preparations. Another double-blind crossover trial compared the uptake of DHA and EPA levels in plasma PL within 72 h following the administration of a single fish oil dose (1.01 g EPA and 0.67 g DHA as TG) in uncoated or coated fish oil capsules [121]. EPA levels rose steadily, reached a peak, and then declined steadily, while the rise in DHA levels was only slightly pronounced and its $AUC_{0–72}$ curve presents a biphasic profile. After an initial increase, the levels decreased rapidly to almost baseline level and then rise again after 6–8 h. However, DHA levels remained high in both groups 72 h after ingestion of the capsules probably due to a delayed DHA uptake from tissues and clearance from circulating blood. Similar kinetic profiles of EPA and DHA in plasma were described in a two-week kinetic study where 7 volunteers ingested 4 g/day of EPA for 2 weeks [109]. There was a more rapid rise in the concentration of EPA than in DHA levels in all lipid fractions, but there was a disproportionate rise in DHA relative EPA in plasma lipids compared with the ratio in the supplement. The authors proposed that the low plasma EPA values relative to DHA were the result of differences in intestinal absorption, faster elimination of EPA from the plasma pool into tissues and a higher β-oxidation rate of EPA compared to DHA.

Cansell et al. [124] compared the absorption of n-3 PUFA in thoracic lymph duct-cannulated rats, after intragastric feeding with EPA and DHA esterified in PL as liposomes or in triglycerides TG as oil. n-3 PUFA absorption was favored by liposomes (mean 98 %) compared to fish oil (73 %) and the DHA proportion in lymph was higher after liposome ingestion (78 %) than after fish oil ingestion (47 %). After 3 days of both treatments, liposomes compared with fish oil, increased the liver content in DHA and much lower in EPA. Moreover, liposomes increased the activity and mRNA levels of carnitine palmitoyltransferase (CPT) I, the mRNA levels of acyl-CoA oxidase and the activity of the peroxisomal FA-oxidizing system, while fish oil exerted opposite effects on CPT I and increased the gene expression of lipogenic enzymes. mRNA levels of hepatic lipoprotein receptors were increased with both diets, but intracellular proteins involved in free FA uptake and lipid synthesis were up-regulated only in liposome-treated rats [125]. The quasi absence of EPA in hepatic PL of liposome-treated rats on the short term could result from increased β-oxidation activities through metabolic regulations induced by more available free EPA and other PUFA.

Distribution

In Plasma Lipids and Red Blood Cells

Single- and multiple-dose studies found that n-3 PUFA are well absorbed from the intestine (>95 %) and can be detected in various plasma lipid fractions such as TG, PL, FFA and CE, which in turn are included in lipoproteins [1, 2, 8, 88]. Animal studies comparing essential pure PL preparations with TG forms consistently showed different patterns of incorporation of n-3 PUFA into different chylomicron and plasma lipids or lipoprotein fractions [88] with a greater incorporation of the n-3 PUFA into tissues from PL compared with TG formulations.

Dietary Supplementation with Fish Oils

It results in a dose-dependent increase in the content of EPA and DHA in plasma lipids, platelets, RBC, leukocytes, heart, liver and colonic tissue [14, 114, 115, 126–130]. Additionally, in all these tissues EPA and DHA often replace n-6 PUFA such as AA. Some interventional trials performed in healthy volunteers, showed that fish consumption was more effective in increasing serum EPA and DHA than supplementing the diet with fish oil [14, 98, 99]. In a pilot retrospective study, 16 volunteers were given for 6 weeks either 100 g/day of salmon (providing 383 mg of EPA and 544 mg of DHA, esterified in glycerol lipids) or 1 or 3 capsules of fish oil/day, providing 150 mg of EPA and 106 mg of DHA or 450 mg of EPA and 318 mg of DHA as EE [98]. Both treatments produced a marked dose-dependent increase in plasma EPA and DHA levels, but net increments were higher after salmon intake than when n-3 PUFA were administered as EE. In fact, the same increments were obtained with 2-fold and 9-fold higher doses of EPA and DHA, respectively, if administered with capsules rather than salmon. However, this study was small and not randomized, and EPA and DHA intakes from these 2 sources were not matched.

A larger study in 71 volunteers compared the impact of the source of n-3 PUFA on their incorporation in serum, blood lipid composition, and cellular activation. Three groups were given 400 g smoked salmon (n = 14), cooked salmon (n = 15), or cooked cod (n = 13) per week for 8 weeks. A fourth group was given 15 mL/day of cod liver oil (CLO) (n = 15), and a fifth group served as control (n = 14) without supplementation. The increase of EPA and DHA in plasma lipids was higher in the cooked salmon group (129 % rise in EPA and 45 % rise in DHA) as compared with CLO (106 and 25 %, respectively) even though the intake of EPA and DHA in the CLO group of 3.0 g/day compared with 1.2 g/day in the cooked salmon group [99]. Since the n-3 PUFA were predominantly in TG in fish as well as CLO, it was suggested that the larger uptake from fish than CLO is due to differences in physiochemical structure of the lipids. Similar effects were observed on plasma lipids and lipoproteins after the consumption of fish or fish oil by hyperlipidemic subjects [131]. However, another study, which used the n-3 index as a marker, found no difference between fish meal and fish oil [100]. The main problem in these studies is that the amount of n-3 PUFA contained in any given fish varies widely, and therefore, the EPA and DHA intakes were not prospectively matched. A recent review discusses the possible difference between whole fish as a nutrient package and fish oil supplements as a source of n-3 PUFA with respect to cardiovascular disease prevention [15].

The rate of incorporation and clearance of n-3 PUFA in RBC membranes and plasma was studied in 20 individuals receiving supplementation with either fish oil (1296 mg EPA + 864 mg DHA per day) or flaxseed oil (3510 mg ALA + 900 mg linoleic acid per day) [102]. After 8 weeks of fish oil supplementation, RBC membrane EPA and DHA increased 300 and 42 %, respectively. The mean erythrocyte n-3 index reached a near optimal value of 7.8 %, and remained relatively high until week 12. EPA and DHA showed greater increases and more rapid washout period decreases in plasma PL than in RBC membranes. Flaxseed oil supplementation increased RBC EPA to 133 % and DPA to 120 % of baseline, but DHA was unchanged. In plasma PL, EPA, DPA, and DHA showed a slight but statistically insignificant increase. Thus, RBC membrane EPA + DHA

content increases during relatively short intervals in response to supplementation at rates related to amount of supplementation.

A randomized, controlled study analyzed the dose–response effects of daily supplementation with marine lipids containing a 2:3 ratio of DHA + EPA as EE at doses of 1.5, 3, and 6 g/day for 12 weeks in 45 healthy normotriglyceridemic male volunteers. Supplementation with marine lipids produced a near linear increase in DPA and DHA in plasma PL [126]. The apparent DHA saturation dose was 1.2 g/day, which is much lower than when pure DHA is provided as a supplement suggesting a possible displacement with EPA in plasma PL. ALA concentrations, however, decreased dose-dependently in response to the supplementation. Other studies found that marine PL were more efficient carriers of n-3 PUFA than TG (normal fish oils) in terms of n-3 PUFA absorption in different tissues [10, 132]. This may be due to the amphiphilic properties of PL resulting in better water dispersibility and their greater reactivity toward phospholipases compared to the glycerolysis of triglycerides [10].

A double-blind controlled 2 × 2 factorial 8-week intervention investigated the effects of high and low 18:2n-6 intake in combination with FO supplementation on tissue fatty acid composition [133]. Sixty-four healthy young men were randomized to capsules with FO or olive oil (control) (4.4 mL/day) and to either sunflower oil and margarine or rapeseed oil and a butter spread to provide a high or a low 18:2n-6 intake. Fish oil capsules resulted in higher DHA, DPA and EPA content in human peripheral blood mononuclear cells (PBMC) as compared with olive oil. In a mouse model, fish oil supplementation (4 % wt/wt) produced a modest enrichment of DHA and DPA in mouse serum total PL but increased EPA and DHA levels in membrane rafts (4-fold) and non-rafts (1.9-fold) of $CD4^+$ T cells compared to T cells from 5 % corn oil fed mice [134]. After one week of feeding B10.RIII and B10.G mice with a diet in which all the fat (5 % by weight) was supplied as either fish oil (17 % EPA, 12 % DHA and 2 % linoleic acid) or corn oil (0 % EPA, 0 % DHA, 0 % AA, and 65 % linoleic acid) macrophages from these mice were highly enriched in DHA (9.8 mol%) and in total n-3 PUFA (22.3 mol%), compared to corn oil fed control mice [135].

ALA Supplementation

Several studies analyzed the effects of ALA supplementation on FA composition of plasma or cell lipids [26, 41]. Chronic increased consumption of ALA produced a linear dose-dependent increase in ALA in both plasma PL, cell lipids and circulating cells such as leukocytes and platelets. ALA supplementation also increased EPA and DPA concentrations in plasma, cell lipids and cell pools, while its effects on DHA was less clear, as some studies reported a decrease while other reported no changes in DHA status, which is consistent with the high oxidation rate and the low fractional conversion rates of ALA previously discussed [26, 27, 136–138]. In men, [^{13}C]-ALA was detected in plasma non-esterified fatty acid (NEFA) pool within 2 h and reached a peak at 6 h [36] and 6 h after the ingestion of the tracer it was estimated that 15–81 % of the administered dose of the tracer was present in adipose tissue. In another study, the administration of ALA or EPA (0.75 g/day and then 1.5 g for periods of 3 weeks each) increased EPA and DPA concentrations but not DHA concentrations in RBC and plasma PL [139].

The long-term effects of ALA supplementation were studied in 20 elderly Japanese individuals by replacing soybean cooking oil with perilla oil-PO (i.e., increasing 3 g/day of ALA). ALA in total serum lipids increased after 3 month on the PO diet, but EPA and DHA increased 10 months after the PO diet, and then returned to baseline within 3 months after being switched back to soy [140]. Brenna et al. [141] reviewed 21 clinical studies analyzing the changes in blood EPA and DHA after ALA supplementation. In most cases, there was a significant increase in plasma EPA and DPA content upon ALA supplementation, but only in 4 studies was there an increase in DHA in plasma fractions and in circulating blood cells. These results agree with the tracer studies which found that the conversion of ALA to DPA is limited (0.13–6 %) and the conversion to DHA is highly constrained ($\leq$0.05 %). All these results confirmed that the conversion of ALA to DHA is insufficient to increase the concentration of this n-3 PUFA and meet the demands of the body. It is possible that increasing dietary ALA increases substrate competition, inhibiting the desaturation of tetracosapentaenoic acid (24:6n-3) to DHA [142]. However, because the size of the plasma DHA pool is far greater than that of EPA, it may take longer until a small contribution of ALA conversion to the plasma DHA pool is detected.

EPA and DHA Supplementation

High dietary intakes of EPA and DHA increased dose-dependently the EPA and DHA content in plasma PL and decreased tissue content of AA [27]. EPA- and DHA-containing products increase the concentration of EPA and DPA in plasma and in RBCs [14, 27, 109, 114, 126–128, 143], but EPA accumulates more rapidly and to a greater extent in plasma and RBCs than DHA [71, 90, 113, 144, 145]. EPA is incorporated into PL, TG and CE, while DHA is taken up into TG to a much greater extent than into CE, and only small amounts of EPA and DHA are present in their non-esterified free fatty acid (NEFA) form [14, 109, 128, 144, 146–149]. Several short-term studies analyzed the rate of incorporation of EPA after a single dose of oral EPA

as TG or EE formulations and found that the maximal incorporation of EPA into TG occurred after 4–6 h [73, 74, 76, 90]. After prolonged supplementation with n-3 PUFA as EE or TAG forms, EPA reached steady state levels within 1 week, while the rise in DHA continued for several weeks [71, 109].

Supplementation trials with purified EPA-EE (~4 g/day) to healthy volunteers significantly increased both EPA and DPA concentrations in plasma and platelet and RBC PL, while blood DHA concentrations remained unchanged or decreased, confirming the poor enzymatic conversion of DPA to DHA [141, 146–150]. In a double-blind, placebo-controlled trial, 52 subjects with type 2 diabetes were randomly assigned to consume EPA, DHA, or olive oil (4 g/day) for 6 weeks while continuing to consume their usual diet. EPA supplementation increased EPA and DPA in plasma PL (540 and 69 %, respectively) without significantly changing DHA (7 % decrease). In the DHA group, DHA and EPA increased by 156 and 64 %, respectively, whereas DPA decreased by 17 %. In another double-blind, placebo-controlled trial of parallel design, 59 overweight, mildly hyperlipidemic men were randomly assigned to receive 4 g/daily purified EPA, DHA, or olive oil (placebo) for 6 weeks [147]. EPA supplementation markedly increased EPA and DPA levels in plasma and in platelets, whereas DHA was not affected. Similar results were obtained in another study performed in schizophrenics; EPA supplementation (1–4 g/day for 12 weeks) significantly increased EPA (but not DHA) content in RBC membranes [151]. In another study, 22 healthy subjects were randomly assigned to receive 4 g of EE of either EPA or DHA for 4 weeks [149]. EPA significantly increased platelet EPA and DPA, but not DHA concentrations. However, DHA treatment significantly increased platelet DHA and DPA concentrations, but not EPA.

The uptake and incorporation of DPA and EPA into human plasma and RBC lipids was studied in 10 females receiving 8 g of pure DPA or pure EPA in randomized crossover double-blind manner over a 7-day period; placebo treatment was olive oil [152]. DPA supplementation significantly increased the proportions of DPA in the plasma PL (2-fold) and TG fractions (2.3-fold, day 4). DPA supplementation also significantly increased the proportions of EPA in TG (3.1-fold, day 4) and CE fractions (2.0-fold, day 7) and of DHA in TG (3.1-fold, day 4). However, DPA proportions in RBC PL did not change following supplementation. Supplementation with EPA significantly increased the proportion of EPA in plasma CE and PL fractions (both by 2.7-fold, day 4 and day 7) and in RBC PL (1.9-fold, day 4 and day 7). However, EPA supplementation did not alter the proportions of DPA or DHA in any lipid fraction. The results of this short-term study suggested that DPA may act as a reservoir of the major n-3 PUFA in humans.

Harris et al. [153] compared the rate and extent of enrichment of RBC membranes and plasma PL with EPA and DHA in healthy premenopausal females randomly assigned to consume 485 mg EPA and DHA either from 2 servings of oily fish (i.e., salmon and albacore tuna) per week or from 1 to 2 capsules/daily for 16 weeks. They found that the EPA + DHA content of RBCs or plasma PL did not differ significantly when equivalent doses of n-3 PUFA were provided. EPA + DHA contents stabilized in plasma PL after 4 weeks but continued to rise through week 16 in RBCs. EPA in RBCs increased significantly more rapidly in the fish group than in the capsule group during the first 4 weeks, but rates did not differ significantly between groups thereafter.

Lindenborg et al. [154] demonstrated pure DPA and EPA exhibited different postprandial metabolic fates. In a double-blind crossover study, pure DPA and EPA were incorporated in the meals of 10 healthy female volunteers. DPA almost completely eliminated the incorporation of FA in chylomicrons within 5 h, possibly because DPA was acting as a pancreatic lipase inhibitor, but EPA did not produce this effect. Plasma chylomicronemia was significantly reduced after the meal containing DPA compared with the meal containing EPA or olive oil only. Both EPA and DPA were incorporated into the chylomicrons TG to different extents (EPA more than DPA), while there was less incorporation into chylomicron PL. Lipidomic analysis showed that the main TG species that EPA and DPA were incorporated into were EPA/18:1/18:1, DPA/18:1/16:0, and DPA/18:1/18:1, with a significantly lower incorporation of DPA compared with EPA in chylomicron TG. There was very limited conversion of EPA and DPA to DHA and there were no increases in EPA levels during the 5-h postprandial period after the DPA meal. Thus, EPA and DPA were incorporated into the chylomicrons TG to different extents and into different TAG molecular species.

DHA Supplementation

In healthy North Americans, DHA represents ~4 % of total lipid in RBC, while mean plasma or serum PL DHA content ranged from 1.5 to 7.5 % of total FA [27, 97, 148, 155]. Plasma PL concentrations of DHA increased rapidly in a dose-dependent manner after daily supplementation, reached equilibrium within 1 month after and once steady state concentrations were attained they were maintained throughout the supplementation period [27, 113, 148]. In 12 different studies, supplementation with DHA (0.2–6 g DHA/day for 1–6 months) resulted in a rapid, dose-dependent and saturable increase in plasma PL and RBC DHA levels at doses up to ~1.5–2 g/day; at higher

doses, plasma DHA concentrations approach saturation [26, 27, 33, 146, 149, 156–158]. In RBC, steady state DHA concentrations were reached after 4–6 months, which is consistent with the slower turnover of these cells. DHA supplementation also produced an apparent linear increase in EPA concentrations (~0.4 g/100 g for each 1 g of DHA intake) presumably through retroconversion or by inhibition of further metabolism of the EPA formed from ALA [27]. These changes were associated with a dose-dependent and saturable reduction in plasma PL ALA concentrations, although this reduction was quite variable among studies. Conquer and Holub [148] reported the changes in n-3 PUFA in serum PL and NEFA in healthy subjects of Asian Indian background. The subjects consumed 8 capsules daily of placebo (DHA-free), low DHA (0.75 g/day), or high DHA (1.5 g/day) over 6 weeks. Plasma DHA levels in PL rose by 167 % overall with low-dose supplementation and only by an additional 23 % upon doubling the dose to 1.50 g/day. After 6 weeks of supplementation with 0.75 or 1.5 g DHA/day, absolute concentrations of DHA as PL were not significantly different from the corresponding 3-week values. Interestingly, the absolute concentrations of serum DHA as NEFA showed a marked rise with low-dose supplementation (212 %) and a further 70 % rise upon doubling the supplementation to 1.5 g/day and the 6-week concentrations were significantly different from the corresponding 3-week values at both dose levels.

