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# Gert Folkerts • Johan Garssen 

Editors

## Pharma-Nutrition

An Overview

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Prof. Dr. Gert Folkerts started his career in the Rudolf Magnus Institute for Pharmacology at the Utrecht University. He did his thesis on viral infections and asthma and is appointed as full professor (Chair COPD and In Vivo Pharmacology) in the division of Pharmacology, Department of Pharmacy, Faculty of Sciences. He spent some time at the University of Edmonton (Canada), the Wellcome Research Institute in Beckenham (UK) and Janssen Research Foundation in Beerse (Belgium). He has successfully guided a great number of research projects concerning inflammatory diseases. These activities have resulted in more than 160 scientific publications in well-respected international journals. He is a full-time professor at the Utrecht Institute for Pharmaceutical Sciences, Utrecht University, the Netherlands. He is also an associate editor of the European Journal of Pharmacology and is CEO of the contract research company Curax BV.

Prof. Dr. Johan Garssen studied medicine and biology at the Free University, Amsterdam, the Netherlands. He specialized in immunology and pharmacology. He completed his PhD thesis at the University of Utrecht on the role of T cells in respiratory allergy, an immunopharmacological approach. This PhD program was partly performed at Yale University, New Haven, USA. After a postdoc period, he became senior scientist at the National Institute of Public Health in the Netherlands. There he coached many research projects, both preclinical as well as clinical research, in the field of immunomodulation induced by a.o. nutritional ingredients, drugs, and environmental agents. He became head of the immunology section at NumicoResearch, Wageningen, the Netherlands, and director of the immunology platform of Nutricia Research (formerly Numico and Danone), medical nutrition and infant nutrition. He now is a full professor in immunopharmacology at the Utrecht Institute for Pharmaceutical Sciences, Utrecht University. He has published more than 370 peer-reviewed papers in the field of "immunomodulation."

Part I
General

# Chapter 1 <br> Pharma-Nutrition 

Seil Sagar, Gert Folkerts, and Johan Garssen

Nutrition has the primary goal to maintain, or if possible to improve, health. Pharmaceuticals, on the other hand, are generally developed to treat, cure or to prevent diseases [1]. In the last few years the disciplines of pharma and nutrition have evolved separately. Nonetheless, worldwide increased incidence of complex multifactorial disorders, particularly chronic and degenerative diseases and their growing burden in modern society have narrowed the gap between pharmacology and nutrition science [2]. Hence, multifactorial diseases probably require multi-pathway understanding and multi-targeting approaches which might lead to compound combinations [2]. This rediscovered common ground between the complementary values of pharma and nutrition can be conceptualized in the term "pharma-nutrition." In this respect, functional foods or dietary supplements, i.e., those that carry substantial health claims, can be helpful in reducing health risk factors and may thereby prevent chronic diseases [1]. Various chapters in this book highlight the aspects of molecular characteristics of food ingredients towards clinical effectiveness and relevance.

The interaction between microbial community and human host plays a crucial role in regulating immune homeostasis [3-5]. Moreover, alterations in these gut microbial communities were demonstrated to cause immune dysregulation, leading to autoimmune disorders [5]. In this respect, nutrition and orally ingested drugs that pass the gastrointestinal mucosa can affect the balance between the mucosal immune system and microbial community herein. This in turn may affect the

[^0]microbial composition and/or mucosal integrity [4]. Over the last decade, there has been a growing interest in the use of interventions that target the intestinal microbiota as a treatment approach for allergic inflammation. There is now increasing evidence that changes in the gut microbiota contribute to the development of allergies and asthma [6-10]. Additionally, the potential role of beneficial bacteria as modulators of the intestinal microbiota and mucosal immune responses has been extensively investigated and discussed in the last few years [11, 12]. It is now well established that beneficial bacteria can have an impact on the epithelial barrier and immune functions by interacting with the host's epithelial and immune cells within the gastrointestinal system [13]. Besides their impact on the gastrointestinal system, beneficial bacteria were also reported to modulate immune responses in the airways. Hence, Bifidobacteria and Lactobacilli, which are a part of the gut microbiota, suppressed both allergic and autoimmune responses by reducing allergic symptoms and inhibiting allergic airway response in murine models of acute airway inflammation [14-18] and murine models of chronic allergic asthma [19]. Moreover, a combination of Bifidobacterium breve with a specific mixture of nondigestible oligosaccharides reduced allergic responses in mice [20]. The same combination also proved to be useful in allergic asthmatic adults and infants with atopic dermatitis [21, 22]. Additionally, specific combinations of nondigestible oligosaccharides were more effective in improving the immune responses and reducing disease parameters of allergic asthma in mice than either of the oligosaccharides alone [23-25]. Interestingly, we have recently demonstrated that the combination of Bifidobacterium breve with a specific mixture of nondigestible oligosaccharides suppressed airway inflammation in a murine model for chronic asthma [26].

This book reviews the impact and effects of natural products and functional/ medical foods (nutritional programming) on disease management, specifically focusing on diseases related to (1) Inflammation and Immunity, (2) Cancer, COPD, and Cachexia, (3) Allergy, and (4) Brain Neuro/Immune. As both pharmacologists and nutritionists are recognizing that the one disease-one target-one drug (or nutrient) concept will be less successful than in the past, this book aims to stress the importance of a multi-target approach versus a single-target approach. This book also reviews the connection between the microbiome, within the intestine, and the outcome of diseases. Hence, animal and human studies have demonstrated that natural products and functional/medical foods can impact the microbiome.

In Part I the concept of pharma-nutrition is introduced. The definitions and characteristics of the pharma-nutrition industry in the European health and life science sector are explored, and developmental trends of the medical nutrition industry are exemplified. Patenting behavior in the medical nutrition industry is also described. Additionally, recent and ongoing innovations in food allergy safety assessment and innovations in toxicological safety assessment of food are addressed. Regarding the toxicological safety assessment of food, an innovative new toxicological safety assessment approach for complex food matrices based on the Threshold of Toxicological Concern concept is presented and discussed.

The effects of antibiotics and beneficial bacteria on the intestinal microbiota composition in irritable bowel syndrome are discussed in Part II (Inflammation and Immunity). Colonic cleansing and fecal microbiota transplantation may be a promising novel treatment option for irritable bowel syndrome. Furthermore, the role of nutrition in immune system development and the protective and predisposing effects of early nutrition on healthy development of the immune system are also explored. Nutritional intervention using prebiotics, probiotics, and long chain polyunsaturated fatty acids support an optimal immune development and hence may provide a better defense against infections. Additionally, the immunologic basis by which nondigestible oligosaccharides may affect the mucosal and systemic immune system is discussed. Combining oral delivery of nondigestible oligosaccharides with allergenic epitopes might improve effectiveness and may add to future oral immunotherapy strategies. In regard to inflammatory bowel disease, the role of omega-6 and omega-3 fatty acids in inflammatory bowel disease is discussed. High dietary n-3 fatty acids intake and low n-6 fatty acids intake with a more balanced n-6-n-3 fatty acid ratio seems a promising therapeutic approach for inflammatory bowel disease. Furthermore, the protective role of omega-3 polyunsaturated fatty acids against age-related neuroinflammation is discussed. Polyunsaturated fatty acids seem to play an important role in controlling inflammation in the brain because of their abundance in this organ and their modulatory effects on inflammation and cell functions. In addition, the endocannabinoid system which forms a molecular connection between nutrition and pharmacology is reviewed. The possible pharmacokinetic and pharmacodynamic interactions of natural products, such as Ginkgo biloba extract, saw palmetto extract, Coleus forskohlii extract, grapefruit juice, and green tea, with drugs are also discussed in this part. Heme oxygenase- 1 is a widely accepted cytoprotective molecule with various medical benefits. It functions primarily as an antioxidant and has anti-inflammatory, pro- and anti-proliferative properties; and it can act as an immunomodulatory enzyme. The ability of natural substances, such as curcumin, flavonoids and isothiocyanates to increase the level of heme oxygenase is described in Part II. In regard to Parkinson's disease, the potential of nutrition and gastrointestinal health as modulators of Parkinson's disease is also discussed. Therapeutic intervention through whole foods, dietary patterns, and supplemental nutrition (probiotics, prebiotics, and synbiotics) may positively impact intestinal milieu and result in reduced inflammation and oxidation and reduced risk for Parkinson's disease.

In Part III (Cancer and Cachexia) recent therapeutic approaches for cachexia are reviewed. As nutritional strategies are insufficient to reverse the cachexia syndrome, a combination with pharmacological strategies may be a more effective approach than nutrition alone. In this respect, the beneficial impact of nutrition on the nutritional status of cancer cachexia patients is discussed. Furthermore, novel possibilities and biomarkers for individualized tumor therapy with natural products are reviewed. Custom-tailored combination treatments seem to become a reality soon and each individual cancer patient may be treated based on his or her individual molecular tumor architecture. Additionally, the potential
of translational research and challenges, particularly the use of nutraceuticals, as novel strategies to reduce cancer incidence, prevalence, and mortality and social economic impact is stressed.

Recent insights into the pathophysiology of the onset of atopic dermatitis, particularly the interplay between skin barrier abnormalities, inflammation, and skin microbiota are highlighted in Part IV (Allergy). Potential strategies for prevention and treatment are also addressed. Additionally, the use of biologicals, allergen-specific immunotherapy and dietary compounds to actively reduce the allergic responses is discussed. To date, the future direction in allergy management is shifting away from the classical allergen avoidance into active tolerance induction. It is likely that the way forward lies in the combination of life style management, nutritional support to preset the immune system towards tolerance induction and allergen-specific immunotherapies based either on whole proteins or on tolerance-inducing peptides. Additionally, the pros and cons of the different nutritional options chosen as a replacement feeding for children with cow's milk protein allergy are reviewed. To date, the choice for first an extensive hydrolyzed formulae and then an amino-acid-based formulae seems the best approach for cow's milk allergy.

In Part $V$ (Brain, Neuro/Immune) the role of the neuro-immune axis and its targetability in relation to neurological disorders, such as depression, neurodegenerative diseases, and autism are discussed. Additionally, the challenges and potential pitfalls of randomized controlled trials involving nutritional interventions for the prevention or treatment of cognitive decline in older people are examined. Moreover, the therapeutic effects of nutraceuticals in immune disorders are reviewed. Besides their effects on the immune system, nutritional elements such as probiotics, $\mathrm{n}-3$ polyunsaturated fatty acids, vitamin D , and zinc can alter components of the neuroendocrine system that in turn play a critical role in regulating systemic immunity. Additionally, nutritional approaches for healthy aging of the brain and the prevention of neurodegenerative diseases are also discussed. Rice bran, curcumin, anthocyanin-rich fruits, and olive polyphenols represent promising nutraceuticals for modulating mitochondrial function in the brain. Hence, mitochondrial dysfunction plays an important role in brain aging and in the pathogenesis of neurodegenerative diseases, including Alzheimer's Disease.