The metabolic fate of DHA differs substantially when ingested as TG compared with PC, in terms of both bioavailability of DHA in plasma and accumulation in target tissues [132]. The distribution of DHA in plasma, platelet, and RBC lipid classes was studied in a 72 h postprandial study in 3 young adults after ingestion of a single dose of ^{13}C-DHA esterified in a PC form. ^{13}C-DHA first appeared in plasma NEFA and TG, reaching a maximum at 6 h and then further declined; the abundance at 6 h was 2.47-fold higher in the TG than in NEFA. In contrast, the labeling of PE and PC was more progressive until 9 h and plateaued from 9 to 72 h where the [^{13}C]-DHA abundance was higher in PE than in PC. The labeling of RBC and platelet PL exhibited different kinetics, probably involving different metabolic pathways for ^{13}C-DHA incorporation in cell membranes. The increase in the labeling in platelet PC occurred till 24 h post-ingestion, followed by a plateau between 24 and 72 h. In erythrocytes, a slow and progressive increase of PC labeling was observed as a function of time; the incorporation of [^{13}C]-DHA into PE was weak and a small amount of [^{13}C]-DHA appeared in erythrocyte LPC reaching a plateau after 9 h. The supply of [^{13}C]-DHA to platelets occurred through NEFA, while [^{13}C]-DHA was carried by both LPC and NEFA to erythrocytes. These results contradicted previous results from the same group which observed, after intake of TG labeled with ^{13}C-DHA, that lyso-PC was the only source of ^{13}C-DHA for erythrocytes [30]. These results confirmed that the lipid form of ingested DHA affects markedly its kinetics and partly its metabolic fate.

Zuijdgeest-van Leeuwen et al. [114] studied the incorporation of EPA and DHA in different plasma lipid fractions in 5 healthy volunteers following during supplementation with n-3 PUFA EE (EPA 6 g/day and DHA 5.3 g/day) for 7 days. EPA-EE were rapidly incorporated into plasma lipids, especially into PL and CE, as well as into TG. The proportion of EPA in PL showed a 15-fold increase after 7 days, while DHA showed a smaller increase. In CE, EPA also increased, while DHA did not increase at all, while incorporation of DHA into TG was even higher and faster than that of EPA. Half-life of EPA in PL ranged from 1.6 to 2.3 days, whereas mean half-life of EPA in CE was 3.2 days. The higher incorporation of EPA in plasma lipids is consistent with results from other studies investigating the incorporation of n-3 PUFA in TG [71, 90] and EE formulations [113, 144, 145]. Only Blonk et al. [126], who used a supplement of n-3 fatty acid EE, did not find any difference in incorporation between EPA and DHA into plasma PL after 12 weeks of supplementation with EPA and DHA as EE. Similarly, the decline during washout was more rapid for EPA than for DHA [114]. Thus, EPA concentrations decreased rapidly, returning to baseline values after 7 days. The washout from plasma PL presented a half-life of 1.6–2.3 days, whereas mean half-life of EPA in CE ranged from 1.6 to 4.1 days. The decrease of EPA in plasma TG was initially fitted by the monoexponential with a half-life of 0.47–1.6 days. In three subjects, however, washout of EPA from TG followed a biexponential function, with a short half-life (0.11–0.77 days) in the initial phase and a half-life of 1.29–4.17 days in the second phase. For the decrease of DHA in plasma TG, the half-life using the monoexponential model was 0.9–2.6 days and using the biexponential model the short and long half-lives ranged from 0.2 to 0.4 days and 3.8 to 5.3 days, respectively. In conclusion, EPA-EE are rapidly incorporated into plasma lipids, especially into PL. Other studies have confirmed that EPA accumulated more rapidly in plasma and RBC and cleared more rapidly than does DHA after the completion of supplementation [109, 113, 128, 159]. Marangoni et al. [159] reported that whole plasma DHA concentrations decreased slowly once n-3 PUFA supplementation had stopped and even after 4 weeks of washout were not entirely back to baseline levels; conversely, plasma EPA concentrations decreased rapidly after supplementation was stopped. Thus, DHA appears to have the longest half-life of all n-3 PUFA. The differences in the accumulation of DHA and EPA may be related to the lipid moieties in which they are stored. DHA is carried predominantly in PL, with lesser portions in TG and EE, whereas EPA is more equally distributed among EE, TG and PL [113, 114, 143].

OM-3 EE (Lovaza) induced significant, dose-dependent increases in serum PL EPA content, but increases in DHA content were less marked and not dose-dependent [160]. Uptake of EPA and DHA into serum PL in subjects treated with Lovaza was independent of age (<49 vs. ≥ 49 years), but females tended to have more uptake of EPA into serum PL than males. Following a single 4-g dose of Epanova under fasted conditions, the majority of EPA and DHA in plasma was incorporated in PL, TG and CE, and the free NEFA represent approximately 0.8 and 1.1 % of the total measured amount for EPA and DHA, respectively [92]. Following repeat dosing under low-fat meal conditions, the total apparent plasma clearance (CL/F) and half-life of baseline-adjusted EPA from Epanova at steady state were 548 mL/h and 37 h, respectively, and the CL/F and half-life of baseline-adjusted DHA were 518 mL/h and approximately 46 h, respectively.

In a 4-year placebo-controlled trial, 44 male patients with X-linked retinitis pigmentosa were randomized to receive DHA (400 mg/day) or placebo. Plasma DHA content increased within the first 6 months of supplementation (average of 2.5-fold) compared with baseline levels [161]. RBC kinetics followed a similar pattern, although it took 4–6 months after the start of DHA supplementation to reach new steady state concentrations, which is consistent with the slower turnover of these cells. RBC DHA concentrations were maintained thereafter throughout the supplementation period.

Incorporation into the PL of Cell Membrane as a Major Structural Component

The blood transports n-3 PUFA to the target tissues (liver, heart, brain/nervous system and retina, vessels, adipose tissue, kidney), where they are primarily incorporated primarily into PL, sphingolipids and plasmalogens (Fig. 39.3) [27]. Dietary supplementation with n-3 PUFA (as fish, fish oil supplements or food fortified products) dose-dependently increase the content of EPA and DHA into the cell membranes of all organ tissues of the body where they modulate their functional and structural properties [27, 141, 162, 163]. Cellular membranes from some tissues (e.g., retina, brain, myocardium) are particularly enriched in n-3 PUFA; indeed, ~30 % of all fatty acids in the outer segment membrane of retinal photoreceptors are omega-3 fatty acids [164].

DHA is the most abundant n-3 PUFA in cell membranes, being particularly abundant in the heart, brain and retina, so that DHA content generally exceeds EPA 5- to 30-fold in most organs. Conversely, small quantities of ALA and EPA are present in tissues [27]. Of note, DHA is several hundred-fold more abundant than EPA in membrane structural lipids of brain and retina [163]. Indeed, it has been found that DHA is required in the nervous system for optimal neuronal and retinal function and influences signaling events which are vital for neuronal survival and differentiation [165].

Incorporation of n-3 PUFA in the PL of the cell membrane modulates its fatty acid composition and contributes to regulate cell and organ function as well as a variety of biological processes as [see 7, 162, 166] (a) they assure the correct structure and composition of cell membranes. (b) They maintain the integrity and order (fluidity) of the membrane which is an important determinant for the correct hormone-receptor binding and membrane protein-mediated responses. Indeed, DHA is highly flexible within the membrane and is particularly effective at accommodating transitional changes associated with transmembrane protein activation [167]. (c) They affect different transport mechanisms (including ion channels and pumps) and, thus, regulate membrane permeability and cellular excitability. (d) They exert a second messenger action when intercalated in the cell membrane and modulate signal transduction [168]. Binding of neurotransmitters, hormones and growth factors to their membrane receptors activates PLA2 which catalyzes the hydrolysis of the sn-2 position of membrane glycerophospholipids to release AA, EPA and DHA. These molecules become substrates for eicosanoid biosynthesis, depending on the activities of cyclooxygenases (COX-1 and COX-2), lipoxygenases (5-, 12-, or 15-LOX), or cytochrome P450 monooxygenases. (e) They can also act as ligands for members of nuclear receptors of transcription factors such as peroxisome proliferator-activated receptors (PPARs), liver X receptors (LXRs) and sterol regulatory element-binding protein 1c (SREBP-1c) that regulate FA metabolism and gene expression [169]. (f) They can be incorporated into lipid rafts, subdomains of the plasma membrane that contain high concentrations of cholesterol, glycosphingolipids, and protein receptors organized in glycolipoprotein microdomains [25, 170]. Fish oil supplementation increased mainly DHA levels in T-cell membrane rafts (4-fold) and non-rafts (1.9-fold) of $CD4^+$ T cells compared to T cells from corn oil fed mice [134]. Similarly, macrophages from mice fed a diet containing 5 % fish oil were highly enriched in DHA (9.8 mol%), as well as in total n-3 PUFA (22.3 mol%), compared to 5 % corn oil fed control mice [135]. (g) They can act as reservoirs for potent biologically active molecules. In fact, DAG is a lipid second messenger generated by members of the phospholipase C superfamily that does not only bind to and activate protein kinase C enzymes but also regulates a variety of other target proteins such as small G proteins. Because the acyl chain length and degree of saturation may affect the function of n-3 PUFA in biological membranes, it is expected that alterations in the content of these FA will differentially affect membrane structure and function. Indeed, different effects of EPA, DHA, and DPA on enzyme activity, gene expression, and platelet aggregation have been described in vitro [29, 168].

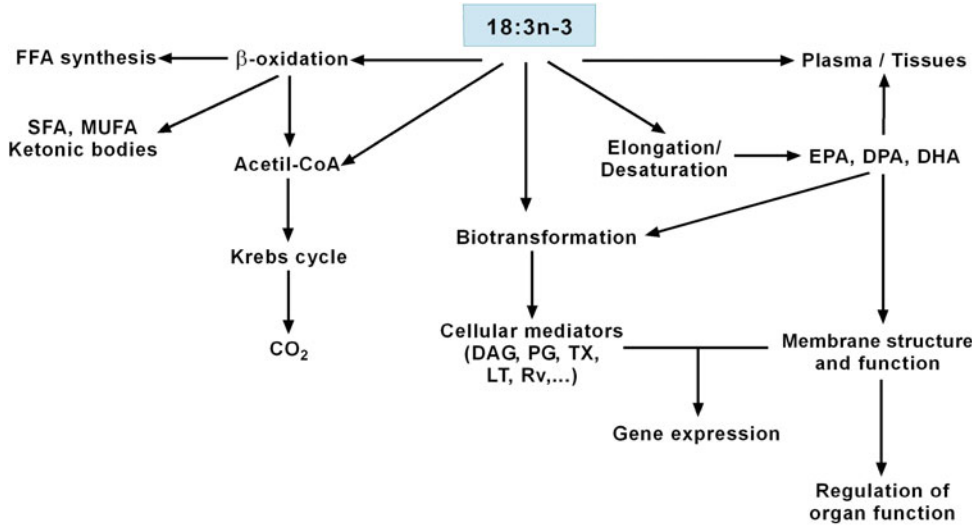

Fig. 39.3 Distribution and biotransformation of n-3 PUFA. They are incorporated into the phospholipid bilayer where they modify membrane structure and function and gene expression and produce numerous bioactive signaling molecules that play a key role in the regulation of platelet aggregation, smooth muscle contraction, inflammation, immunity, and organ function. *EPA*, eicosapentaenoic acid; *FFA*, free fatty acids; *MUFA*, monounsaturated fatty acids; *PGs*, prostaglandins; *SFA*, saturated fatty acids; *TXs*, thromboxanes; *LTs*, leukotrienes; *Rvs*, resolvins

It is very important that DHA, EPA, and ALA compete with AA for incorporation into cell membranes as well as for binding and conversion of AA by the enzymes [cyclooxygenases (COX1 and COX2), lipoxygenases (LOX), and cytochrome P450 (CYP)] that catalyze the biosynthesis of alternative, physiologically active prostaglandins (PG), thromboxanes (TX), and leukotrienes (LTs) which participate in the regulation of blood pressure, renal function, blood coagulation, inflammatory and immunological reactions, and other functions in tissues [7, 166, 169–172]. Thus, the net effect of consuming foods enriched in n-3 PUFA is a diminished cellular potential to synthesize these powerful AA-derived mediators of inflammation and a diminished potential for platelets to produce the vasoconstrictor and prothrombotic agent thromboxane A2 (TXA2) (Fig. 39.3) [7, 26, 162]. EPA can also be metabolized by COX into PGH3 which is then converted by COX1/2 and LOX into the alternative 3-series PGs (PGD3, PGI3, PGE3, PGJ3, and PGF3α) and TXs (thromboxane A3, with three double bonds in the carbon chain), and 5-series LTs (with five double bonds in the carbon chain: LTB5, C5, and E5); other metabolites of EPA and DHA (resolvins, protectins) modulate the inflammatory response. 3-series prostaglandins present platelet antiaggregant properties and less inflammatory activities compared with those produced from ARA [173, 174], while thromboxanes A3 presents reduced pro-aggregant properties and LTB5 is at least 30 times less potent than LTB4 [150]. PGD3 is a potential circulating antithrombotic agent that antagonizes the migration of neutrophils across endothelial cells mediated by PGD2, while PGE3 is less proinflammatory than PGE2 [7, 175]. Additionally, 5-LOX metabolizes EPA to LTB5, a metabolite at least 30 times less potent than LTB4 [150]. DPA can interfere with COX-1 activity, resulting in the suppression of TX synthesis from AA and can also cause an acceleration of lipoxygenase pathway, thus inhibiting platelet aggregation [176].

Storage in Adipose Tissue for Later Use

Adipose tissue represents 15 % (men) to 23 % (women) of body mass. At this level, n-3 PUFA are stored as TG and ALA is the predominant n-3 FA (~0.7 % of total FA), while only small amounts of EPA or DHA are present [177–179]. It was estimated that in a 75-kg man the whole body ALA reserve in adipose tissue would be approximately 79 g (roughly equivalent to typical intake over 53 days) and in a 65-kg woman would be approximately 105 g (roughly equivalent to typical intake over 70 days) [26]. The adipose tissue stored only very small amounts of DHA or EPA (<0.3 % of total FA content), which implies the need for a continuous supply through the diet [177–179].

Effects of DHA and EPA Supplementation on Tissue Content

Although the absorption of n-3 PUFA in the small intestine is equivalent, PL and TG formulations can influence the distribution of n-3 PUFA into different tissues [26, 27]. The blood transports n-3 PUFA to different body tissues, where they are primarily incorporated in membranes PL. DHA is the most abundant n-3 PUFA in cell membranes, while only

minute quantities of ALA and EPA are generally present in tissues; in fact, DHA generally exceeds EPA 5- to 30-fold in most organs [27]. DHA is particularly abundant in neural tissue (such as brain and retina), where DHA is several hundred-fold more abundant than EPA. The exceptionally low levels of EPA may be attributed to the high beta-oxidation rates of EPA after its uptake into the brain [135]. The rates of incorporation of EPA and DHA into cell membranes and tissues are higher for PL; conversely, comparable and lower rates of incorporation of EE and TG forms of EPA and DHA have been reported. Indeed, there is some evidence that PL might be a more efficient delivery form of PUFA to the brain than TG [180, 181].

In rats, after DHA supplementation for 2–3 months the contents of DHA and EPA increased in a dose-dependent manner in the brain, heart, liver, RBC, and bone marrow, whereas ALA content decreased in a dose-dependent manner [27, 182]. In guinea pigs fed for 3 weeks from weaning with a high-ALA diet (0.3–17 %), the ALA levels significantly increased in all tissues except the brain and the levels of n-3 PUFA in all tissues except intestines, brain, carcass, and skin [183]. The n-3 PUFA content of the whole body was less than 5 % of that of the ALA content, and the major n-3 PUFA (>66 % of total) in the body was DPA, the brain being the only tissue where the DHA content exceeded that of DPA. Moreover, in guinea pigs high-ALA diets or dietary DHA supplementation produced moderate increases in DHA levels in the retina and brain PL. In the liver and heart, dietary ALA had little effect on tissue DHA content, but dietary DHA supplements led to large increase (up to 10-fold) in DHA content in both tissues [184]. The results confirmed that dietary ALA was less effective than dietary DHA supplementation in increasing tissue DHA levels because ALA is rapidly beta-oxidized to acetyl-CoA and CO_2 or excretion via the skin and fur rather than metabolism to DHA and that tissues vary greatly in their response to exogenous DHA.

In rats, both EPA and DPA supplementations significantly increased the EPA content in plasma and an increased level of DPA was observed in the liver and heart 2 days after the feeding of the DPA ceased [185]. There was also an increased level of EPA in the liver in the DPA-fed group, which confirmed the retroconversion of DPA to EPA. However, DPA supplementation increased heart DPA levels, whereas EPA did not increase heart EPA levels. These results showed that EPA and DPA, both provided in the free form, are metabolized differently. Furthermore, both EPA and DPA feeding increased the total n-3 PUFA levels even though the total amount of DPA excreted in feces was 4.6-fold greater than that of EPA. A possible explanation of this finding is that part of the absorbed EPA is used in other metabolic (production of eicosanoids) or catabolic (β-oxidation) processes, so that the EPA reached a similar tissue deposition to DPA, which was less absorbed, but more efficiently retained. Similarly, in rats, short-term supplementation with pure DPA significantly increased the hepatic concentration of DHA and the concentration of EPA in the liver, heart and skeletal muscle, presumably by the process of retroconversion [186]. Another study assessed the effect of oral supplementation with DPA on the levels of serum and tissue lipid classes and their FA compositions including individual PL types in the liver, heart, and kidney from rats receiving daily oral gavage over 10 days as corn oil without (controls) or with purified DPA in FFA formulation (21.2 mg/day) [28]. The DPA group exhibited significantly lower serum lipid concentrations, but the concentrations of DPA in the total lipid were significantly higher in the DPA group of serum, liver, heart, and kidney, with the PL being the major DPA reservoir (45.2–52.1 % of the DPA in the total lipid). However, no significant differences in DHA (22:6n-3) amounts in total lipids were observed. The highest relative mol% values as DPA were in heart tissue and lowest in the kidney. The EPA concentrations were markedly higher in the DPA group and most pronounced in the kidney relative to liver and heart yielding an estimated apparent % conversion of DPA to EPA of 67 % and EPA:DPA ratios reaching 5.74 in kidney PE. In weanling rats, DHA supplementation for 2–3 months increased dose-dependently the DHA content in the liver, brain, heart, skeletal muscle, heart, RBC, and bone marrow with a concomitant reduction in ARA [27, 182]. In the heart, a combination of selective uptake and degradation mechanisms may be responsible for the relative enrichment of DHA.

DHA accounts for 10 % of FA in the human brain and is the main PUFA in structural lipids in the brain and retina where it plays important roles in brain development, learning ability and visual acuity [164, 187]. Lysophosphatidylcholine (lysoPC) could represent a preferential vehicle of DHA to target tissues [30, 188]. Indeed, the brain preferentially takes up DHA from the circulation as lysoPC-DHA compared with non-esterified DHA [188]. In rats, constant basal turnover of esterified DHA in the brain with unesterified DHA in plasma occurs at an estimated rate of 2–8 % per day in adult rats [165]. Generally, it is difficult to deplete DHA from the neural membranes of adult mammals even with a diet low in DHA, presumably because of preferential uptake of DHA into the brain and its low turnover which may allow the adult brain to preserve its FA content much longer than other organs upon dietary changes in n-6/n-3 PUFA ratio [165, 189]. [^{3}H]-DHA esterified at the sn-2 position of lysoPC was preferentially recovered in the brain (4–5 % of the injected radioactivity) as compared to the unesterified form of DHA (0.3–0.4 %), while the lyso-PC form was taken up less than or to the same extent as the

unesterified form by the liver, heart, and kidney [188]. These findings confirmed that lyso-PC was an efficient carrier of n-3 PUFA to the brain [180, 188].

Polozova and Salem [190] found that DHA is highly incorporated in the liver, heart, and brain within 5 min after an intravenous injection of [^{14}C]DHA in mice; however, brain incorporation of ^{14}C-DHA slowly rose to 0.7 % at 24 h. They also found higher radioactivity in PL and NEFA fractions in the liver and lower radioactivity in the TG fraction as compared with oleic acid-injected rats. DHA was deposited in significantly higher amounts compared with OA, EPA and DPA in the liver, heart, brain, and kidney. In the heart, both DPA- and DHA-treated groups showed significantly higher label compared with OA (by 11-fold) and EPA (by 3-fold) groups. In the brain and kidney, the EPA, DPA, and DHA groups had significantly higher amounts of radioactivity compared with the OA group. In skeletal muscle, the DPA and DHA groups had a significantly higher incorporation of label compared with the OA and EPA groups. The preservation of DPA from β-oxidation and the higher incorporation of DPA (and DHA) in the heart and muscle, compared with EPA, suggest that DPA might have a specific role in these tissues.

Graf et al. [110] quantified the age-dependent DHA incorporation into the brain of 2-, 4-, or 10-week-old rats after a bolus dose of different DHA-esters. Animals were gavaged with [^{14}C]-DHA-TG, [^{14}C]-DHA-PL, [^{14}C]-DHA-TG + PL at 2 mg/kg. After 24 h, the distribution of radioactivity in body and brain regions was determined using quantitative whole body autoradiography. The tissular amount of radioactivity was dependent on the tissue, age of the animals and type of treatment. In all age groups and after all treatments, the highest accumulation of ^{14}C-DHA-derived radioactivity was found in the liver (14–30 % of the dose). However, no ^{14}C-DHA-derived radioactivity was present in bone and very low concentrations were found in the rat brain after 24 h (0.07–0.67 %), which is in agreement with another study where 0.5 % of ^{11}C-DHA arrived in the brain of rhesus monkeys after intravenous administration of ^{11}C-DHA [191]. Higher DHA incorporation (>2-fold) was also found in the skeletal muscle, large intestine wall, kidney, heart, adrenal gland, brown fat, lung, testes, thyroid gland, and uveal tract/retina. Interestingly, in the brain the DHA-PL uptake was significantly increased in 11 out of 14 brain regions compared to supplementation in the TG formulation [110]. The highest DHA concentrations were found in the inferior and superior colliculus, pituitary gland, hippocampus, thalamus, pons, olfactory lobe, medulla, and occipital cortex. The age of the animals significantly influenced accumulation of ^{14}C-DHA for most of the analyzed tissues, but some tissues were more affected by age than others. Brain, spinal cord, and testes contained ~9 times more radioactivity at 2 weeks compared with 10 weeks, and ~3 times more radioactivity at 4 weeks compared with 10 weeks. However, in the heart, liver, spleen, rectum, and stomach, no age-dependent variations of radioactivity inclusion were found. Effects of different esters on the transport of ^{14}C-DHA to body tissues were most evident in 10-week-old rats, where oral ^{14}C-DHA-PL delivered a 2-fold higher accretion of radioactivity in the brain, liver, and kidney compared with ^{14}C-DHA-TG, indicating that optimal efficacy of DHA-containing PL may occur in the adult rat. In 10-week-old rats, tissues such as liver, brain, kidney and anterior uveal tract accumulated 2- to 3-fold more [^{14}C]-DHA-derived radioactivity after [^{14}C]-DHA-PL dosing compared with [^{14}C]-DHA-TAG dosing. However, in baboons DHA and ALA were most concentrated in structures local to the brain stem and diencephalon, particularly the basal ganglia, limbic regions, thalamus, and midbrain, and comparatively lower in white matter [192]. These data revealed that DHA and ALA concentrations in the CNS are highly region-specific and are unexpectedly high in the deep central nervous system regions embedded in white matter of much lower DHA and ARA concentration.

Carrie et al. [187] examined the effects of dietary ALA deficiency followed or not at the age of 7 weeks by PL supplementation on the fatty acid composition of total PL in 11 brain regions in mice. In control mice, DHA levels were significantly higher in the cerebral cortex (mainly the frontal cortex) compared to all regions, while in mice with dietary ALA deficiency the pituitary gland, frontal cortex, striatum, olfactory bulb, occipital cortex, and hippocampus were the most markedly affected with 40 % reduction of DHA. In ALA-deficient mice, supplementation for 2 months with egg yolk PL or cerebral PL restored a normal FA composition in brain regions except for the frontal cortex. These results indicate that there is a species-dependent regional distribution of n-3 PUFA, and these differences in brain distribution should be taken into consideration for the optimal formulation of functional foods aimed to support brain development and function.

A study in obese Zucker rats compared n-3 PUFA given for 4 weeks in the form of either fish oil (FO; n-3 PUFA in TG) or krill oil (KO, majority of n-3 PUFA in PL) balanced for EPA and DHA content with a control diet [193]. EPA and DHA concentrations were higher in plasma and peritoneal macrophages in the FO and KO groups compared with the control group. Additionally, KO led to a significantly higher incorporation of EPA and DHA into tissue (liver and heart) PL compared to FO supplementation, which is in agreement with previous studies in rats and baboons [110, 194]. A significantly higher presence of DHA in the brain was observed after KO supplementation as compared to FO supplementation [195].

Differences in the accumulation and retention of DHA and EPA may be related to the lipid moieties in which these

n-3 PUFA are stored. DHA is carried predominantly in PL, a more stable lipid fraction in plasma, with lesser portions in TG and EE, whereas EPA is more equally distributed between neutral lipids (sterol esters and TG) and PL [113, 114, 143]. Only small amounts of each of these FA are present in their NEFA form [148]. The differential distribution of DHA and EPA may be linked to differences in the kinetics of washout as well as their saturation dynamics in plasma and availability to tissues.

Metabolism

n-3 PUFA can undergo different processes, including β-oxidation to produce energy, recycling into saturated (SFA) and monounsaturated fatty acids (MUFA), formation of ketonic bodies, and metabolic biotransformation (Fig. 39.3). EPA and DHA from Epanova are mainly oxidized in the liver similar to other FA derived from dietary sources. Following repeat dosing under low-fat meal conditions, the total apparent plasma clearance (CL/F) and half-life of baseline-adjusted EPA and DHA at steady state are 548 mL/h and 37 h and 518 mL/h and approximately 46 h, respectively [92].

Beta-Oxidation of n-3 PUFA

We already mentioned that conversion of ALA to DHA is very limited (<5 %) in humans because ALA is rapidly β-oxidized, a process splitting the carbon chain of fatty acids into smaller fragments to produce energy releasing carbon dioxide (CO_2) in exhaled breath (Fig. 39.3) [43, 46, 196]. In fact, in guinea pigs, ALA is more prone to β-oxidation or excretion via the skin rather than metabolism to DHA [183]. Moreover, a diet high in LA increases its rate of β-oxidation, limits its accumulation in plasma, and reduces its conversion to long-chain derivatives by 40 %, leading to a net reduction of 70 % in n-3 PUFA [38–40, 45, 49]. Similarly to ALA, EPA also undergoes a rapid β-oxidation in some tissues, which may explain the low levels of ALA and EPA tissular level as compared with DHA [136, 186, 197]. Kaur et al. [186] studied the extent to which EPA, DPA, and DHA are catabolized to CO_2 or, conversely, incorporate into tissue lipids in rats treated with a single oral dose of ^{14}C-DPA, ^{14}C-EPA, ^{14}C-DHA or ^{14}C-oleic acid (OA). DPA and DHA were conserved from β-oxidation to a greater extent than EPA and OA at 6 h. Similarly, in healthy volunteers, following the ingestion of [^{13}C]-ALA around 15–35 % of dietary ALA is rapidly catabolized to CO_2 in the first 24 h, reaching 60 % after 7 days [36–38, 45]. However, these figures may underestimate by ∼30 % the amount of dietary ALA undergoing β-oxidation due to the trapping of labeled CO_2 in bicarbonate pools [26, 27]. Additionally, EPA-CoA was a good substrate for mitochondrial carnitine acyl-transferase-I, the rate-limiting enzyme in mitochondrial FA β-oxidation, while DHA is a poor substrate for both mitochondrial and peroxisomal β-oxidation, which could explain the high rate of β-oxidation of EPA [198]. Thus, in rats and humans DPA and DHA are conserved from β-oxidation to a significantly greater extent than EPA and are efficiently stored in various tissues including the liver, heart, retina, and brain [29, 186, 190, 197, 198].

Following ingestion of [^{13}C]-labeled n-3 PUFA, the recovery of $^{13}CO_2$ in breath is higher in men than in women (24–33 vs. 19–22 % of an ingested dose, respectively), which is in agreement with the greater use of carbohydrate as an energy source in women compared to men [26, 36, 37, 45, 199]. The greater β-oxidation of ALA in men may reflect differences in the mass of tissues with high active fatty acid β-oxidation (skeletal and cardiac muscle, liver, and kidney) compared with women.

Recycling of Carbon from ALA into SFA and MUFA

Carbon fragments produced during the β-oxidation of ALA enter the cellular acetyl-CoA pool and then the Krebs cycle or can be recycled into SFA and MUFA acids, so that very little is stored as ALA (Fig. 39.3) [26, 43, 141]. The recycling of carbon from ALA into SFA and MUFA may be important as a source of FA in pregnant animals [26]. Burdge et al. [36] analyzed the recycling of carbon released by β-oxidation in adult humans. Following the administration of 700 mg ^{13}C-ALA, labeled 16:0, 18:0, 16:1n-7, and 18:1n-9 were detected in plasma PC and TG, but not in other plasma lipid pools. The total proportion of label recovered in plasma PC was 6-fold greater than in TG in men and 25-fold greater in women [26]. In agreement with the greater partitioning of ALA toward β-oxidation in men, the total concentration of labeled SFA and MUFA in plasma lipids was 20 % greater in men compared with women [36, 37]. Thus, in men, carbon recycling into SFA and MUFA greatly exceeded conversion to n-3 PUFA.

Formation of Ketonic Bodies

ALA can be used as the substrate for ketone bodies which represent an alternative energy source for the brain during fasting, aging, or illness (Fig. 39.3). It has been suggested that ALA supplementation may produce a mild ketonemia that could help to retain or restore cognitive function during aging.

Metabolic Biotransformation

All n-3 PUFA have at least two methylene-interrupted double bonds, the molecular moiety (1,4-*cis*,*cis*/Z, Z-pentadiene) to be oxygenated by lipoxygenases (LOX) or by non-enzymatic peroxidation [175]. Figure 39.4 summarizes the main pathways for the metabolism of n-3 PUFA.

ALA is a substrate of 15-LOX, leading to the formation of 13(*S*)-hydroxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid [13(*S*)-HOTE]. In addition, four conjugated triene end products

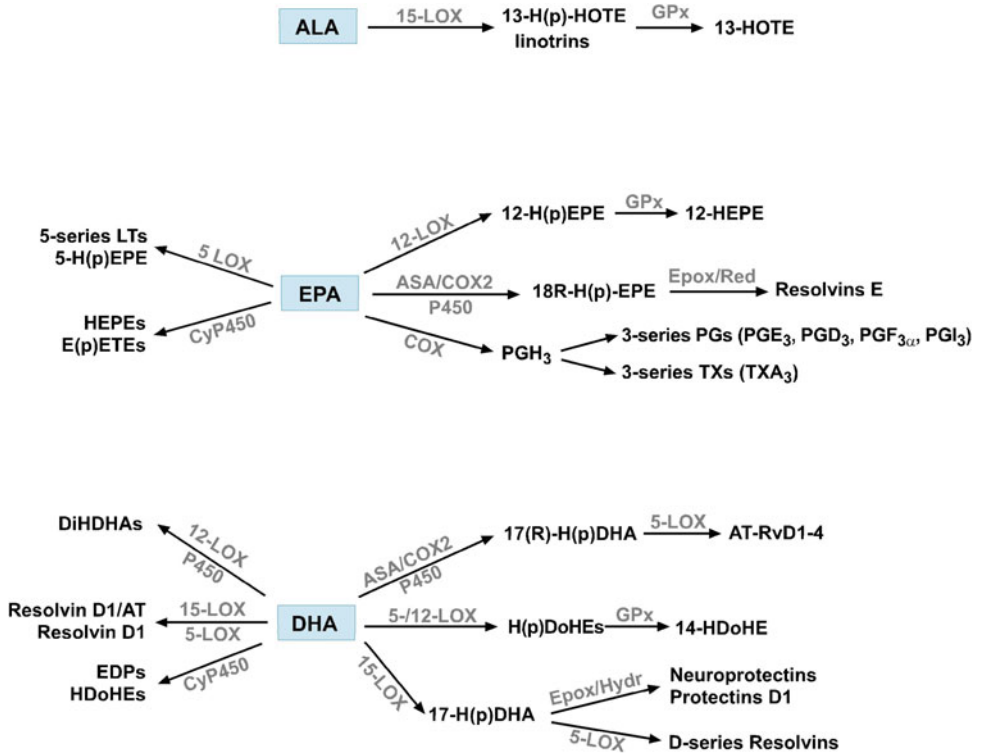

Fig. 39.4 Main biotransformation pathways of EPA, DPA, and DHA. COX: cyclooxygenase. CYP: cytochrome P-450 enzymes. DiHETE: dihydroxyeicosatrienoic acid. EEQ: epoxyeicosatetraenoic acid. EET: epoxyeicosatrienoic acid. EpODEs: epoxyeicosatrienoic acid. Epox/Hydr: epoxidase + hydrolase. GPx: glutathione peroxidase. HDoHE: hydroxydocosahexaenoic acid. HEPE: hydroxyeicosapentaenoic acid. HETEs: hydroxyeicosatetraenoic acids. HOTE: hydroxyoctadecatrienoic acid. Hp: hydroperoxy. H(p)EPE: hydro(peroxy) eicosapentaenoic acid. H(p)ETE: hydro(peroxy)eicosatetraenoic acid. HpODE: hydroperoxy-octadecadienoic acid. Lox: lipoxygenase. LT: leukotriene. PG: prostaglandin. Resolvins D: DHA-derived resolvins. Resolvins E: EPA-derived resolvins. Tx: thromboxane

were identified after reduction: 9(*R*),16(*S*)-dihydroxy-10*E*,12*E*,14*E*-, 9(*S*),16(*S*)-dihydroxy-10*E*,12*E*,14*E*-, 9(*S*),16(*S*)-dihydroxy-10*E*,12*Z*,14*E*-, and 9(*R*),16(*S*)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acids. The latter two have the *tras/cis/trans/E,Z,E* conjugated triene feature of poxytrins. From ALA and DHA, poxytrins are named linotrins and protectin DX, respectively. Both linotrins and protectin DX inhibit human blood platelet aggregation induced by both collagen and TXA2, and exhibited anti-inflammatory properties [200]. Linotrin 9(*R*),16(*S*) (linotrin-1) appeared slightly more potent to inhibit the two cyclooxygenase isoforms (COX-1 and COX-2) and platelet aggregation than linotrin 9(*S*),16(*S*) (linotrin-2), but both might account, in part, for the anti-inflammatory and anti-atherothrombotic properties of ALA [200]. EPA and DHA are substrates of platelet 12-LOX to produce monohydroxy derivatives including 12 hydroxy-eicosapentaenoic acid (12-HEPE) and 11- and 14-hydroxy-docosahexaenoic acid (14-HDoHE), respectively, which inhibit TXA2-induced platelet aggregation and aortic contraction [201] and may antagonize the TXA2 action by interfering with its receptor sites [175, 202]. DHA is a good substrate for other LOX leading to 4- and 7-HDoHE through 5-LOX and 17-HDoHE through 15-LOX. Furthermore, DHA can also undergo a double oxygenation by 15-LOX leading to 10(S),17(S)-dihydroxydocosa-(4Z,7Z,11E,13Z,15E,19Z)-hexaenoic acid, also called protectin DX (PDX), which inhibits platelet aggregation induced by both collagen and TXA2 and neutrophil activation in vitro [200, 203]. In activated macrophages, DHA is converted via 12-LOX and cytochrome P450 oxygenase into 14S,21S- and 14S,21R-dihydroxydocosa-4Z,7Z,10Z,12E,16Z,19Z-hexaenoic acids (diHDHA); in skin wounds, these metabolites enhanced wound healing, re-epithelialization, granulation tissue growth, and capillary vasculature formation of murine wounds [10]. Thus, these metabolites may represent a novel mechanism that regulates wound healing.