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# Chapter 2 <br> New Developments in Food Safety Assessment: Innovations in Food Allergy and Toxicological Safety Assessment 

Geert Houben, Marty Blom, Jolanda van Bilsen, and Lisette Krul

## 1 Introduction

Food preferably has to be tasteful, healthy, attractive, and affordable, but should above all be safe. The safety of our daily food nowadays is at a reasonable level, but is far from obvious. With a certain frequency, food safety incidents come up, and the increasing complexity and globalization of our food production networks contribute to an easy outgrow of such incidents to international crises. There is an increased food demand due to a growing world population, whereas existing food sources are limited. To meet the increasing food demand, industry continues developing new ingredients, sources, and products like novel protein-based products (e.g., insect-derived food proteins); extracts from fruits, vegetables, and herbs; natural fragrances; and flavorings but also introduces new processing methods and innovative food packaging concepts. Furthermore, new food concepts are under development in an attempt to reduce the health burden due to overweight and other (related) western food-related conditions or to specifically treat or prevent foodrelated diseases such as food allergy. Finally, our current food is still found to pose natural or process-induced hazards, sometimes newly discovered (e.g., acrylamide several years ago), and not seldom its safety is jeopardized due to malpractice such as fraud.

During the past half century, food risk assessment and risk management approaches have developed, particularly with respect to microbiological and chemical safety aspects of food and food production and processing. Yet, several white spots or areas of inefficiency still exist. Until recently, methodologies for assessing and efficiently managing risks posed by allergens in food were lacking. Furthermore, assuring the chemical safety of current and increasingly complex new foods and food concepts and innovations in food as mentioned above requires a

[^1]continuous improvement of our toxicological food safety assessment and management approaches. These should be pragmatic and prevent unnecessary spending of time, money, and animals for safety testing. In this chapter, recent and ongoing innovations in two areas are addressed: innovations in food allergy safety assessment and innovations in toxicological safety assessment of food.

## 2 Food Allergy Safety Assessment

### 2.1 Food Allergy

Food allergies are adverse reactions to an otherwise harmless food or food component that involves an abnormal response of the body's immune system to specific protein(s) in foods. True food allergies may involve several types of immunological responses [1]. The most common type of food allergies are mediated by allergenspecific immunoglobulin $\mathrm{E}(\mathrm{IgE})$ antibodies which bind to receptors on circulating basophils and mast cells in mucosal tissues. Upon recurrent exposure to the same allergen, cross-linking of cell-bound IgE induces an allergic response by mediator release [2,3]. The clinical picture of food allergy is pleomorphic and can range from gastrointestinal symptoms to severe anaphylaxis [4]. This adverse hypersensitivity response to food poses a serious public health concern [5-7]. The etiology of food allergy or tolerance, however, continues to be poorly understood. For a more detailed review of the mechanisms of allergy, in particular food allergy, the reader is referred to several excellent review articles [8-10].

### 2.2 Food Allergens

Virtually all food allergens are proteins, although only a small percentage of proteins are major allergens [11, 12]. Any food that contains protein has the potential to cause allergic reactions in some individuals. However only a few foods or food groups are known to cause allergies on a more frequent base than other foods. The majority (approximately $90 \%$ ) of food allergic reactions are caused by eight foods: milk, egg, peanuts, tree nuts, fish, soya, wheat, and shellfish [13]. Although controversy exists as to whether the prevalence of food allergy is increasing, it nonetheless remains an important health issue, affecting approximately $1-2 \%$ of adults and 6-8 \% of children [14].

### 2.3 Safety Assessment and Risk Management of Known Major Allergenic Foods

The only option for food allergic individuals to manage their food allergy is the strict avoidance of allergenic food. Medication is available to suppress symptoms, and an increasing number of studies are being published on oral tolerance induction protocols for peanut, milk, egg, and wheat, though these procedures have not yet become standard practice. The majority of the food allergic population therefore relies on rigorous elimination of the allergen in their diet. Legislations in many regions of the world, as for instance for the EU laid down in EU directives 2003/89/EC and 2006/ 42/EC, prescribe the labeling of food products for several major allergenic foods or products derived from that allergen when added as ingredients to food. In addition, many food producers have incorporated allergen auditing programs and voluntarily warn the allergic consumer as to the potential presence of allergens by using precautionary labeling of food products, e.g., "may contain xxx." However, despite this, several retrospective studies [15-17] show that many allergic individuals experience an accidental allergic reaction due to hidden allergens or inappropriate labeling. Surveys of commercially available products demonstrate that the presence or the absence of a precautionary warning corresponds poorly with the actual presence of the allergen in the product [18, 19], which can lead to potentially dangerous situations [15, 20]. A recent study in Canada showed that approximately $17 \%$ of allergic individuals experiencing an accidental exposure attributed this to products with unintentional cross-contamination during manufacturing and no precautionary statement on the label [17]. Conversely, many products do not contain the allergen to which the precautionary warning on the label refers. As a consequence, a precautionary warning on products is not always valuable to allergic consumers and they increasingly seem to ignore precautionary labels [21, 22].

To improve this situation, quantitative guidance is needed with advice on maximum levels of unintended allergens in foods (also called action levels) to improve the precautionary labeling. Several initiatives have been set up by both food industry and enforcement bodies with the involvement of various stakeholders to improve allergen management and to introduce more uniform and transparent risk information [23-25]. One of the ultimate goals may be to establish internationally harmonized guidance that includes action levels for labeling unintended allergens. In contrast to many other risk assessment situations, human data on threshold doses to allergenic foods is available and can be used to establish action levels. In very sensitive patients small amounts may elicit severe reactions, but also thresholds for allergic reactions of micrograms or grams of allergenic food have been reported [26-29]. The US FDA Threshold Workgroup [30] therefore concluded that a single threshold level for any of the major food allergens might yield thresholds that are unnecessarily protective and further that additional data were required. A recent study by Taylor et al. [31] combined the threshold dose of peanut allergic individuals from different centers into a peanut threshold distribution curve which can be used to derive the