EPA, DPA, and DHA are metabolized into epoxidized and hydroxylated derivatives by the cytochrome P450 (CYP) mixed function oxidase system in microsomes from liver, kidney, small intestine, skin, brain and other tissues [204, 205]. In fact, most CYP isoforms can metabolize EPA and DHA with significantly higher catalytic efficiency than AA. Studies in rats [28, 29, 186] and humans [152, 154] suggest that EPA and DPA are metabolized differently. In the majority of CYP isoforms, EPA is the preferred substrate

and is converted with significantly higher rates than other n-3 PUFA which explains the exceptionally low tissular levels of EPA. DHA is metabolized with higher (CYP2E1 and CYP4F2) or similar rates (CYP2C8 and CYP2C19) compared to AA, but is a poor substrate for other isoforms (2C23, 4A11, 4A12A, and 4A12B) [206]. DHA is generally metabolized with higher rates than DPA, but the same enzymes which showed already weak activity toward DPA are also relatively inactive with DHA. CYP2C9/2C19, 2C8, 2J2, and 1A2 were the most efficient epoxygenases of EPA and DHA [206–208]. The n-3 double bond was a major site of epoxidation, as catalyzed by CYP2J2 and most of the CYP2C isoforms. Usually the preferentially oxidized double bond was the last one, i.e., 17–18 or 19–20 double bond, respectively, in EPA and DHA, whereas the epoxidation of the first double bond, i.e., 5–6 in eicosa series and 4–5 in docosa series, was very low. However, other double bonds can be epoxidized, particularly by CYP2C enzymes. The principal CYP-dependent metabolites derived from EPA include five epoxyeicosatetraenoic acids (8,9-, 11,12-, 14,15-, and 17,18-EpETE) and two n/(n-1)-hydroxy-eicosapentaenoic acids (20- and 19-HEPE), whereas DHA can be metabolized to several epoxydocosapentaenoic acids (7,8-, 10,11-, 13,14-, 16,17-, and 19,20-EDPs) and n/(n-1)-hydroxyldocosahexaenoic acids (22- and 21-HDoHE) [175, 204, 205, 207, 209].

CYP1A1 presents both epoxygenase and n-1 hydroxylase activities, converting EPA into 17,18-EpETE and 19-OH-EPA, which represent 68 and 31 % of total metabolites, respectively [206, 209]. With DHA as substrate, CYP1A1 exclusively epoxidizes the n-3 double bond and produces 19,20-EDP as main metabolite [207]. Human CYP4A1 and CYP4A11 displayed significant epoxygenase activities when a n-3 double bond in the fatty acid was available. High n-3 epoxygenase activities toward EPA and DHA were also observed with CYP1A1 [210] and CYP2E1. All CYP2C isoforms epoxidize the n-3 double bonds of EPA and DHA, CYP2C19 being the most efficient in epoxidation. Most CYP2C isoforms (2C8, 2C9, and 2C18) show a 1.5- to 2-fold increase in catalytic activities when converting EPA instead of AA. CYP2C8 metabolizes EPA producing 11,12- and 14,15-EpETE [207, 208] and DHA producing 19,20-EDP as the main metabolite [207]. CYP2C11 and CYP2C23 are the predominant enzymes producing EETs in rat liver and kidney, respectively. With EPA as substrate, the 17,18-double bond is clearly the preferred site of epoxidation (about 60 % of total EpETEs) [206, 209]. CYP2E1 also exhibits n-3 epoxygenase activity and preferentially produces 17,18-EpETE and 19,20-EDP when using EPA and DHA as substrates [207]. CYP2J2 is the major EET producing isoform in the human heart, converting EPA preferentially to 17,18-EpETE (~40 % of the total epoxygenase product) and DHA almost exclusively to 19,20-EDP [206–209]. CYP4A/CYP4F isoforms show (n-1)-hydroxylase activities when utilizing EPA and DHA and are the major 20-HETE producing enzymes in mammals. CYP4A1 hydroxylates EPA to 20- and 19-HEPE and additionally epoxidizes the n-3 double bond to yield 17,18-EpETE. CYP4A11 hydroxylates EPA to 20-and 19-HEPE and DHA to 22- and 21-HDoHE [211]. CYP4F subfamily members (CYP4F2, 4F3A, and 4F3B) have the ability to produce 20-HEPE and 22-HDoHE from EPA and DHA, respectively [212]. CYP4F2 hydroxylates DHA with more than 3 times higher rates than EPA, suggesting that DHA may be the preferred substrate [211]. CYP4F3A and CYP4F3B show high hydroxylase activities with DHA but are significantly less active with EPA. CYP2U1 expressed in thymus and brain functions as an n/(n-1)-hydroxylase of ALA, EPA, and DHA. CYP4F8 and CYP4F12 metabolize EPA and DHA by epoxidation of the n-3 double bond.

Mice fed n-3 PUFA showed increased circulating and tissular accumulation of epoxides derived from EPA and DHA [17,18-epoxyeicosatetraenoic acids (EEQs) and 19,20-epoxydocosapentaenoic acids EDPs, respectively] in plasma and in membrane PL of heart, kidney, liver, lung, and pancreas, whereas the levels of epoxyeicosatrienoic acids (EETs) produced by CYP-dependent epoxidation of AA were largely reduced significantly [206, 209]. These epoxides exhibited antiarrhythmic actions at nanomolar concentrations.

EPA and DHA are also enzymatically converted to a new family of lipid mediators termed resolvins (resolution phase interaction products), denoted E series (RvE) and D series (RvD), respectively [213]. Human endothelial cells expressing aspirin-acetylated COX-2 transform EPA to 18R-hydro(peroxy)eicosapentaenoic acid [18R-H(p)EPE] which can then be further transformed by leukocyte 5-LOX to RvE1 (5*S*,12*R*,18*R*-trihydroxy-6*Z*,8*E*,10*E*,14*Z*,16*E*-eicosapentaenoic acid) [214]. Resolvin E1 increases the endothelial production of nitric oxide and prostacyclin, decreases adhesion receptors and the formation of ROS and proinflammatory cytokines. DHA is the precursor for two groups of resolvins, referred to as the 17S- and 17R-resolvin D series [215].

Generation of RvD1-4 from DHA involves sequential oxygenations by 15-LOX and 5-LOX; DHA is converted by 15-LOX to 17S-H(p)-DHA, which on subsequent oxygenation by 5-LOX results in production of RvD1-4. Aspirin-acetylated COX-2 generates 17*R*-hydroxy-DHA, which following sequential oxygenation by 5-LOX results in production of 17-epi-RvD1 or aspirin-triggered RvD1 (AT-RvD1: 7*S*,8*R*,17*R*-trihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid) [216].

All these enzymes are strongly induced by acute inflammation. Indeed, resolvins exhibit potent anti-inflammatory [they inhibit transendothelial trafficking

and activation of polymorphonuclear neutrophils, macrophages, and lymphocytes], antioxidant, antinociceptive, and pro-resolution properties [7, 164, 169, 175, 213, 215]. Resolvin D2 is mainly produced by dendritic cells. It decreases the activation of PMN and its infiltration into inflamed tissues and promotes phagocytosis and clearance of apoptotic cells. On the other hand, DHA is also converted into protectin D1 (PD1) which exhibits potent anti-inflammatory, antiapoptotic and neuroprotective activities [162, 164]. PD1 exerts potent agonist actions on macrophages and vascular endothelial cells that can control the magnitude of the local inflammatory response involved in atherosclerosis.

Excretion

In rats with diet-induced hyperlipidemia supplemented with DHA, given as DG rich in DHA (DHA-DG) or TG rich in DHA (DHA-TG), the amount of DHA excreted into the feces was ~0.4 % of the DHA administered [217]. Similar results were observed in human ileostomates [123]. In rats treated with EPA + DHA (250 mg/day) and fed with a semisynthetic high-fat diet for 9 days, dietary EPA and DPA appeared in the feces on days 6, 7, and 8, but the amount of DPA excreted was 4.6-fold greater than that of EPA (0.4 vs. 0.07 % of the dose fed) [185]. Epanova does not undergo renal excretion [92].

Adverse Effects

The European Food Safety Authority (EFSA) considers that dietary intakes of EPA + DHA up to 5 g/day, and supplemental intakes of EPA alone up to 1.8 g/day or of DHA alone up to ca. 1 g/day, are safe for adults in the general population [168], and the US Food and Drug Administration (FDA) considers that a daily intake of up to 3 g EPA + DHA is safe (Docket No. 91N-0103) [218]. However, dietary recommendations for EPA and DHA range between 250 and 500 mg/day for European adults, i.e., well within safety ranges.

n-3 PUFA present good tolerability in clinical trials with a low rate of adverse events [72]. Adverse events reported in ≥ 1 % of patients treated with Lovaza (4 g/day) or placebo in 8 randomized, placebo-controlled, double-blind, parallel-group studies are summarized in Table 39.4. Adverse events led to discontinuation of treatment in 3.5 % of patients treated with Lovaza and 2.6 % of patients treated with placebo. In 23 placebo-controlled clinical trials (n = 1025), the adverse reactions occurring at incidence >3 % were eructation (4 %), taste perversion (4 %), and dyspepsia (3 %) [160]. Similarly, in patients (n = 6975) with chronic heart failure (New York Heart Association class II-IV) there were no differences in the discontinuation rate due to adverse events between the treatment and placebo groups [1, 2]. Similarly, in a pool of two long-term (≥ 52 weeks) placebo-controlled trials involving 748 patients, the adverse effects reported in at least 3 % of patients treated with Epanova as compared to placebo (olive oil) were diarrhea, nausea, abdominal pain or discomfort, and eructation [92]. However, safety of n-3 PUFA in pediatric patients (<18 years of age) has not been established.

The main adverse reactions described with n-3 PUFA are as follows:

- Gastrointestinal: eructation, dyspepsia, epigastric or upper abdominal pain, loose or watery stools, abdominal distension, constipation, flatulence, nausea or vomiting, and dysgeusia.
- Cardiovascular: chest pain.
- Dermatologic: rash, pruritus.
- Other: fever, chills, flu symptoms, body aches

In some patients, n-3 PUFA may increase low-density lipoprotein cholesterol (LDL-C) levels as well as alanine aminotransferase (ALT) levels without a concurrent increase in aspartate aminotransferase (AST) levels. Thus, LDL-C and ALT levels should be monitored periodically during the treatment. However, the EFSA Panel concludes that supplemental intakes of mostly EPA at doses up to 4 g/day have no significant effect on LDL-cholesterol concentrations [168].

Adverse effects described in humans in association with high intakes of EPA and DHA include bleeding episodes, impaired immune function, increased lipid peroxidation, and impaired lipid and glucose metabolism [168]. However, the EFSA Panel concluded that (1) supplemental intakes of EPA and DHA combined at doses up to 5 g/day consumed for up to 12 weeks did not significantly affect glucose homeostasis in healthy or diabetic subjects or induce changes in immune functions which might raise concern in relation to the risk of infections or inappropriate activation of inflammatory responses. (2) Supplemental intakes of EPA and DHA combined at doses up to 2–6 g/day, and supplemental intakes of mostly DHA of 2–4 g/day, increase LDL-cholesterol plasma levels by about 3 %, whereas EPA at doses up to 4 g/day has no significant effect on LDL-cholesterol; this effect is accompanied by a decrease in TG with no changes in total (or non-HDL) cholesterol concentrations. It is important that the Panel considered that this small increase in LDL-cholesterol levels at the doses mentioned above may not be adverse in relation to CVD risk.

Bleeding Complications

In vitro, n-3 PUFA increase the bleeding times and inhibit platelet aggregation and an increased tendency to bleed from

Table 39.4 Adverse events reported in 8 randomized, placebo-controlled, double-blind, parallel–group studies for hypertriglyceridemia that used Lovaza® 4 g per day (taken from Ref. [160])

Body system	Omacor (N = 226)		Placebo* (N = 228)	
Adverse event	n	%	n	%
Subjects with at least 1 adverse event	80	35.4	63	27.6
Body as a whole:				
– Back pain	5	2.2	3	1.3
– Flu syndrome	8	3.5	3	1.3
– Pain	4	1.8	3	1.3
Cardiovascular:				
– Angina pectoris	3	1.3	2	0.9
Digestive:				
– Dyspepsia	7	3.1	6	2.6
– Eructation	11	4.9	5	2.2
Skin:				
– Rashes	4	1.8	1	0.4
Taste perversion	6	2.7	0	0.0

*Placebo was corn oil for all studies

the nose and urinary tract, and an increased mortality from hemorrhagic stroke was reported in Greenlandic Inuits with high intakes of fatty fish (mean intakes of n-3 PUFA ~6.5 g/day) [168]. Therefore, the hypothesis that n-3 PUFA supplementation can modify platelet function and eventually might increase the risk of spontaneous bleeding and hemorrhagic stroke was addressed in several controlled clinical trials.

A prospective randomized open-label, blinded end point trial study (*Japan EPA Lipid Intervention Study*, JELIS) compared the effects of EPA-EE (1.8 g/day) consumed for five years in combination with statins (n = 9326) versus statins alone (n = 9319) [219]. After a mean follow-up of 4.6 years the primary end point (major coronary event, including sudden cardiac death, fatal and non-fatal myocardial infarction, and other non-fatal events including unstable angina pectoris, angioplasty, stenting, or coronary artery bypass grafting) in the EPA group was 19 % lower (2.8 vs. 3.5 %; P = 0.011). The risk of unstable angina and non-fatal coronary events was also significantly reduced (24 and 19 %, respectively) in the EPA group, but sudden cardiac death and coronary death did not differ between groups. In this study, no significant intergroup differences were observed for the risks of cerebral embolism, transient ischemic attack, undetermined cerebral infarction or cerebral and subarachnoid hemorrhage, but there was a borderline significant reduction in cerebral thrombosis [220]. Although self-reported epistaxis and subcutaneous bleeding was more frequently reported in the EPA group than in controls, the EFSA Panel [168] recognized that self-reported side effects are subject to high reporting bias in open-label studies. The Panel remarked that no significant differences in the total incidence of stroke, or cerebral or subarachnoid hemorrhage, which were objectively assessed, were observed between groups. In several prospective cohort studies that analyzed the relationship between dietary intake of n-3 PUFA and risk of stroke, increased risk of hemorrhagic stroke has not been reported [221, 222]. In these studies, mean dietary intakes of n-3 PUFA at the highest quintiles were <1 g/day.

A Cochrane review [223] analyzed 7 RCT (3 in normotensives, 2 in hypertensives, 1 in a mixed population of normo- and hypertensives, and 1 in heart failure patients) which used EPA + DHA at doses of 1.8–6.9 g/day for 6–24 months, and no difference in the risk of bleeding between the intervention (n = 17/949) and control (or placebo, n = 13/836) groups was observed. Harris et al. [224] reviewed 19 controlled intervention studies (n = 4397) in patients who underwent coronary artery bypass grafting (n = 2 studies), carotid endarterectomy (n = 2), and femoral artery catheterization (n = 15) who received n-3 PUFA (1.4–21 g/day) from different sources (fish oil or capsules with EPA and DHA as TG or EE) in addition to anticoagulant medications. In 14 of these trials, n-3 PUFA were administered 1–42 days prior to surgery, and in 5 studies, postoperatively. Interestingly, none of the studies observed an increased frequency or severity of bleeding complications associated with EPA and DHA supplementation. Another meta-analysis of RCT [225] reviewed the effects of n-3 PUFA (EPA + DHA 0.9–6.9 g/day for 1–55 months) in 29 RCT recruiting 35,144 high-risk cardiovascular patients. The primary outcome was all-cause mortality and secondary outcomes were coronary restenosis following percutaneous

coronary intervention and safety. However, n-3 PUFA were not associated with an increase risk of bleeding (RR 0.85; 95 % CI 0.52, 1.27).

Leaf et al. [226] randomized 551 candidates for percutaneous intraluminal coronary angioplasty to high doses of EPA (4.1 g/day) and DHA (2.8 g/day) or placebo for 14 days before, and 6 months following percutaneous intraluminal coronary angioplasty. Although all patients received 325 mg of aspirin for 6 months post-angioplasty there were 3 % bleeding episodes in each treatment group. A retrospective review of bleeding complications was performed in 182 subjects treated with high-dose fish oil (mean dose 3 g/day), aspirin (mean dose 161 mg/day), and clopidogrel (mean dose 75 mg/day) and 182 controls on aspirin and clopidogrel alone [227]. During a mean follow-up period of 33 months, 1 major bleeding episode occurred in the treatment group and no major bleeding episodes occurred in the control group. Therefore, it was concluded that high-dose fish oil is safe in combination with aspirin and clopidogrel. Salisbury et al. [228] studied the relation between n-3 index and bleeding in 1523 patients hospitalized with acute myocardial infarction (when patients are at high risk of bleeding due to the use of potent antithrombotic medications and invasive management) and found that there were no differences in bleeding across n-3 index categories. Thus, there is little reason for concern about excessive bleeding in patients who take fish oil supplements concurrent with modern medical therapy for acute myocardial infarction.

Harris et al. [229] examined the effects of n-3 PUFA and aspirin on platelet function in 8 healthy men treated with aspirin over 3 days (325 mg on day one followed by 80 mg daily on days 2 and 3) before beginning 2 weeks of fish oil supplementation (4.5 g/day). Aspirin alone prolonged bleeding time by 34 % and fish oil only by 9 %, whereas their combination prolonged the bleeding time by 78 %, a value which was not significantly different than the sum of the individual increases, but did not increase the antiaggregant effect of aspirin. Another review analyzed 31 supplementation studies with n-3 PUFA in 485 patients with end-stage renal disease undergoing dialysis and treated with antithrombotic drugs (mostly aspirin). The majority of trials were small (ranging between 9 and 25 subjects) and uncontrolled, lasted 4–24 weeks and all except two (which provided EPA at doses 1.8 and 3 g/day, respectively) used fish oil (1.4–7.6 g/day) [230]. The risk of increased bleeding times was observed, primarily with >3 g/day fish oil, and serious bleeding complications were isolated to a single patient in one uncontrolled study consuming 3 g/day of EPA, although the event could not be attributed to the EPA treatment [231].

One single-arm intervention study recruited 16 children, 7–8 years of age, with hyperlipidemia and end-stage renal disease on dialysis and at high risk of bleeding. They were supplemented with fish oil (3–8 g/day weight-adjusted dose; 0.3–2.4 g/day of EPA + DHA) for 8 weeks and then followed up for one month thereafter [232]. Platelet counts were normal and no spontaneous bleedings were observed. Similarly, no bleedings were found in another open-label trial in 9 children with attention-deficit/hyperactivity disorder receiving high doses of EPA + DHA (10.8 g and 5.4 g/day) for 4 weeks [233]. The Fish oil trials in pregnancy (FOTIP) included 1040 women with high-risk pregnancies who were randomly assigned to receive fish oil (32 % EPA, 23 % DHA) or olive oil from around 20 weeks (prophylactic trials) or 33 weeks (therapeutic trials) until delivery [234]. No increased risk of bleeding complications at delivery were observed in these six studies; indeed, intracranial hemorrhage occurred in 7 and 3 infants in the fish oil and olive oil group, respectively. This corresponded to a relative risk of 2.4 (95 % CI 0.6–11.6, $P = 0.22$).

Conversely, Clarke et al. [235] described an increased incidence of epistaxis in a single-arm intervention study enrolling 11 children and adolescents with familial hypercholesterolemia treated with fish oil for six months (starting at 1 g/day the first month and increasing by 1 g/day monthly up to 5 g/day). However, the publication did not mention the time at which 8 of the 9 episodes of epistaxis occurred or the dose of EPA and DHA consumed at the time of the events. These bleeding episodes were not observed in other studies using higher doses of EPA and DHA in children at low [233] or high [232] risk of bleeding, or in a number of controlled intervention studies in adults at high risk of bleeding.

Thus, the EFSA Panel considers that long-term supplemental dietary intakes of EPA + DHA up to 5 g/day for up to 2 years and up to about 7 g/day for up to 6 months did not increase the risk of spontaneous bleeding episodes or bleeding complications, even in subjects at high risk of bleeding (e.g., taking acetylsalicylic acid or anticoagulants) [168]. The Panel also considers that intakes of EPA alone at doses up to 1.8 g/day for two years do not raise safety concerns for the adult population.

Carcinogenesis and Mutagenesis

Treatment of rats by oral gavage with OM-3 EE (Lovaza) or OM-3 carboxylic acids (Epanova) even at the dose of 2000 mg/kg/day (5 times human systemic exposures following an oral dose of 4 g/day based on a body surface area comparison) for 101 weeks (males) and 89 weeks (females) did not increase the incidence of tumors [92, 160]. Both OM-3 EE and OM-3 carboxylic acids were not mutagenic or clastogenic in the bacterial mutagenesis (Ames) test with *Salmonella typhimurium* and *Escherichia coli* or in the chromosomal aberration assay in Chinese hamster V79 lung cells or human lymphocytes. They were also negative in the in vivo mouse micronucleus assay [92, 160].

Reproduction Studies

In rat reproduction studies, Lovaza and Epanova did not produce an adverse effect on male or female fertility. Both formulations did not produce adverse effects in female rats given oral gavage doses up to 2000 mg/kg/day beginning 2 weeks prior to mating and continuing through day 6 of gestation. In a fertility study of rats treated with oral gavage doses of 2000 mg/kg/day of Lovaza (males for 10 weeks prior to mating and females for 2 weeks prior to and throughout mating, gestation, and lactation), no adverse effects on fertility were observed. Similar results were obtained in female rats given oral gavage doses of Epanova (up to mg/kg/day) beginning 2 weeks prior to mating and continuing through day 6 of gestation [92, 160].

In pregnant rats given oral gavage doses of Epanova (100, 600, and 2000 mg/kg/day) from gestation day 6 through organogenesis, late embryonic deaths and embryos with skeletal variations were observed. In pregnant rabbits given oral gavage doses of Epanova (from gestation day 6 through organogenesis), skeletal malformations, variations in ossification, and visceral variations were observed in the fetuses in groups given up to 500 mg/kg/day (2 times human systemic exposure following an oral dose of 4 g/day based on a body surface area comparison). At 750 mg/kg/day, several rabbits aborted and evidence of maternal toxicity was observed, and there was an increase in the incidence of fetuses with malformations and variations. In another study, pregnant rats were given oral gavage doses of Lovaza of 1000, 3000, 6000 mg/kg/day from gestation day 6 through day 15, or 2000 mg/kg/day from gestation day 14 through lactation day 21. At doses >3000 mg/kg/day (7 times the human systemic exposure following an oral dose of 4 g/day), Lovaza produced an embryocidal effect, decreasing live births and survival to postnatal day 4 by 20 and 40 %, respectively [160].

In a multigenerational developmental study in pregnant rats given oral gavage doses of Epanova (100, 600, and 2000 mg/kg/day) from gestation day 6 through lactation day 21, difficulties during and shortly after parturition led to morbidity/mortality in 9 of 24 dams given the highest dose. There were no abnormalities observed in offspring (F1) from treated dams, but survival was decreased from day 10 of lactation onward in second generation offspring (F2) from dams given 600 mg/kg/day (1.5 times the human systemic exposure following an oral dose of 4 grams/day based on a body surface area comparison) [92].

Pregnancy and Breast-feeding

There are no well-controlled studies in pregnant women and on the excretion of n-3 PUFA into human milk. Therefore, n-3 PUFA should be used during pregnancy and lactation only if the potential benefit justifies the potential risk to the fetus (Pregnancy Category C).

At low doses (≤ 3 g daily), fish oils are safe in pregnant and breast-feeding women [236]. However, concerns have been raised regarding the potentially dangerous effects of industrial and environmental contaminants (mercury, dioxin-like compounds and polychlorinated biphenyls) found, in particular, in fatty fish [12–15]. Organic mercury is highly lipophilic and accumulates in the body lipids of fatty fish more than in fish oil. Methylmercury accumulates more than inorganic mercury in larger and longer-living predators (e.g., swordfish) and is much more toxic; of note is the neurologic damage to developing fetuses and young children caused by toxic levels of methylmercury [237]. Interestingly, the most commonly consumed dietary sources of n-3 PUFA (salmon, sardines, trout, herring) have low levels of mercury [12, 13, 15]. Nevertheless, contaminants are virtually eliminated during the manufacture and processing of fish oils supplements.

Several studies and meta-analysis concluded that maternal intake of n-3 PUFA or marine oil supplementation during pregnancy resulted in a slightly longer gestation period (1.6–4.5 days) and somewhat higher birthweight [238–240], particularly in newborns with higher concentrations of DHA in umbilical plasma PL [241]. In the Cochrane review of 6 trials involving 2783 women, marine oil supplementation during pregnancy resulted in a mean gestation that was 2.6 days longer and the birthweight was slightly higher (weight mean difference 47 g) [242]. n-3 PUFA derived from fish oil are excreted in human milk at levels higher than plasma, and thus, infant RBC and plasma DHA concentrations at birth are correlated with the maternal n-3 status [243]. Throughout gestation, accretion of maternal, placental, and fetal tissue occurred and consequently the n-3 PUFA requirements of pregnant women and their developing fetuses are high.

DHA availability to the growing fetus and infant is important because this is the period of most rapid brain growth and development and it has been suggested that DHA plays a major role in development of the brain and retina during fetal development and the first 2 years of life [218, 236]. In fact, DHA is found in high concentrations in the retina and accumulates in the brain during early life (from 3 months of gestation to 18 months after delivery in humans) and feeding animals with a n-3 PUFA-deficient diet results in visual and cognitive abnormalities. These observations strongly suggested that an adequate supply of n-3 PUFA and, in particular preformed DHA, is required for the development and function of the central nervous system, including the retina and brain. Studies in preterm infants strongly suggested that formulas containing DHA improve visual function early in infancy [244]; furthermore, formulas

containing DHA have been shown in some studies, although not all, to improve cognitive function in term infants [245].

Supplementation with flaxseed oil rich in ALA (10.7 g/day for 4 weeks) increased breast milk, plasma, and RBC ALA levels over time [246] and EPA supplementation also increased DPA in plasma, RBC and breast milk. However, because conversion of ALA into DHA is limited (1–5 %), no significant changes were observed in breast milk, plasma, or RBC DHA contents after flaxseed oil supplementation [26, 27]. Thus, adequate intakes of preformed DHA in pregnant women, lactating women, and young children should be considered conditionally essential. Maternal DHA levels decline during pregnancy, but maternal plasma DHA levels increased dose-dependently in response to DHA supplementation [247, 248], and then, DHA is transferred from the mother to the fetus through the placenta and to the infant postpartum through breast milk [249]. DHA levels decreased by ~50 % in plasma PL and RBC within 4 months after birth, but human milk DHA content increased in a linear, dose-dependent manner in response to DHA supplementation or in infants fed with DHA-rich formula [250, 251]. Rat pups fed from the first day of life with a n-3-deficient formula exhibited a loss of more than 70 % of their brain DHA content by 29 days of age (and retinal level of DHA by 69 %) [252]. Interestingly, repletion of brain and retinal DHA was attained after oral supplementation of n-3-deficient animals. Human autopsy studies also showed that infants consuming formulas without preformed DHA have lower brain DHA than infants receiving DHA via breast milk [244, 249, 253]. Together, these studies confirmed that tissue concentrations of DHA and EPA can be elevated through dietary supplementation.

At 21 weeks of gestation, 144 women were enrolled into a randomized, double-blind clinical trial and treated with a basic vitamin–mineral supplement (V/M group), V/M plus 4.5 g fructooligosaccharide (FOS group) and V/M plus FOS plus 200 mg fish oil-derived DHA (DHA-FOS group) [254]. At 37 weeks of gestation, and 3 months after delivery, RBC-DHA were significantly higher in the DHA-FOS group. The breast milk DHA% was twice as high in the DHA-FOS group than in the other two groups, and the RBC-DHA% of the infants in the DHA-FOS group was also significantly higher and correlated significantly with maternal RBC-DHA%. In another randomized, double-blind, placebo-controlled study, 98 women received either 4 g/day of fish oil (56 % DHA and 28 % EPA) or placebo (olive oil) from 20 weeks of gestation until delivery [255]. Compared to the control group, maternal EPA and DHA were significantly higher in the fish oil group at 30 and 37 weeks of gestation, and remained elevated at 6 weeks postpartum. Similarly, the proportions of EPA and DHA were significantly higher in RBC from neonates in the fish oil group, compared to those in the control group. In another study, 590 pregnant women in weeks 17–19 of pregnancy were given either 10 mL cod liver oil (n-3 PUFA) or corn oil (n-6 PUFA) daily until three months after delivery. Cod oil increased the concentration of DHA in maternal as well as infant plasma and umbilical tissue PL, as compared to corn oil [256]. Moreover, the maternal plasma TG increase during pregnancy was less pronounced in women supplemented with cod liver oil as compared to corn oil. These results confirmed that maternal supplementation with n-3 PUFA during pregnancy and lactation provides more DHA to the infant and reduces the maternal plasma lipid levels compared to supplementation with n-6 PUFA.

Contraindications

Fish-derived supplements are contraindicated in individuals with known hypersensitivity to any component of the formulation and should be used with caution in patients with known hypersensitivity to fish and/or shellfish or sensitive to seafood, nuts, seeds, or plants from which n-3 PUFA are derived. The EFSA panel [168] considers that supplemental intakes of EPA + DHA of up to 5 g/day consumed for up to 12–16 weeks do not significantly affect glucose homeostasis in healthy or diabetic subjects and do not induce changes in lipid peroxidation which might raise concern in relation to cardiovascular disease risk as long as the oxidative stability of these n-3 PUFA is guaranteed. Moreover, supplemental intakes of EPA and DHA up to about 5 g/day are unlikely to induce changes in immune functions which might raise concern in relation to the risk of infections or inappropriate activation of inflammatory responses.

Possible Drug Interactions

Antiplatelet Agents

EPA, DPA, and DHA inhibited platelet aggregation induced by several agonists [collagen and platelet-activating factor (PAF)] and reduced the release of TXA_2 and TXB_2 from collagen-aggregated platelets and the plasma levels of fibrinogen and coagulation factors V and VII [71, 257, 258]. Therefore, patients taking antiplatelet and/or antiaggregant drugs were excluded from treatment in patients with hypertriglyceridemia. A double-blind, placebo-controlled, parallel trial assessed whether DHA and EPA could have differential effects on platelet aggregation [259]. They randomized 59 hypertensives with type 2 diabetes mellitus and postmenopausal women to 4 g/day of EPA, DHA or olive oil (placebo) for six weeks. DHA, but not EPA, supplementation significantly reduced collagen aggregation (−16.9 %) and TXB2 production (−18.8 %), whereas no

significant changes were reported on PAF-stimulated platelet aggregation, fibrinolytic function or vascular function in either the EPA or DHA groups relative to placebo. Another study compared the inhibition of platelet function produced by EPA-EE (4 g/day) or a concentrated fish oil extract (6 g/day). After 4 weeks, both supplements increased the amount of EPA in platelet PL. EPA-EE increased the bleeding time by 57 % and the threshold dose of collagen needed to induce platelet aggregation by 46 % and decreased the TXA2 synthesis in response to collagen by 65 %, thus being more effective in decreasing platelet reactivity than a concentrated fish oil extract providing an equivalent amount of n-3 PUFA [260].

Violi et al. [261] reviewed 7 studies (3 on healthy subjects and 4 on patients at risk of hypercholesterolemia, hypertension, type 2 diabetes, or a combination of these) that analyzed the effects of EPA and DHA supplementation (1–4 g/day for 30 days to 1 year) on platelet function. Among these studies, 2 trials of shorter and longer duration showed no effect of n-3 PUFA on platelet aggregation, while 5 showed inhibition of platelet function or prolongation of platelet survival. Interestingly, the inhibitory effect did not appear to be dose-related because it was observed with 1 or 4 g/day. They also identified 8 studies that specifically investigated the effect of ALA (0.86–5.9 g/day) on platelet function in healthy subjects or patients at risk of atherosclerosis. In general, the studies did not consistently show an inhibitory effect of ALA on platelet function. In another meta-analysis of 15 randomized controlled trials, n-3 PUFA supplementation significantly reduced adenosine diphosphate (ADP)-induced platelet aggregation and there was a trend toward decreased collagen- and AA-induced platelet aggregation compared with controls [262].

In a placebo-controlled, randomized, double-blind study, 11 patients with prosthetic heart valves, cardiomyopathy or deep vein thrombosis treated with warfarin and with stable international normalized ratio (INR) values for at least 4 weeks were assigned to receive fish oil (3 or 6 g/day) for 4 weeks. There were no significant differences in INR values between the groups during the trial, which indicated that there was not a clinically significant interaction between warfarin and fish oil supplements up to 6 g/day [263]. In another randomized, double-blind, placebo-controlled trial enrolling 44 patients undergoing elective major abdominal surgery, fish oil administered up to 0.2 g/kg/day did not modify platelet aggregation and had no effect on bleeding events [264].