Fig. 2.1 Elaboration of a reference dose protecting the vast majority of allergic individuals (from Blom M.W. et al. Action limits for the potential cross-contamination of a food product with Allergens. Netherlands Journal of Allergy \& Asthma (2013) 13: 74-80)
eliciting dose to which a certain portion of the population might respond with objective allergic reactions (EDp): an approach that was used to establish reference doses for many major allergenic foods by a scientific expert panel that reviewed all published and unpublished threshold data available at the Food Allergy Research and Resources Program (FARRP) of the University of Nebraska and the Netherlands Organization of Applied Scientific Research TNO for the Australian-New Zealand Allergen Bureau [32, 33]. Figure 2.1 illustrates the approach followed by this expert panel for elaboration of reference doses. Starting point for the elaboration of these reference doses was an accepted risk of less than $1 \%$ mild objective reactions, chosen in consultation with stakeholders as an acceptable compromise from the perspectives of food safety objectives, practical feasibility, and scientific feasibility. These reference doses were developed into a guidance for establishing action levels for precautionary labeling by the Allergen Bureau. An incidental reaction, that may occur in view of the accepted remaining risk, will in general be mild and transitory, generally requiring no medical intervention. This quantitative guidance is a big step forward and welcomed by the different stakeholders and increasingly taken up by food-producing companies all over the world. For the allergic consumer this will lead to a greater choice of products and a growing confidence in precautionary labeling.

### 2.4 Safety Assessment of Novel Proteins/Protein Sources

Any food that contains proteins has or will have the potential to cause allergic reactions in some individuals. In order to prevent novel foods from posing a high risk of inducing new allergies, regulatory bodies require the assessment of safety, including the assessment of allergenicity. In Europe the Novel Food Law defines novel foods and novel food ingredients as those that have no history of safe significant use within the EU before 15 May 1997. The absence of a history of safe use can be the result of the food being new to the European Union (e.g., exotic fruits, insects, and algae) or of novel processing techniques. Food existing of or derived from genetically modified (GM) organisms may also have an allergenic hazard and thus also need to be assessed on safety, including allergenic potential.

Even though novel non-GM foods meet less consumer resistance than GM foods [34, 35], reality is that with respect to allergenic potential, it does not matter whether the novel food is from a GM source or a non-GM source; both may have the potential to add to the allergenic burden of the diet of the consumer. However, frameworks for the assessment of the allergenic potential of GM foods have been extensively developed and are more structured than those for other novel proteins/ protein sources, for which the assessment will necessarily be more subjective.

### 2.4.1 Allergenic Potential of GM Foods

In the early 1990s it became clear that developments in gene technology might have significant implications for the food supply, particularly in terms of their potential to increase the quantity and quality of available foods. Currently, crops are genetically modified by incorporating new proteins to target deficiencies in nutrients and improve insect or salt resistance so that less pesticide can be used or plants can grow under non-optimal conditions and can often be produced in larger quantities on less land.

Theoretically there are four scenarios in which a novel GM food may be a risk for allergenicity: (1) The transfer of a known allergen or cross-reacting allergen into a food crop: It is generally accepted that the current in silico homology searches, combined with serology where appropriate, are sufficient to predict clinically relevant cross-reactivity with known allergens [36]. (2) The potential to increase endogenous allergenicity of the target food by increasing the level of expression of endogenous allergens: This risk to the population is somewhat controversial since it could be argued that at-risk allergic individuals will be avoiding that crop already, although the counterargument is that increasing levels of allergens could increase the number of individuals likely to acquire de novo sensitization. (3) There is an unexpected expression of novel proteins/peptides as a result of the introduction of unintended open reading frames (reviewed by [37]). (4) The novel protein may be a de novo allergen that has not been previously been experienced by the human population. Where there is no previous history of dietary exposure, such as for those
target proteins that are isolated from alternative sources (fungi, bacteria, etc.) or where significant changes have been introduced to the amino acid sequence to confer particular benefits, or cross-linking the proteins results in protein structures with new characteristics, the current battery of tests available will not be sufficient to identify a truly novel allergen. As a consequence, there has been a growing interest in the design and development of appropriate animal models and their potential integration into safety assessment paradigms. In 2010, EFSA's Genetically Modified Organisms (GMO) Panel has adopted a scientific opinion on strategies for assessing the risk of allergenicity of GM plants and microorganisms and derived food and feed which is an update of the 2001 FAO/WHO Decision Tree that was recommended by the joint Food and Agriculture Organization and World Health Organization (FAO/WHO) Expert Consultation on Allergenicity of Foods Derived from Biotechnology [38].

The EFSA panel considers the weight-of-evidence, case-by-case approach the most appropriate way of assessing the allergenicity of GM food and feed [39]. In summary, it is recommended that with regard to the search for sequence homology and structural similarities, the local alignment method with a known allergen with a threshold of $35 \%$ sequence identity over a window of at least 80 amino acids is considered a minimal requirement. When IgE binding tests are considered necessary, e.g., when there is sequence homology and/or structure similarity with known allergens, the use of individual sera from allergic individuals rather than pooled sera is recommended. In addition to the pepsin resistance test, it is recommended that the resistance to digestion of the newly expressed proteins is evaluated using other in vitro digestibility tests mimicking physiological conditions of humans; some protein is likely to survive intact into the lower intestine because of the following: (1) Protein does not enter the acid environment of the stomach as a pure test solution, but rather as part of a complex food matrix. Within a bolus of food passing through the stomach, it is unlikely that all protein is exposed to the extremes of acid pH . (2) Upon entering the stomach, proteins continuously leave the acid environment of the stomach, in a non-, partially, and fully digested state (Verhoeckx et al., in prep). In addition, proposals have been made with regard to other additional testing that may improve the assessment, cell-based tests (basophil activation tests (BAT), rat basophil leukemia (RBL) cell line transfected with human IgE receptor activation tests), and sharing of T cell epitopes between transgene-encoded proteins and allergens, e.g., animal models. Even though MHC restrictions in immune responses of animal models currently preclude any conclusions, the use of animal models can be considered as an enhancing step in the weight-of-evidence approach if they are further developed and validated. Animal models can be useful to investigate the capacity to elicit an allergic reaction and/or to act as an adjuvant in different environmental/exposure conditions and analyze the underlying mechanisms. Moreover, animal models can be used as a substitute for allergic human sera for a (pre-)screening of the immunological cross-reactivity of the novel protein with known allergens and may also be appropriate for studying the allergenicity of whole GM foods.