The effect of fish oil supplementation (EPA 2 g/day + DHA 1.3 g/day) on hemostatic parameters and bleeding episodes was investigated in a placebo-controlled study in 521 patients undergoing coronary artery bypass surgery treated with either aspirin (300 mg/day) or warfarin (INR 2.5–4.2) [265]. During a follow-up of 9 months, no excess of bleeding episodes attributed to fish oil were observed. Another study analyzed in 10 healthy volunteers the effects of a prescription omega-3 FA product (OM-3 FA) and aspirin, alone and in combination, on platelet aggregation [266]. Blood samples were taken on day 1 (baseline), day 2 (one day after taking aspirin 325 mg bid), day 29 (after 28 days of P-OM3, 4 capsules/day) and day 30, after one day of combined OM-3 FA and aspirin. Platelet aggregation was not affected by OM-3 FA alone, but increased in the groups treated with aspirin. The OMEGA-PCI (*OMEGA-3 Fatty Acids After PCI to Modify Responsiveness to Dual Antiplatelet Therapy*), an investigator-initiated, prospective, single-center, double-blind, placebo-controlled, randomized study, recruited 63 patients undergoing percutaneous coronary intervention who received standard therapy (including aspirin 75 mg/day and clopidogrel 600 mg loading dose followed by 75 mg/day). Patients were randomly assigned to the treatment with 1 g/day n-3 PUFA (460 mg EPA + 380 mg DPA as EE) or placebo for 1 month. In these patients, the $P2Y_{12}$ reactivity index was significantly lower (22.2 %) after 1 month of treatment with n-3 PUFA compared with placebo, which indicated that the addition of OM-3 EE to the combination of aspirin and clopidogrel significantly potentiated platelet response to clopidogrel after percutaneous coronary intervention. In another 14-day study of 52 healthy adult subjects, Epanova (4 g/day) at steady state did not significantly change the single-dose AUC or C_{max} of R- and S-warfarin or the anticoagulation pharmacodynamics of 25 mg warfarin [92].

The EFSA Panel [168] concluded that the changes on platelet function observed at supplemental intakes of EPA and DHA (either alone or in combination) up to about 4 g/day are not considered to be adverse as they are not associated with an increased risk of clinical complications (e.g., spontaneous bleeding). Moreover, the Panel recognized that even when some trials found that n-3 PUFA can produce a prolongation of bleeding time, it did not exceed normal limits and did not produce clinically significant bleeding complications. Nevertheless, studies have not been performed to thoroughly examine the effect of the combination of Lovaza or Epanova with anticoagulant and antiaggregant drugs. Thus, the manufacturers of Lovaza or Epanova (4 g/day) advise caution when both n-3 PUFA formulations are administered in patients at high risk of bleeding and/or treated with oral anticoagulants, heparins (unfractionated, low-molecular-weight heparins), factor Xa inhibitors (rivaroxaban, apixaban, and edoxaban), direct thrombin inhibitors (bivalirudin, dabigatran) or with herbs and supplements that increase the risk of bleeding (danshen, dong quai, garlic pills, ginger, ginkgo biloba, horse chestnut, willow bark). Therefore, patients receiving this combination should be monitored periodically [92, 160].

HMG-CoA Reductase Inhibitors: Statins

The combination of n-3 PUFA with a statin represents a treatment option for patients with mixed hyperlipidemia. In a randomized crossover trial, simvastatin (80 mg/day) was administered under fasted conditions with or without Lovaza (4 g/day) for two 14-day periods in 24 healthy adults. At steady state, Lovaza (4 g/day) did not affect the extent (AUC) or rate (C_{max}) of exposure to simvastatin or its major active metabolite, beta-hydroxy simvastatin [267]. In another randomized, open-label, repeated-dose study in 50 healthy adults, the coadministration of atorvastatin (80 mg/day) with Lovaza (4 g/day) for 14 days did not affect the AUC or C_{max} of exposure to atorvastatin and its active metabolites (2- and 4-hydroxy-atorvastatin) at steady state [268]. Similarly, in 48 healthy adults, the combination of Lovaza (4 g/day) with rosuvastatin (40 mg/day) for 14 days did not affect the AUC or C_{max} of exposure to rosuvastatin at steady state [269].

Of note, recent evidence suggests that statins may inhibit the cardioprotective effects of n-3 PUFA [270]. A meta-analysis of 14 randomized, double-blind, placebo-controlled trials involving 20,485 patients with a history of cardiovascular diseases found no beneficial effect of n-3 PUFA supplements on several cardiovascular events, such as sudden cardiac death, myocardial infarction (fatal or non-fatal), stable and unstable angina pectoris, congestive heart failure, transient ischemic attack and stroke, or on all-cause mortality [3]. Furthermore, no significant preventive effect was observed in subgroup analyses by history of cardiovascular disease, country location, geographic area, duration of treatment, dosage of EPA or DHA, concomitant medication use, type of placebo material in the trial, methodological quality of the trial, or use of fish oil supplementation only as treatment. However, a subgroup analysis for concomitant medication use found a non-significant preventive effect against the risk of cardiovascular events (RR 0.74, 95 % CI 0.54–1.03) among patients not receiving statins, whereas those receiving statins had no protection at all (RR 1.02, 95 % CI 0.92–1.12). A similar finding was observed in patients with a history of myocardial infarction, so that in those who were not treated with statins, low-dose supplementation with n-3 fatty acids may reduce major cardiovascular events [271]. These data suggest strong interactions between n-3 PUFA and statins which may, at least partly, explain the discrepancy between recent and early randomized clinical trials as in recent RCT most patients (>90 %) are treated with statins, whereas they were prescribed in a reduced number of patients in early RCT. It has been proposed that statins increase AA in cell membranes which may, in turn, inhibit the cardioprotective effects of n-3 PUFA; additionally, statins inhibit the synthesis of coenzyme Q10, a key component in mitochondrial bioenergy transfer [270]. More importantly, in contrast to old trials enrolling high-risk n-3 PUFA-deficient patients, most patients included in recent RCT were not severely n-3-deficient. Therefore, it would be expected that n-3 PUFA supplements would be protective only in patients who are deficient in n-3 PUFA and not in those at high risk for reasons other than n-3 deficiency. In this latter group of patients, the expected benefit of n-3 PUFA supplementation will not be high and statins could almost totally eliminate the small benefits expected from n-3 PUFA supplements.

Antihypertensive Drugs

n-3 PUFA produce arterial vasodilation and reduce arterial blood pressure. Thus, caution should be exercised in patients treated with antihypertensive agents to avoid an excessive reduction in blood pressure. In a clinical trial, 16 male hypertensive patients received propranolol (80 mg/day) for 36 weeks; 15 patients received a supplement of encapsulated fish oil (9 g/day, equivalent to 1.8 g/day of EPA + 1.1 g/day of DHA) for 36 weeks; and another 16 patients received propranolol for 12 weeks, propranolol plus fish oil capsules for 12 weeks and propranolol plus fish oil placebo for another 12 weeks [272]. The blood pressure-lowering effect of fish oil was comparable to that of propranolol and the simultaneous intake of fish oil plus propranolol was more effective to reduce blood pressure than propranolol or fish oil alone. In another double-blind placebo-controlled crossover trial, 43 hypertensives treated with diuretics or beta-blockers were randomly assigned to take either Lovaza (EPA 1.9 g + DHA 1.5 g daily) or corn oil (4 g/day) for 6 weeks [273]. Lovaza decreased systolic/diastolic blood pressures and the mean within-individual difference in blood pressure compared with corn oil supplementation was $3.1 \pm 1.0/1.8 \pm 0.6$ mmHg, $P < 0.01$).

Other Interactions

n-3 PUFA supplements may increase fasting glucose plasma levels. Thus, in diabetic patients it may be necessary to monitor plasma glucose levels and to adjust the doses of glipizide, glyburide, metformin, or insulin. In patients after cardiac transplantation treated with cyclosporine, n-3 PUFA may reduce the increase in blood pressure and the kidney damage produced by this immunosuppressant drug [274]. Orlistat, a pancreatic lipase inhibitor that prevents dietary fats from being absorbed in the small intestine, might inhibit the gastrointestinal absorption of fish oil when they are taken together [275]. To avoid this potential interaction orlistat and

fish oil should be administered at least 2 h apart. Cigarette smoking increases the oral bioavailability of n-3 PUFA from plasma counteracting in part the losses occurring through β-oxidation, enhances the percent conversion of EPA to DPA and the fractional synthetic rate for formation of DHA among women smokers was triple that of non-smokers [35]. Since the free forms of the EPA and DHA are undetectable in the circulation (<1 μM), clinically significant drug–drug interactions due to inhibition/induction of P450-mediated metabolism EPA/DHA combinations are not expected in clinical practice.

Conclusions

Mammalian cells are not able to synthesize de novo α-linolenic acid, and it can only be converted to a limited extent to EPA and to a very small extent to DHA. At the present time, there are several formulations of n-3 PUFA (nTG, rTG, EE, FFA and KO) both for dietary supplementation and for therapeutic administration, containing greatly variable amounts of EPA and DHA which present important differences in their pharmacokinetic properties. The pharmacokinetic information is of great interest given increasing public interest for n-3 PUFA supplementation, and the addition of n-3 PUFA to many foodstuffs. Both short-term (postprandial) and long-term studies have analyzed the oral bioavailability of n-3 PUFA in healthy volunteers. Unfortunately, these studies present multiple important methodological limitations which explain why contradictory results are frequently reported and the difficulty to extrapolate the results from the short-term to those reported in long-term studies. Due to ethical reasons the study of n-3 PUFA distribution in human tissues is severely limited and the biotransformation is extrapolated from animal models. Clearly, further studies with a larger sample size are needed to identify the pharmacokinetic differences in n-3 PUFA bioavailability among the most common formulations. The goal is to develop new formulations with increased oral bioavailability and reduced intersubject variability. Whether greater bioavailability translates into differences in plasma TG in subjects with hypertriglyceridemia and greater cardiovascular protection is presently uncertain. Finally, most of the studies have been performed in healthy young volunteers, while the pharmacokinetic profile of n-3 PUFA in elderly people and in patients with common comorbidities (i.e., hypertension, diabetes, obesity, hypertriglyceridemia) and receiving polypharmacy, i.e., those that can benefit most from n-3 PUFA supplementation, remains unknown. Therefore, pharmacokinetic studies of the n-3 PUFA should be performed in the next future in these populations.

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Fish Oil Fatty Acids for Aging Disorders

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Jyothi Maria Veigas and Gabriel Fernandes

Introduction

Aging is inevitable and so is death. Aging in humans begins between the age of 40 and 50, and the survival of an organism depends on the balance between damage and processes of maintenance and repair systems of the body. Therefore, in order to achieve healthy aging, interventions aimed at strengthening body's maintenance and repair systems rather than treating the damage [1] may help prevent or delay the damage to begin with. It is every individual's desire, as Row and Kahn [2] would put it, to age successfully, that is, to grow older avoiding disease and disability, maintaining high physical and cognitive function, and conducting sustained engagement in social and productive activities. Exercising control over what we eat and how much we eat plays an important role in reaching this goal.

Long-chain polyunsaturated fatty acids (PUFAs) such as n-3 fatty acids and n-6 fatty acids have long been known to be essential for optimal health. n-3 fatty acids can be obtained from both plant and marine sources. Biologically, active n-3 fatty acids, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are found in marine fish, the richest sources being anchovy, herring, farmed and wild salmon, and mackerel along with other fish such as sardines, tuna, and bluefish. Certain algae also produce high levels of EPA and DHA. The chief n-3 fatty acid found in plant sources is alpha-linolenic acid (ALA) which is found mostly in walnuts, flax seed, canola, etc. In order to exert benefits similar to EPA, ALA must be first converted to EPA by a series of elongation enzymes which only occurs to a small extent in humans. This conversion is also affected in the presence of high linoleic acid (LA), an n-6 fatty acid, in the diet which competes with these enzymes for its conversion to arachidonic acid [3] reducing the conversion of ALA by 40–50 % [4]. Arachidonic acid (AA) is a precursor of 4-series leukotrienes which is an important mediator of inflammation [5, 6]. In the past few decades, there has been an increase in the consumption of LA in the American diet which constitutes to about 7 % of daily caloric intake and 20 % of total dietary fatty acids [7]. While an n-6/n-3 ratio of 4:1 is presumed to be ideal, the current Western diet is largely deficient in n-3 fatty acids with the ratio ranging from 10:1 to 25:1 [8–10]. This higher ratio of n-6/n-3 fatty acids is recognized to be associated with chronic inflammatory disorders such as non-alcoholic fatty liver disease, cardiovascular disease, obesity, inflammatory bowel disease, rheumatoid arthritis, and Alzheimer's disease [11]. Further, lowering n-6 PUFA intake was associated with an increased bioavailability of esterified and non-esterified n-3 PUFA in humans [12]. Therefore, a diet comprising of a good amount of n-3 fatty acids may prevent or delay the onset of inflammatory disorders. In this context, fatty fish, fish oil supplements, and purified DHA and EPA appear to be the best sources of bioavailable n-3 fatty acids.

Since the original report of Bang et al. [13] suggesting the relationship between diet and the reduced incidence of cardiovascular disease (CVD) and complete absence of diabetes mellitus in Greenland Eskimos, there has been a considerable interest in the use of n-3 fatty acids as dietary supplements attracting much attention toward their potential in clinical uses. Several studies have shown promising results against a variety of age-related disorders such as autoimmune diseases, cardiovascular diseases, cancer, and diabetes. An increased intake ratio of n-3/n-6 fatty acids is suggested to have an inverse association with risk of breast cancer among women [14]. This finding could be of significant importance in the prevention and treatment of breast cancer. Older individuals have been shown to have increased accumulation of EPA and

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DHA [15]. A study conducted in New Zealand showed an increase in serum phospholipid EPA and DHA with age which could be attributed to higher fish consumption in older population. The study also described a gender difference in the proportion of EPA and DHA where EPA content was lower in women compared to men while it was the opposite for DHA. In another study in Finland, higher fish consumption was associated with a decreased risk of congestive heart disease (CHD) among women but not in men [16]. These data suggest that separate considerations may be given to men and women when examining the association between disease risk and biomarkers of n-3 PUFA [15] and developing treatment strategies.

There is mounting clinical evidence supporting the benefits of fish oil fatty acids in maintaining health and alleviating diseases. Developing health-oriented and preventative strategies rather than disease-oriented research and treatment approaches should be encouraged for upholding well-being of general population. This chapter focuses on the positive effects of n-3 PUFA on cardiovascular health and age-related bone loss.

Fish Oil Fatty Acids and Cardiovascular Health

Cardiovascular diseases top the list of leading causes of death worldwide. A healthy diet and lifestyle are key to maintaining a healthy heart and a healthy body. Aging is considered one of the major risk factors for CVD. It is widely accepted that fish oil, rich in n-3 PUFA, protects against CVD risk factors, CHD, myocardial infarction (MI), atherosclerosis, and hypertension [17–20]. American Heart Association (AHA) recommends at least 2 servings of fish-containing n-3 fatty acids per week for persons without documented heart disease and about 1 g of EPA + DHA per day for patients with documented heart disease. AHA also recommends 2–4 g EPA + DHA, under physicians care, for people with high triglycerides levels [21].

Report of Bang et al. [13] on food composition of Greenland Eskimos and a low CVD incidence evoked a flurry of animal studies and clinical trials to support the cardioprotective effects of n-3 fatty acids. However, cardiovascular benefits of n-3 PUFA continue to raise qualms and queries regarding their validity. In a recent review, Fodor et al. [22] refute the hypothesis of Bang et al. [13] stating that they arrived at the proposition based on the annual reports produced by the Chief Medical Officer (CMO) in Greenland, without actually examining the cardiovascular status of Greenland Eskimos. Based on the evidence they found through a review of literature and the limited validity of CMO reports [23], Fodor et al. [22] conclude that incidence of coronary artery disease (CAD) in the Eskimos is similar to that in non-Eskimos. Despite this opposition, the report of Bang et al. [13] did evoke a tremendous interest in the scientific community with regard to benefits of n-3 fatty acids. This has resulted in the colossal information supporting their anti-inflammatory and inflammation-resolving properties [24] which can be extended to their ability to alleviate inflammation-mediated disorders, including cardiovascular diseases.

Mechanism(s) of Action

The precise mechanisms by which fish oil offers cardio protection are unclear and are thought to be brought about by synergism between multiple complex mechanisms. A detailed account of these mechanisms is reviewed by Adkins and Kelly [25]. In their article, Adkins and Kelly [25] describe that n-3 fatty acids mitigate atherogenesis by a direct regulation of transcription factors involved in inflammation and through production of 3- and 5-series eicosanoids and lipid mediators such as resolvins. It is now common knowledge that n-6 fatty acids such as arachidonic acid are pro-inflammatory in nature due to their conversion to two-series prostaglandins (PGE2), prostacyclins (PGI), and thromboxanes (TXA2) and to four-series leukotrienes and hydroxyl eicosatetraenoic acids (HETEs) by the enzymes cyclooxygenases (COX) and 5-lipoxygenases (5-LOX), respectively. On the contrary, the n-3 fatty acids DHA and EPA exert anti-inflammatory properties due to the production of eicosanoids—3 series PGE2, PGI, and TXA2 as well as 5 series leukotrienes (LT)—through their inhibitory actions on COX-2 and 5-LOX. DHA and EPA are also precursors of resolvins and protectins which possess potent anti-inflammatory properties [26]. Other factors that influence the cardioprotective actions of n-3 fatty acids are stimulation of peroxisome proliferator-activated receptor (PPARγ) and inhibition of Toll-like receptor 4 activation and NFκB activation [25]. Another possible mechanism is their direct effect on vascular smooth muscle cell function. EPA was shown to exert its endothelium-independent vasorelaxant effects in WKY rat aortae through the production of prostanoids which activate K^+ATP channels [27]. Recent studies strongly suggest that DHA has more potent and beneficial effects than EPA. DHA has been reported to decrease plasma triglycerides (TG) and DHA, but not EPA supplementation, significantly increased serum HDL cholesterol [28, 29]. Atheist a portion of the hypotriglyceridemic effect of n-3 FA has been attributed to increasing circulating apoE levels [30]. n-3 PUFAs are also shown to inhibit adhesion molecules such as ICAM-1, VCAM-1, E-selectin, and P-selectin which are involved in recruitment and platelet adhesion during thrombosis and inflammation and are expressed as a result of cytokine-induced endothelial activation [25, 31–33].