### 2.4.2 Allergenic Potential of Non-GM Foods

Detailed guidance on how to assess the allergenic potential of novel foods is mainly available for GM foods as described above, but not for "natural" non-GM foods that are newly introduced into the diet, such as alternatively processed proteins or new alternative food protein sources. Since new alternative protein sources (e.g., beet leaves, algae, and insects) are increasingly explored for a sustainable food production, this area will become more important. The assessment of these novel foods will have some similarities to EFSA's weight-of-evidence approach for novel GM foods, in that the source of the protein needs to be defined. Homology searching is less appropriate for novel proteins/protein sources because there is no specific transgene to sequence. Gubesch et al. [40] designed a methodology to screen novel plant-derived foods for the presence of pan-allergens, IgE binding of food allergens, and clinical relevance of $\operatorname{IgE}$, which illustrates a stepwise approach which could be adopted for the allergenicity assessment of other protein sources as well. Using this approach, cross-reactive allergens can be identified that possibly elicit an allergic reaction in a consumer already sensitized to a known food allergen.

Pan-allergens are ubiquitous proteins responsible for $\operatorname{IgE}$ cross-reactivity to a wide variety of related and unrelated allergenic sources. Usually the IgE crossreactivity is a consequence of structural similarity between homologous proteins, which is translated into conserved sequence regions, three-dimensional folding, and function [41]. However, it has been shown that antibodies also can contribute to cross-reactivity by means of conformational diversity [42] and T cells may also display a cross-reactivity [43].

Although usually considered as minor allergens, sensitization to pan-allergens might be problematic as it bears the risk of developing multiple sensitizations. This may explain the phenomenon that the majority of patients seem to display adverse reactions upon contact to multiple allergen sources. For example, profilin is a pan-allergen that is recognized by IgE from about $20 \%$ of the patients with allergies to birch pollen and plant food [44]. Therefore, as a first screening step, the presence of proteins homologous to known allergens needs to be confirmed by specific animal antibodies or antisera. Thereafter, a specific serum screen to identify potential IgE-binding capacity is appropriate. In this targeted serum screening, it is important to use sera from allergic patients with $\operatorname{IgE}$ reactivity to known pan-allergens.

Where the protein in the novel food is unrelated to any major food allergen or comes from an exotic source for which there is little information, e.g., insects or imported fruits, then an investigation into the phylogenetic relationships of the food source with other known foods should first be conducted. This would lead to the design of a targeted serum screen, in which sera from individuals previously sensitized against phylogenetically related foods should be screened for potential cross-reactivity (i.e., to peanuts if screening for a novel legume or to shrimp if screening for mealworms).

Finally, the clinical relevance of in vitro $\operatorname{IgE}$ binding should be verified by provocation tests (skin-prick tests or a double-blind placebo-controlled food challenge) in a clinical environment. The meaningfulness of such studies with special regard to market authorization of novel proteins/protein sources may be questionable when considering that in vitro IgE-binding properties in targeted serum screening and even clinical reactivity in preselected allergic patient groups may be observed with any novel vegetable or fruit [40]. Nonetheless, the continuing performance of comparable studies with novel foods can improve our knowledge about the allergenic potential of novel foods. Having sets of data on different novel foods, those foods with an extraordinary allergenic potential may be easier to identify.

Using the stepwise approach as mentioned above, cross-reactive allergens can be identified. However, this approach will not identify the potency to sensitize a predisposed individual de novo. To this end, the assessment should be supplemented with several assays. Since there is no single test available that predicts the de novo sensitizing potency of protein (sources), a set of assays should be conducted as described in the previous paragraph. Together with the possible allergen crossreactivity data, the risk assessment can be performed on a weight-of-evidence base. TNO drafted a generic allergenicity risk assessment flow chart for novel food proteins and protein sources summarizing the key elements to be assessed (Fig. 2.2).

### 2.4.3 Allergenic Potential Hypoallergenic Food Proteins

In marketing, the term hypoallergenic should only be used when there is little likelihood that a food will cause an allergic reaction. A well-known example is the hypoallergenic infant milk formula. In this chapter, the term hypoallergenic refers to the significant reduction or elimination of individual known allergens from foods which may prove beneficial to human health.

Different approaches may be chosen to reduce the allergenic burden in foods: (1) physical removal of the targeted allergen, (2) genetic modification (RNA silencing or mutational knockout gene expression), and (3) food processing.

In plants, genetic modification may be used, provided that the elimination of allergenic proteins is not deleterious to the plant since such proteins may have a function in the plan or contribute to the nutritional value. To this end, RNA silencing (knockdown gene expression) and mutational knockout gene expression techniques are used. Several examples exist of genetically modified foods with reduced levels of allergenic proteins, such as rice [45], soybean [46], apple [47], peanuts [48], and tomato [49].

However, genetic modification is not the only approach which can be applied to the development of hypoallergenic foods and ingredients. Other novel processing techniques, such as high-pressure processing or extreme heat application, may reduce the allergenicity of problematic foods and ingredients [50]. Needless to say that genetically modified or alternatively processed foods should be assessed for allergenicity with methods and approaches described previously in this chapter.

*Not on the market yet, so no serum from allergic patient is available
Fig. 2.2 Flow chart of allergenicity risk assessment novel "natural" non-GM protein/sources

### 2.5 Desirable Improvements in Allergenicity Assessment

Currently, the weight-of-evidence, case-by-case approach is considered the most appropriate way of assessing the allergenicity of GM food and feed. This assessment results in a "yes" or "no" verdict to the likelihood of being an allergenic protein (source), which results often in an oversimplification in terms of risk management: for example some novel allergenic protein (sources) may hardly have a significant impact on public health and not require active management but may become unnecessarily banned from the market. For risk management, it is desired to classify novel foods/proteins in a more subtle way. A promising approach is to compare the allergenicity of novel protein (sources) to known allergens with low, intermediate, and high allergenic potency. This relative allergenicity scaling
helps regulatory bodies to decide their priorities and thus improve allergy management by focusing resources to where they are needed.

In order to design a relative allergenicity scale of known allergens, one should first decide what defines allergenicity, i.e., which criteria should be included to identify allergenic foods of public health importance. To this end expert groups under the aegis of the ILSI Europe Food Allergy Task Force [51, 52] have previously proposed and evaluated the following as important criteria: (1) IgE-mediated character of adverse reactions, (2) the required dose of allergenic food to elicit adverse reaction in an already sensitized individual (threshold dose), (3) severity of adverse reactions, and (4) prevalence. It must be acknowledged that the severity of the adverse reactions is a result of the nature, extent, and duration of exposure rather than the inherent allergenic potency of the protein per se. Therefore in order to design a relative allergenicity scale, a risk-scoring system should be developed in which all available data on the IgE-mediated character, threshold dose, and prevalence of a panel of known low/intermediate/high allergens should be collected. Eventually, risk scores should be attributed to the combined available information for each newly introduced food. Such an overall scoring system is currently being developed by TNO as a useful tool for proper risk assessment and management of novel allergens.

## 3 Toxicological Safety Assessment of Food

### 3.1 The Toxicological Food Safety Assessment and the Threshold of Toxicological Concern

A toxicological food safety assessment is generally performed by a sequential approach. In this sequential approach traditionally four steps are taken. In the first step the composition of a food product should be completely identified and quantified. Based on this information, the potential hazard of each substance present in the food product should be separately assessed in the second step. For each substance a toxicological threshold has to be derived. Mostly this threshold is derived from animal toxicity data and converted using safety factors to thresholds for human exposure. Subsequently, in the third step an assessment of the past and the current or the expected exposure is performed. Finally, in the fourth step, a risk assessment is performed for each substance by comparing the calculated exposure to the threshold calculated to determine if the product is expected to pose a health concern. It should be noted that it is likely that not each single substance can be identified in a complex food matrix, resulting in data gaps which forces to perform (animal) toxicity testing using the product as a whole.

Foods are chemically complex food matrices (CCFM) and may consist of many substances. Therefore, the abovementioned sequential approach may lead to unnecessary detailed research and as such to a waste of time, animals, and resources. It is therefore essential to make better use of existing toxicological
information. In the past decades, alternative approaches have been developed for the safety assessment of substances. An example of an alternative approach is the Threshold of Toxicological Concern (TTC) concept. Based on a large toxicological dataset, containing chronic toxicity data of a wide variety of substances, the TTC concept provides a predictive safety assessment tool for substances for which no toxicological information is available. Depending on the molecular structure of a substance, a human exposure threshold value can be derived for most substances, below which there is a very low probability of a risk to human health.

The history of development and application of the TTC concept has been extensively reported in several publications [53-59]. Kroes et al. [54] published a decision tree in which different TTC values are proposed for different groups of substances. The decision tree distinguishes between genotoxic and/or high-potency carcinogens, organophosphates, and Cramer class III, II, and I substances. For all these classes of substances a conservative threshold of concern is determined based on a large set of chronic toxicity data. These thresholds are derived for lifetime daily exposure to the substances. Proteins, heavy metals, and polyhalogenated dibenzo- $p$-dioxins and related substances were excluded from the decision tree. Using the decision tree, for many substances a threshold can be derived which can be used in safety assessment (confirmed by the EFSA).

The main difference between the TTC concept and the traditional sequential approach is that the TTC concept is an exposure-driven approach. Since the past decades, exposure-driven safety assessment concepts are applied or developed in several frameworks, e.g., indirect food additives endorsed by the US FDA [60], flavoring components endorsed by JECFA [61], genotoxic impurities in pharmaceuticals endorsed by EMEA [62], contaminants in foods proposed by ILSI [53], and exposure-based waiving under REACH [63]. This is considered as a shift in safety assessment mindset away from the preferred traditional approach to investigate the exact toxicological profile of a substance after which the exposure is considered.

### 3.2 New Toxicological Safety Assessment Approach for Complex Food Matrices

Many of the compounds present in a complex food matrix might be present at low concentrations resulting in intakes below the TTC thresholds. In order to increase the efficiency of the assessment process of complex food matrices, without making concession to the safety aspects, TNO has developed a complex matrix safety assessment strategy (CoMSAS) which is a stepwise multidisciplinary strategy combining high-end sample preparation, fractionation, and analytical techniques with the TTC concept [64, 65]. The strategy concerns a stepwise analytical approach based on the exclusion of the presence of specific groups of substances using target analytical techniques following the Kroes et al. [54] decision tree and modifications as proposed by Munro et al. [58]. Also the recent conclusions of the EFSA opinion on the TTC concept [59] are taken into consideration. The major


Fig. 2.3 Stepwise approach to assess safety of complex food matrices using CoMSAS
advantage of this strategy is that a full identification of all compounds in the matrix and concurrent compound-specific safety assessment is not needed for substances at low exposure levels. For a detailed description of categories of substances and their thresholds that are considered in the TTC and CoMSAS approach it is referred to Kroes et al. [54], Munro [58], and EFSA [59]. The CoMSAS is schematically presented in Fig. 2.3 and will be further explained below using a stepwise approach.