The cardioprotective action of n-3 fatty acids may also be linked to their ability to slow telomere shortening. Recently, interest in the association between telomere length, cellular aging, and age-related somatic diseases [34, 35] is gaining popularity. Blood levels of EPA and DHA have been linked to temporal changes in telomere length. Farzaneh-Far et al. [36] demonstrated an inverse relationship between telomere shortening and blood levels of n-3 fatty acids in individuals with CAD. Author reported a significantly faster rate of telomere shortening in individuals in the lowest quartile of EPA + DHA (0.13 telomere-to-single-copy gene ratio [T/S] units over 5 years; 95 % confidence interval [CI], 0.09–0.17), compared to those in the highest quartile (0.05 T/S units over 5 years; 95 % CI, 0.02–0.08; $p < 0.001$) [36]. Telomere length has been suggested to be an important biomarker of aging. Though it is not solely implicated in aging, persons with longer telomeres have been observed to live longer than the ones with shorter telomeres [37] and healthy centenarians had longer telomeres than the unhealthy centenarians with cardiovascular and other morbidities [38]. This study reveals that complex structures such as the telomeres are amenable to influence by simple nutrients such as n-3 fatty acids to bring about change in aging dynamics in promoting health and opens up avenues to understand the intricate mechanisms involved in this role.

Clinical Evidence

A large number of clinical data support as well as refute the cardioprotective effects of n-3 fatty acids in the global population (Table 40.1). A recent review of 21 randomized control trials and clinical trials involving n-3 intervention mainly in persons with high cardiovascular risk revealed a reduction in cardiovascular events (10 %, $p = 0.001$), cardiac death (9 %, $p = 0.03$), and coronary events (18 %, $p < 0.0001$) and a slight reduction in total mortality (5 %, $p = 0.13$) in the n-3 fatty acid group compared to the control group [39]. A systematic review revealed a direct relationship between consumption of fish or fish oil and reduced all-cause mortality and cardiac and sudden death [40]. A low EPA + DHA level is associated with a risk of sudden cardiac death [41]. n-3 fatty acids have shown to decrease heart rate [42] and blood pressure in hypertriglyceridemia patients with normal to high blood pressure [43, 44]. DHA has been found to be more potent in reducing plasma triglycerides compared to EPA and only DHA supplementation significantly increased serum high-density lipoproteins (HDLs), which is associated with more efficient reverse cholesterol transport and reduced CHD [28, 45, 46]. A meta-analysis revealed decreased heart rate following fish oil intake [47]. High triglyceride level in the blood is associated with metabolic syndrome and increases the risk of diabetes, stroke, and heart disease. Prescription grade fish oil supplements containing EPA and DHA in their ethyl ester forms (Lovaza®, Omacor®, Omtryg™) and free forms (Epanova™) and an EPA-only formulation (Vascepa) are gaining significance in the treatment of high triglyceride levels [48]. They contain high concentrations of DHA and/or EPA compared to the regular, over the counter fish oils. According to the ECLIPSE II study, free fatty acid formulations of EPA and DHA are more bioavailable than the ethyl ester forms [49] suggesting a better triglyceride lowering effect at a lower dose by the former compared to the latter. Fish oil fatty acids have also shown to reduce cardiovascular adverse events associated with rosiglitazone therapy in aging insulin-resistant C57BL/6J mice. Supplementing rosiglitazone-treated mice with 5 % fish oil inhibits left ventricular (LV) hypertrophy and attenuates cardiac remodeling through reduced LV expression of atrial and brain natriuretic peptides, fibronectin and interleukin-6 (IL-6), and tumor necrosis factor-α (TNFα), concomitant with increased IL-10 and adiponectin levels [50]. However, the OMEGA trial failed to observe any improvements in the rates of sudden cardiac death, total mortality, revascularization, and major cardiovascular and cerebrovascular adverse events in a population of acute MI survivors [51]. On the contrary, the OMEGA-PCI study (Table 40.1) aimed at investigating whether n-3 fatty acids can modify responsiveness to dual antiplatelet therapy in stable CAD patients undergoing percutaneous coronary intervention showed that addition of ethyl esters of n-3 fatty acids to aspirin and clopidogrel therapy potentiated platelet response to clopidogrel in these patients [52].

The balance between n-3 and n-6 fatty acids plays an important role in determining cardiovascular health. A significant negative correlation has been associated with EPA/AA ratio and acute coronary syndrome (ACS) [53]. An analysis of the serum EPA, DHA, and arachidonic acid (n-6) in 1119 patients with cardiovascular complaints revealed that a low EPA/AA but not DHA/AA ratio was associated with an increased incidence of ACS and CAD [53, 54]. Serum n-3 fatty acid levels were inversely correlated with coronary heart disease [55]. According to Superko et al. [56], blood levels of n-3 fatty acids are a more reliable indicator of CVD risk or benefit rather than a fixed dose. For example, as they point out in the JELIS study, the risk of major coronary events was significantly decreased at high plasma EPA level (>133 μg/mL) or an EPA/AA ratio of >0.75 was found to be most protective [57]. However, there are limitations to the clinical trials involving omega-3 fatty acid intervention. According to a recent review, in most of the interventional clinical trials, subjects were recruited regardless of their base line EPA and DHA level. Since individuals with high baseline levels are less likely to develop cardiovascular events, the results may be negatively

Table 40.1 List of select clinical trials assessing the cardiovascular benefits of n-3 fatty acids in older adults

Study (Ref)	Sample/intervention	Intervention	Outcome		
GISSI-Prevenzione [67]	Patients surviving recent MI	1 g/day n-3 PUFA (n = 2836) 0.3 g/day Vitamin E (n = 2830) n-3 PUFA + Vitamin E (n = 2830) Control (n = 2828) for 3.5 years	n-3 PUFA, but not vitamin E, reduced the risk of death and cardiovascular death		
OMEGA [51]	Acute myocardial infarction survivors	1 g/day n-3 fatty acid ethyl esters (n = 1940) or control (n = 1911) for 1 year in addition to guideline-adjusted treatments	Event (after 365 days)	N-3	Control
			Sudden cardiac death, p = 0.84 Total mortality, p = 0.18 Revascularization, p = 0.34 Major adverse events, p = 0.1	1.5 % 4.6 % 27.6 % 10.4 %	1.5 % 3.7 % 29.1 % 8.8 %
			n-3 supplementation did not improve the outcomes compared to guideline-adjusted treatments		
JELIS [57]	Patients with total cholesterol level of $\geq$250 mg/dL; LDL-cholesterol $\geq$170 mg/dL at baseline	6 capsules of 1800 mg EPA containing 300 mg pure EPA ethyl ester (n = 8321) Conrol (n = 8076)	Increased plasma EPA concentration following intervention was associated with significantly reduced coronary events		
VASCAZEN-REVEAL [125]	Cohort 1: TG = 1.02–2.25 mM/L (n = 90) Cohort 2: TG = 2.25–5.65 mM/L (n = 56)	4 g/day VASCAZEN, (6:1 EPA:DHA) or Corn oil for 8 weeks Cohort 1: Vascazen (n = 49); Corn oil (n = 41) Cohort 2: Vascazen (n = 26); Corn oil (n = 30)	Increased Omega Score[a] (132 % $p < 0.0001$) Reduced AA:EPA ratio (82 %, $p < 0.0001$) Cohort 2: Improved HDL cholesterol (9 %, p = 0.0069) Reduced TG (48 %, $p < 0.0001$) Reduced VLDL cholesterol (30 %, p = 0.0023)		
Arrhythmia trials					
Darghosian et al. [69]	Patients with paroxysmal or persistent AF	4 g/day Lovaza (n = 126) or placebo (n = 64) for 6 months	AF recurred in 58.7 % patients on n-3 PUFA and 46.9 % patients on placebo No difference in time to recurrence of AF between groups No clinically meaningful difference in inflammatory markers between groups		
Kowey et al. [71]	Participants with confirmed symptomatic paroxysmal or persistent AF, with no substantial structural heart disease	8 g/day Lovaza or placebo for the first 7 days followed by 4 g/day Lovaza or placebo for 23 weeks (n = 663) Cohort 1: Paroxysmal AF: Lovaza (n = 266); Placebo (n = 276) Cohort 2: Persistent AF: Lovaza (n = 66); Placebo (n = 55)	In the paroxysmal AF stratum, 24-week treatment with prescription n-3 (Lovaza) compared with placebo did not reduce recurrent AF over 6 months. In the persistent AF stratum, 50 and 33 % patients on Lovaza and placebo, respectively, had documented symptomatic AF or flutter event		
OPERA trial [72]	Patients scheduled for cardiac surgery	Preoperative loading of 8–10 g over 2–5 days and postoperatively 2 g/day for up to 10 days	AF recurred postoperatively in 30 % of patients in both groups Perioperative supplementation with n-3-PUFAs, compared with placebo, did not reduce the risk of postoperative AF		

(continued)

Table 40.1 (continued)

Study (Ref)	Sample/intervention	Intervention	Outcome
AFFORD [126]	Patients with symptomatic paroxysmal or persistent AF within 6 months of enrollment	4 g/day fish oil (n = 165) or placebo (172) for 3 weeks	AF recurred in 64.1 % in fish oil arm and 63.02 % patients in placebo arm High-sensitivity c-reactive protein and myeloperoxidase reduced from baseline level with no significant difference between groups
FORWARD [127]	Patients with symptomatic paroxysmal AF that required cardioversion, at least 2 episodes of AF in the 6 months before randomization, or both	1 g/day n-3 PUFA (n = 289) or Placebo (n = 289) for 12 months	AF recurred in 24.0 % patients on n-3 PUFA and 18.9 % patients on placebo No difference in composite all-cause mortality, nonfatal stroke, nonfatal acute myocardial infarction, systemic embolism, heart failure, or severe bleeding

[a]Omega Score = blood EPA + DHA + Docosapentaenoic acid; *AA* Arachidonic acid; *AF* Atrial fibrillation; *Hs-CRP* High-sensitivity C-reactive protein; *MPO* Myeloperoxidase; *TG* Triglyceride; *HDL* High-density lipoprotein; *VLDL* Very low-density lipoprotein; *MI* Myocardial infarction; *PUFA* polyunsaturated fatty acids

impacted by the overlap of EPA + DHA levels in control subjects with high baseline levels and subjects in intervention group who might have low baseline levels [58].

The Case with Arrhythmia

The role of n-3 PUFA in arrhythmia has been a subject of much debate. Though most studies in animal models [59–61] and isolated organ and cell cultures [62–64] report an antiarrhythmic property of n-3 PUFA, a recent study points to their pro-arrhythmogenic property in a dog model of MI. Belevych et al. [65] observed that the increased n-3 PUFA content in the left ventricles of mixed breed dogs on a EPA + DHA ethyl ester diet was associated with disturbances in Ca^{2+} cycling leading to an increased risk of ventricular tachyarrhythmia in post-MI hearts [65]. The evidence in humans, however, is limited and often controversial. The first claim that n-3 PUFA lower the incidence of atrial fibrillation (AF) in the elderly came from Mozaffarian et al. [66]. They correlated consumption of broiled or baked tuna and higher plasma phospholipid EPA + DHA levels with a lower incidence of AF. This study included participants with no prevalent AF and was mostly based on their dietary consumption of tuna and other broiled or baked fish versus fried fish or fish sandwich. In the absence of a strict account on adherence to dietary guidelines, n-3 content of fish, and possibility of missed diagnosis of asymptomatic paroxysmal AF, the results may be an overestimation or underestimation of the outcome [66]. The Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione study demonstrated significant benefits of long-term n-3 PUFA consumption against risk of death and cardiovascular death in high risk patients which was correlated to their antiarrhythmic effect [67]. n-3 PUFA was shown to improve maintenance of sinus rhythm after direct current cardioversion in patients with persistent AF who are on amiodarone and a renin-angiotensin-aldosterone inhibitor [68]. However, this study involved a small patient population (n = 254) who are already on antiarrhythmia therapy and it is difficult to distinguish whether the effect was due to n-3 PUFA or other factors related to antiarrhythmia drugs or any baseline variables.

Arguably, there are more reports rejecting the antiarrhythmic claims of n-3 PUFA than there are supporting them; some even report an increased risk of arrhythmia. For example, the n-3 PUFA did not prevent the recurrence of AF in patients with paroxysmal and persistent AF at a high dose of 4 g/day [69]. While n-3 PUFA is believed to be associated with a lower risk of fatal coronary heart disease, lower incidence of stroke and reduced biomarkers of ventricular fibrillation, they have not shown to reduce the risk of AF [70]. In a prospective, double-blind randomized placebo-controlled trial involving 663 participants with confirmed paroxysmal and persistent atrial fibrillation, a high dose of n-3 PUFA (4 g/day prescription grade) over 6 months failed to reduce the recurrence of symptomatic AF [71]. Similarly, though Mozaffarian et al. [66] earlier reported an antiarrhythmic potential of n-3 PUFA in a smaller population with no prevalent AF, their subsequent, large multicenter trial (n = 1516) among patients undergoing cardiac surgery, perioperative supplementation of n-3 PUFA did not reduce the risk of postoperative AF when compared to placebo [72]. Extensive review of existing literature does not support the use of n-3 PUFA against postoperative or recurrent AF [73]. Further, long-term fish oil supplementation did not suppress paroxysmal atrial tachycardia/atrial fibrillation burden in older patients (age > 60 years) with sinoatrial node disease and dual chamber pacemakers [74].

Albert [75] argues that n-3 PUFA might be useful as agents that might prevent the development of AF rather than reverse existing AF due to their pleiotropic actions and

suggest the necessity of longer term therapy for a significant impact on atrial remodeling in patients with established AF. However, considering the inconsistent benefits of n-3 PUFA against cardiovascular diseases, particularly against existing AF or incidence of new arrhythmia, caution must be exercised while prescribing them and recommendations to increase fish intake or to take fish oil supplements must be reconsidered [76]. Further, since aspirin is usually added to the medication regimen of arrhythmia patients, high dose of n-3 fatty acids must be used with caution in this population due to their gastrointestinal side effects which is aggravated in the presence of blood-thinning agents such as aspirin.

Aging, Bone Health, and n-3 Fatty Acids

Age-related progressive decline in bone density predisposes older adults to fractures owing to intrinsic and extrinsic factors that accelerate bone loss. During early years of development, there exists a fine balance between bone formation and bone resorption by specialized cells called osteoblasts and osteoclasts, respectively, reaching a peak bone mass around the age of 18 years in women and 20 years in men. This balance is regulated by a myriad of proteins including receptor activator of nuclear factor kappa-B (RANK), its ligand RANKL, and osteoprotegerin (OPG) which are expressed or produced by cells responsible for bone turnover [77]. While RANK and RANKL are predominantly osteoclastogenic causing bone resorption, OPG is osteoblastogenic triggering bone formation [77]. As a person ages, the bone resorption takes precedence over bone formation leading to bone loss which is further accelerated in menopausal and postmenopausal women due to reduced levels of estrogen.

Most common type of bone loss disorder is osteoporosis and is one of the most debilitating disorders leading to significant disability. According to the Office of the Surgeon General, one in two Americans over age 50 is expected to have or be at risk of developing osteoporosis of the hip by 2020; even more will be at risk of developing osteoporosis at any site in the skeleton [78]. Osteoporosis, characterized by loss of bone mass, alterations in bone structure, and a net increase in bone resorption relative to bone formation, leads to skeletal fragility and increased fracture risk [79].

Inflammation stands at the root of most chronic diseases including osteoporosis. Chronic inflammation, due in part to increased cytokine expression after menopause and with aging, is one mechanism contributing to the pathogenesis of osteoporosis [80]. In a study involving 1123 persons aged 20–98 years, physiological concentrations of total n-3 PUFA correlated strongly with a low-circulating pro-inflammatory markers such as IL-6, IL-1Ra, TNF-α, and C-reactive protein (CRP) and increased anti-inflammatory markers such as soluble IL-6R, IL-10, and TGF-β [81]. A study conducted in a small population of older men and women (50–79 years) in southern Tasmania revealed that variation in the low levels of interleukin-6 and high-sensitivity CRP predicts bone loss and resorption [82] suggesting a potential for n-3 fatty acid anti-inflammatory therapy for the prevention of osteoporosis.