### 3.2.1 Step 1

The first step concerns a general analytical screening of the complex food matrix. The goal of this step is to detect as many substances as possible present in the complex food matrix, without identification, and to estimate the concentration at which they are present. This step is performed using a nontarget forest-of-peaks (screening) approach in which a number of analytical techniques is being used to cover a wide variety of physical chemical properties which substances present in the CCFM may possess. Based on the estimated concentration of the detected substances, an exposure estimate for each "peak" can be made based on food consumption information of the total food product concerned. For substances below the exposure threshold of $90 \mu \mathrm{~g} / \mathrm{day}$, no further assessment has to be performed when certain categories of substances can be excluded or are below specific thresholds (see further steps). In principle only the substances with an exposure exceeding $90 \mu \mathrm{~g} /$ day have to be identified after which a substance-specific
safety assessment needs to be performed (see step 4). For identification and quantification of these substances analytical methods like gas chromatography coupled to mass spectrometry, combined with LC and high-resolution mass spectrometry or NMR analysis, may be used. It should be noted that the identity of substances that are present in higher amounts in a complex food matrix is in many cases already known, particularly in case these substances are intentionally present for a specific purpose in the food.

### 3.2.2 Step 2

As indicated before, the TTC concept cannot be applied to proteins, heavy metals, and polyhalogenated dibenzo-p-dioxins and related substances, as these substances have a higher toxicity or are not included in the dataset underlying the TTC concept [54]. Also aflatoxin-like, azoxy- or $N$-nitroso substances do not have a TTC threshold and need a substance-specific safety assessment. Moreover, the threshold of $90 \mu \mathrm{~g} /$ day used in the CoMSAS approach is based on the TTC threshold of Cramer class III substances, which is higher than the threshold for organophosphates and carbamates ( $18 \mu \mathrm{~g} /$ day ). Therefore, for the substances present in the food matrix with an estimated exposure below $90 \mu \mathrm{~g} / \mathrm{day}$, it has to be excluded that they belong to the class of substances as indicated above. Using targeted analytical methods the complex food matrix can be assessed for the presence of proteins, nonessential/heavy metals, metal-containing compounds, dioxin-like substances, highly potent genotoxic substances, and organophosphates/carbamates.

### 3.2.3 Step 3

For substances with a genotoxic potency a threshold of $0.15 \mu \mathrm{~g} / \mathrm{day}$ is set in the TTC decision tree. For applying a general TTC threshold of $90 \mu \mathrm{~g} / \mathrm{day}$, the presence of substances with a genotoxic potency needs to be excluded. The best way to assess this is to test the whole food matrix or extracts thereof for the presence of potential genotoxic substances using a biological assay. Conventional state-of-the-art assays such as the AMES test were not developed to test CCFM. Alternative assays such as the BlueScreen HC assay [66], which is sensitive for gene mutations, clastogenicity, and aneugenicity, can be used as an alternative for this purpose. In case a food or an extract thereof is tested positive in such a genotoxicity assay it is unclear which substance(s) actually causes (cause) the genotoxic response. Fractionation of the food matrix using extraction and separation techniques and testing these fractions for their genotoxic potential may help to finally identify the fraction containing the genotoxic substance(s) and subsequently to find the substance (s) responsible for this effect. This can be followed by identification of the responsible substances present in the positive fraction followed by substance-specific risk assessment. A high-throughput genotoxicity assay such as the BlueScreen HC assay
in combination with fractionation and further analytical research is very helpful for this purpose.

### 3.2.4 Step 4

The next steps of the CoMSAS approach are related to substances present at levels resulting in intakes above $90 \mu \mathrm{~g} /$ day and assessment of substances that appeared present in step 2 or 3 . Step 4 requires assessment of these substances. The safety assessment of the substance can be done based on the TTC threshold of the actual Cramer class, based on substance-specific toxicological data (e.g., retrieved from public literature), or related to legal limit values for the substance (e.g., in case of heavy metals and aflatoxins). In case no substance-specific toxicological information is available, the evaluation can also be performed using available toxicological information from comparable chemical substances (in structure and mode of action). Based on the toxicological evaluation a substance-specific human health limit value can be established which can be compared to the estimated daily intake of the substance. In case it cannot be excluded that a health risk may occur, measures might be taken to reduce or prevent the presence of the substance concerned.

### 3.2.5 Step 5

In case proteins appear present in the complex food matrix, an allergenicity assessment may be necessary. In case of known food allergens labeling of the product can be performed, and/or an assessment can be made for the probability of an allergic response in an allergic individual. For new proteins/unknown food allergens, new safety assessment approaches will be developed (see later on in this chapter).