Role of n-3 fatty acids in bone metabolism is known. A cohort of healthy young men 16–22 years exhibited a positive correlation between serum phospholipid n-3 fatty acids and total bone mineral density (BMD) and BMD at the spine [83] suggesting their role in bone accrual. It is believed that n-3 fatty acids participate in bone remodeling through regulation of an array of factors in the bone microenvironment such as prostaglandins and eicosanoids. Increased amounts of n-3 fatty acids in diets have shown to increase insulin-like growth factor (IGF) [84] which in turn enhances bone growth and turnover [85]. In vitro and in vivo studies have demonstrated a protective effect of n-3 fatty acids against bone resorption. They are believed to protect against bone loss through a number of mechanisms. RANKL is an important mediator of osteoclastogenesis. n-3 fatty acids have shown to inhibit bone resorption markers in RANKL-stimulated RAW 264.7 cells with an associated reduction in osteoclast-specific genes such as tartrate-resistant acid phosphatase (TRAP), cathepsin K, calcitonin receptor and matrix metalloprotein-9 and inflammatory marker TNFα, the effects of DHA being more pronounced than that of EPA [86]. A diet supplemented with fish oil was found to increase bone mineral density in aging C57Bl/6 mice compared to those on corn oil supplemented diet [87]. This was associated with a reduction in pro-inflammatory TNF-α and IL-6 in fish oil fed mice. Further, aging is an important risk factor for increased pro-inflammatory cytokines such as TNFα, ILβ, and IL-6 [88] which play a major role in stimulating osteoclast formation and activation, thereby increasing bone loss [89]. Inhibition of osteoclastogenesis by n-3 fatty acids in animal models of osteoporosis has been reported. They attenuate bone loss in ovariectomized mice, a model of postmenopausal, estrogen deficiency osteoporosis, through inhibition of osteoclastogenesis and activation as well as RANKL-induced NF-kB activation in bone marrow macrophages accompanied by a reduction in pro-inflammatory cytokines such as NFα, IL-2, interferon gamma (IFNγ), and an increase in anti-inflammatory IL-6 [90]. A transgenic mouse model (FAT-1) capable of endogenously producing n-3 fatty acids exhibited significantly less bone loss post-ovariectomization compared to their wild-type counterparts [91]. Fish oil supplementation has shown protective effects against autoimmune-induced osteoporosis in MRL/Mpj-Fas^{lpr} mice. When fed a diet containing 10 % fish oil or corn oil for 12 months, these mice showed a significant increase in BMD of distal femur, proximal tibia, and

lumbar spine of mice in the fish oil group compared to those in corn oil group [92]. This increased BMD correlated well with an increased antioxidant enzyme activity in the spleen cells, absence of synovitis in the knee joints, reduced RANKL, and increased OPG expression [92]. Candelario et al. [93] have recently demonstrated a parathyroid hormone 1 receptor (PTH1R) agonistic property of EPA and DHA. Involvement of PTH1R in bone metabolism is well known [94]. EPA and DHA activate PTH1R at concentrations normally found in blood via protein kinase A (PKA) and PKC, and EPA acts synergistically with PTH causing a superagonistic response of ERK1/2 [94].

Further, one of the characteristic features of age-related bone loss is bone marrow adipogenesis [95] which is linked to osteoclastogenesis chiefly mediated through RANKL [96]. Estrogen plays an important role in increasing bone mass through regulation of osteoclast activity. Its deficiency, as it happens during menopause, often leads to an increased bone loss due to higher bone marrow adipogenesis, reduction in osteoclast activity, and reduced osteoblast formation. However, increased bone marrow adiposity is not restricted to estrogen deficiency and is also reported in osteoporotic men [97] and in women with premenopausal idiopathic osteoporosis [98]. Endogenous n-3 fatty acids have shown to reduce bone marrow adiposity and increase bone mineral density, bone mineral content, and bone volume in the distal femoral metaphysis of FAT1 mice [99]. A 5 % fish oil supplementation in the diet caused a remarkable attenuation of bone marrow adiposity accompanied by an increased insulin sensitivity and reduced inflammation and oxidative stress in aging C57Bl/6J mice [100]. On the contrary, n-6 fatty acids have shown to promote differentiation of adipocytes and inhibit osteoblastogenesisin mesenchymal cells [101, 102]. In a case-controlled study involving elderly Mediterranean population (age > 65 years) with low-energy fractures, dietary n-6 fatty acid was associated with higher risk of osteoporotic fractures. Fernandes et al. [103] suggest that n-3 fatty acids in combination with calorie restriction may modulate the aging and autoimmune disease processes through alteration of fatty acid composition, membrane fluidity, and signal transduction and by modulating the lymphokine hormone receptors and their functions.

Clinical Evidence

Despite the wide claims regarding the impact of n-3 fatty acids on markers of inflammation and bone turnover in vivo and in vitro and the protective effects against bone loss in animal models, their clinical significance in the treatment of osteoporosis and ensuing fractures remains unclear (Table 40.2). Evidence based on epidemiological studies does not offer a strong support to these claims. For example, the EPIC-Oxford study did not see any difference in fracture incidence among fish eaters compared to meat eaters or vegetarians [104]. In the Framingham Osteoporosis Study, about 900 patients (mean age ~ 72 years) who were originally enrolled in the Framingham Hear Study were followed for 50 years and the association between their fatty acid intake and hip fracture was assessed following a food frequency questionnaire. The study did not find any association between intakes of fish or EPA, DHA, EPA + DHA, LA, or the n-6/n-3 fatty acid ratio and hip fracture risk in men and women, although an inverse relationship was observed with ALA intake and hip fracture risk [105]. On the contrary, a high ratio of n-6 to n-3 fatty acids was shown to be directly related to a low bone mineral density in older men and women (aged 45–90 years) in the Rancho Bernardo Study suggesting the role of relative amounts of dietary polyunsaturated fatty acids in preserving skeletal integrity in older age. The study also revealed a significant inverse relationship between n-6/n-3 ratio and BMD at the hip in all women and at the spine in women not on hormone therapy [106]. In a randomized control trial, Kruger et al. [107] studied the interaction between calcium and gamma linolenic acid (GLA) + EPA in older African women (n = 65, mean age 79.5) who were on a low-calcium diet at baseline and were osteoporotic/osteopenic. The results showed a positive correlation between the fatty acids and BMD which was associated with a fall in osteocalcin and deoxypyridinoline levels [107]. Orchard et al. [108] suggest that the increased BMD could be attributed to the presence of calcium in the group taking fatty acids [80], since EPA and DHA are known to increase bioavailability of calcium, thus playing a role in bone heath and increased bone calcium content. Breast cancer survivors on aromatase inhibitor (AI) therapy are prone to estrogen depletion and bone loss. In a randomized, double-blind, placebo-controlled short-term study in postmenopausal breast cancer survivors on AI, daily ingestion of 4 g EPA + DHA was shown to reduce bone resorption [109].

Fish Oil and Pain

With the increase in life expectancy by at least 20 years in the last half a century or so, there is also an increase in age-associated morbidities such as diabetes, neurodegenerative disorders, cardiovascular diseases, and cancer [110] where inflammation and pain are a part of the process. Widespread chronic pain is related to disability, reduced quality of life, and increased mortality. The body's ability to resolve inflammation and pain slows down during aging, and this phenomenon was demonstrated recently in Balb/c mice [111]. While the body does need the n-6 fatty acid linoleic acid (LA) for maintaining normal biochemical functioning,

Table 40.2 List[a] of select clinical trials assessing the role of n-3 fatty acids in age-related bone disorders

Study (Ref)	Sample	Intervention	Outcome
Interventional Studies			
Trials assessing bone markers/inflammatory markers as the endpoint			
Hutchins-Wiese et al. [109]	Postmenopausal breast cancer survivors on aromatase inhibitor therapy	4 g/day EPA + DHA (n = 20) or placebo (n = 18) for 3 months. OR Both groups received calcium carbonate (1000 mg/day) and cholecalciferol (800 IU/day)	Serum fatty acids EPA and DHA increased and AA and n-6/n-3 ratio reduced in the treatment group Bone resorption markers: Significant reduction in serum sCTX, BAP, DPD, P1NP at 3 mo compared to baseline No change in inflammatory markers
Salari Sharif et al. [128]	Postmenopausal women with diagnosed osteoporosis at femur neck and lumbar vertebrae	3 capsules (900 mg)/day of fish oil (n = 13) OR 3 capsules of placebo (n = 12) for 6 months	Fish oil group showed a nonsignificant decrease from baseline, in osteocalcin and BAP at the 6 month follow up. Urine pyridinoline was significantly increased ($p < 0.05$) and serum calcium and vit D was nonsignificantly increased in the fish oil group
Martin-Bautista et al. [129]	72 male and female hyperlipidemic patients (35–65 years)	Fortified milk drink group (n = 39) received 500 mL/day of a milk product fortified with EPA, DHA (from fish oils), oleic acid, vitamins A, B6, D, and E, and folic acid. Or Skimmed milk group (control, n = 33) received 500 mL/day of regular semi skimmed milk with added vitamins A and D for 1 year	Patients receiving fortified milk showed a significant increase in plasma eicosapentaenoic acid (42 %), docosahexaenoic acid (60 %), vitamin B6 (38 %), OPG (18 %), RANKL (7 %), OPG/RANKL (10 %), red blood cell folate (21 %), serum folate (53 %), calcium (4 %), vitamin D (11 %), and osteocalcin (22 %) compared to control patients
Trials assessing bone mineral density and/or fractures as the primary outcome			
Kruger et al. [107]	66 women (mean age 79.5) with confirmed osteoporosis or osteopenia and previously on low-calcium diet	6 g of a mixture of evening primrose oil and fish oil [60 % LA, 8 % GLA, 4 % EPA, and 3 % DHA. OR 6 g of coconut oil as placebo (97 % saturated fat; 0.2 % LA) for 18 months. All patients received 600 mg calcium as calcium carbonate per day in addition to the normal diet	Patients on the n-3 fatty acid group showed an increase in lumbar spine density and femoral spine density
Lappe et al. [130]	Early postmenopausal women	geniVida™ bone blend (GBB) consisting of genistein, vitamin D3, vitamin K1, and 1 g of ethyl ester of 2:1 EPA/DHA (n = 30) OR Calcium (n = 28) for 6 months	Significant decrease in femoral neck BMD in control group Significant increase in the BMD at Ward's triangle in patients taking GBB compared to control group ($p < 0.05$) BALP and N-telopeptide significantly increased in GBB group
Trial assessing pain			
Kremer et al. [131]	Patients with definite or classic rheumatoid arthritis and receiving NSAIDs and slow-acting rheumatoid arthritis drugs and/or prednisone	130 mg/kg/day fish oil (n = 23) OR 9 capsules/day corn oil (n = 26)	Fish oil group experienced significant decrease from baseline ($p < 0.0001$), in mean number of tender joints, duration of morning stiffness ($p = 0.08$) and physician's evaluation of pain ($p = 0.004$)

(continued)

Table 40.2 (continued)

Study	Sample	Observation	Outcome
Study	Sample	Observation	Outcome
Observational Studies (based on lifestyle and food frequency questionnaires)			
EPIC-Oxford [104]	57,450 patients aged 20–89 years and classified as meat eaters, fish eaters, vegetarians, and vegans. Followed up at 5 years after recruitment	Self-reported fracture involving bones other than the digits or ribs	Fracture risk was similar for meat eaters, fish eaters and vegetarians. The higher fracture risk in the vegans appeared to be a consequence of their considerably lower mean calcium intake
Weiss et al. [106]	643 men (mean age 72.9) and 903 postmenopausal women (mean age 72.5) who were part of the Rancho Bernardo Study	Role of n-6: n-3 fatty acid ratio on total hip and lumbar spine BMD	An increasing ratio of total dietary n-6 to n-3 fatty acids was significantly and independently associated with lower BMD at the hip in all women and at the spine in women not using hormone therapy
Framingham Osteoporosis Study [132]	854 men and women who were originally enrolled (1988–1989) in the Framingham Heart Study and had their BMD measured and their changes were studied 4 years later in adults (n = 623) with mean age 75 year	Associations between dietary polyunsaturated fatty acid and fish intakes and hip BMD	Higher intake of fish was associated with maintenance of femoral neck BMD in men and women
Viretanen et al. [133]	75,878 women and 46,476 men free of osteoporosis at baseline	Association between dietary n-3 PUFA and risk of hip fracture	During the 24 year follow up, no statistically significant associations were found between intakes of PUFA, total n-3 PUFA, total n-6 PUFA, n-6: n-3 PUFA ratio or individual PUFAs and hip fracture risk
Järvinen et al. [134]	554 subjects were	Associations between dietary polyunsaturated fatty acid and BMD among elderly women	In women who were not on hormone replacement therapy at baseline, the intake of total PUFAs as well the intakes of linoleic and linolenic acids and total n-3 and n-6 fatty acids were significantly associated with BMD at lumbar spine. Significant associations were demonstrated for total body BMD and fatty acids. No significant associations were found among women with HT at baseline

[a]Adapted from Orchard et al. [80]. *BMD* Bone mineral density; *sCTX* serum c-terminal telopeptide; *BAP* Bone-specific alkaline phosphatase; *DPD* Deoxypyridinoline; *P1NP* Procollagen type 1 N-terminal propeptide; *PUFA* Polyunsaturated fatty acids; *OPG* Osteoprotegerin; *RANKL* Receptor activator of nuclear factor kappa-B ligand

in excess it causes a pro-inflammatory environment. Arachidonic acid, a metabolite of LA and a precursor of 4-series leukotrienes (LT), is responsible for a number of events leading up to inflammation, pain, and related disorders. It is also a precursor of inflammatory prostaglandins (PGs) which sensitize pain receptors on nerve endings through their action on ion channels [5, 6, 112]. PGs and LTs increase vascular permeability and sensitize pain receptors [113, 114] leading to increased sensitivity to pain. On the contrary, EPA and DHA are precursors of potent anti-inflammatory lipid mediators. When healthy mice were placed on a diet containing fish oil, there was a delayed pain perception in response to thermal heat in these mice. This delay was directly linked to a reduction in expression of the acid-sensing ion channels (ASIC1a and ASIC 3) and transient receptor potential V1 (TRPV1) as well as c-Fos, a marker of neuronal activation [10]. Involvement of acid-sensing ion channels (ASICs) such as ASIC3 in the maintenance of primary hyperalgesia is known [115]. A recent study revealed that mice and *Caenorhabditis elegans* lacking the TRPV1 pain receptor display increased longevity and a youthful metabolic profile at old age [116]. They found that TRPV1 mutant mice lived more than 100 days longer than the control mice. In this line, pain reduction through inhibition of TRPV1 by EPA and DHA [10] may lead to a better quality of life and an increased life span. The antinociceptive property of n-3 fatty acids was supported by Nobre et al. [117] who reported a reduction in carrageenan-induced rat paw edema and increase in withdrawal threshold in response to thermal stimulation in mice and rats treated with low doses of n-3 fatty acid supplements. They also noted a significant reduction in the TNFα expression in the inflamed rat paw following n-3 supplementation [117].

In a mouse model of neuropathic pain, DHA caused reduced activity and terms of realization of the neuropathic

pain syndrome, induced early stabilization of weight-bearing balance, and abolished the development of dystrophic changes in the tissues of denervated limb [118]. Ko et al. [119] present a case series where five patients with different underlying diagnoses were shown to experience clinically significant pain reduction and improved function when treated with high doses of fish oil. Further, resolvins and protectins, the major anti-inflammatory metabolites of EPA and DHA, respectively, are implicated in resolution of neuropathic pain and antinociception in animal models [120–122]. Arnardottir et al. [111] demonstrated that levels of these specialized lipid mediators are reduced during aging and that supplementing mice with DHA-inhibited acute inflammation induced polymorphonuclear leukocyte migration and shortened inflammation resolution time in mice. They also showed that DHA increases levels of resolvin D1 and resolvin D3 in vivo and DHA and, to a lesser extent EPA, is involved in human monocyte reprogramming to a proresolving phenotype [111]. Further, a diet containing high n-3 and low n-6 fatty acids was shown to significantly reduce chronic headache compared to a diet containing n-6 fatty acids with associated increases in antinociceptive n-3 pathway markers 18-hydroxy-eicosapentaenoic acid and 17-hydroxy-docosahexaenoic acid in the former group [123] suggesting a novel strategy for the treatment of chronic pain. However, with limited studies on pain resolution by n-3 fatty acids in humans, more randomized control trials are warranted to make recommendations for fish oil supplements for the conditions of chronic pain.

Conclusion

Fish oil and fish oil-derived fatty acids such as DHA and EPA have attracted attention for several decades due to their inflammation resolution properties which have been extended to study their role in protecting against inflammation-mediated disorders. While in vitro animal models have shown very promising results, clinical trial results in humans have been mixed, particularly their cardiovascular benefits. While n-3 fatty acids show prominent lipid lowering effects in humans as demonstrated by RCTs in different populations, they fail to prove their effects against arrhythmia. Further, a recent systematic review and meta-analysis of 20 studies involving 68,680 randomized patients showed that n-3 supplementation was not associated with a reduced risk of all-cause mortality, cardiac death, sudden death, myocardial infarction, and stroke [124]. Therefore, caution must be exercised while taking fish oil supplements particularly aimed at treating these conditions. Special consideration may be given while using n-3 fatty acids in arrhythmia patients given the inadequate clinical evidence regarding their antiarrhythmic property and also due to their possible role in worsening arrhythmia. Moreover, n-3 fatty acids are an inherent part of regular diet, and control subjects in randomized controlled trials may not really qualify as pure controls because of the presence of n-3 fatty acids at baseline and throughout the study. Therefore, studies must be planned to make adjustments for the n-3 levels in control participants.

Purity of fish oil is an important factor in achieving good results. Most fish oils in the market do not contain sufficient quantity of EPA and DHA. One may need to take large doses of these products to achieve desirable results. Also, there is a concern regarding the possible presence of environmental toxins such as mercury, polychlorinated biphenyls, and others. Therefore, it is important to choose fish oil supplements from a reliable source.

Further, in the absence of sufficient clinical evidence regarding pain resolution by n-3 fatty acids, inclusion of parameters unraveling pain and inflammation resolution in clinical trials on n-3 fatty acids geared at studying other outcomes would not only save resources and time but also contribute to existing scientific knowledge. In conclusion, we feel that fish oil and fish oil supplements are best considered as a prophylactic therapy geared at preventing or delaying onset of cardiovascular events and bone loss, rather than for the treatment of already existing diseases and as an adjuvant therapy in inflammation-mediated diseases. An exception for this may be the use of prescription grade fish oil formulations for the treatment of hypertriglyceridemia. More importantly, there is no shortcut to successful aging, and adopting a healthy lifestyle making room for healthy diet appears to be the best choice.

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