### 3.3 Discussion on CoMSAS

The CoMSAS approach as described above is most efficient in case in a complex food matrix a limited number of substances are present to which the daily exposure exceeds $90 \mu \mathrm{~g} / \mathrm{day}$. To investigate this, TNO has applied the approach to migrants from food contact materials (non-intentionally added substances), natural food supplements, and processing of herbs. The pilot cases demonstrated that the threshold of $90 \mu \mathrm{~g} /$ day would be sufficiently high for a reasonable applicability in the safety assessment of complex food matrices. It should be noted that the TTC concept is not a static concept, but remains under development as more information is expected to be published in near future. The TTC thresholds may therefore also change depending on the information available. The exclusion scheme and strategy
presented in this chapter are based on the most recent literature on TTC at the time of writing. For example, in its evaluation in 2012 the EFSA concluded that the chronic toxicity dataset underlying the threshold for Cramer class II substance ( $540 \mu \mathrm{~g} / \mathrm{day}$ ) is not sufficient and that therefore the threshold of Cramer class III substances ( $90 \mu \mathrm{~g} / \mathrm{day}$ ) should be applied to Cramer class II substances. Based on this conclusion, the threshold used in the CoMSAS approach was set at $90 \mu \mathrm{~g} / \mathrm{day}$. TNO currently assesses the chronic toxicity dataset underlying the TTC concept to assess whether on a scientifically valid base other thresholds for (sub)classes of the Cramer class III substances can be derived. Details of this study will be submitted for publication upon completion. Based on the outcome of this assessment eventually also the threshold used for the CoMSAS approach may be adapted.

The TTC approach was developed for the evaluation of single substances present in food. The CoMSAS approach is developed for the evaluation of complex food matrices (mixtures). It might occur that in a complex food matrix different substances with similar or interacting toxicological activity are present that will appear as separate "peaks." The aspect of interaction between (toxicity of) substances at low dosages was evaluated by the Dutch National Institute for Public Health and Environment [67] and Boobis [68]. Both concluded that at low doses interaction between substances such as synergy and antagonism is not likely to occur. However, Pieters and Konemann concluded that dose addition even at low concentrations of substances in mixtures cannot be excluded. In most cases a factor of 10 appeared to be sufficient to cover for dose addition. TNO has assessed this further and concluded that "to some extent cumulative effects at exposure levels for each substance at or below $90 \mu \mathrm{~g} / \mathrm{day}$ might occur. However, the health relevance of possible cumulative effects at this dose level is considered to be that low that a need for a correction factor to cover possible cumulative effects is very low to absent" [69].

The current sequential approach in toxicological safety assessment may lead to unnecessary detailed research, especially considering that more and more complex food matrices have to be assessed. To stimulate food innovation and reduce animal toxicity testing, scientifically sound but pragmatic safety assessment strategies are required in which existing relevant information is used optimally. Besides the TTC principle and read across for single substances, CoMSAS provides a pragmatic approach for the assessment of matrices without the need for full identification. The CoMSAS complies with the current accepted state of the art regarding the TTC concept. In future, the TTC concept might be refined or extended with other (higher) thresholds of concerns for classes of substances due to new knowledge and literature. Moreover, new non-testing concepts might be developed. By making more optimal use of existing information, based on large datasets of toxicological data, these data in combination with for example toxicogenomics information can provide even more pragmatic ways of safety assessment in future.

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# Chapter 3 <br> Bridging a Pharma-Like Innovation Gap in Medical Nutrition 

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## 1 The Health and Life Sciences

As the boundaries between many once-distinct industries blur and consequently combine, it gives rise to new industries. This also holds true for the health and life science sector $[6,18,59]$. In the past few years the gap between pharma and nutrition science is closing. One reason is the increasing scientific evidence regarding the potential of nutrition and the role in the prevention or treatment of diseases and/or risk factors for disease [23].

As a result of the rediscovered medical application of nutrition, the traditional boundaries between the pharmaceutical and food industries are fading. It is at this interface where the ideal set of conditions/environment is provided for the development of a new industry segment: pharmanutrition.

Convergence is taking place where (large) pharmaceutical, biotechnology and food companies are merging or forming strategic alliances to maintain long-term profitability [5, 6, 59]. Consequently, the number of companies with multidisciplinary activities eligible to fall under the pharmanutrition industry has increased.

[^2]Especially in an era where the pharmaceutical industry is facing both fewer product approvals in combination with blockbuster patent expirations, such convergence trends offer profitable opportunities [5, 10]. Food industry research programmes slowly start resembling approaches used in the pharmaceutical world, while pharmaceutical companies realise the potential of nutrition slowing down disease progression or improving therapeutic outcome [23].

The resulting new industries present companies with both threats and opportunities. On the one hand, industry convergence increases the risks for developing new knowledge and technologies. Inventors leave the comfort zone of their monodisciplinary area of expertise to venture into unknown discipline-crossing activities. On the other hand, the early stages of industry convergence offer significant opportunities, one of them encompassing the first-mover advantage, and potentially setting the knowledge and technological industry standard in doing so [10].

Compared to the food industry, the pharmaceutical industry is relatively young, and it has developed into a cluster of industries concentrating on developing commercial applications for global health-care markets [69].

Existing pharmaceutical and food companies realise that pharmanutrition is an area filled with opportunities for enhancing discovery, technological and development competencies. [5, 72]. Already during the past few decades, various boundary spanning innovative pharmanutritional products have been granted market approval. These so-called pharmanutrition products claim to provide a form of specific health benefits beyond basic nutrition. Examples of pharmanutrition products resulting from the convergence between the food and pharmaceutical industries are functional foods and medical nutrition (Fig. 3.1).

Nevertheless, both pharmaceutical and food companies also recognise the disadvantages in funding inventions that lead to the commercialization of boundaryspanning products. Especially during the early stages of industry convergence, such products are perceived by the regulatory authorities and legal practices as entities with ambiguous identities. Consequently, the boundary-spanning product is generally misunderstood by the majority of risk-averse consumers and experienced as illegitimate. Additionally, the greatest distinction between food products and medicines is of great significance for legal practice, since medicines are more strictly regulated than foods [20]. Therefore, carefully categorising industries and identifying industry boundaries may lead to better consumer perception and higher market acceptance.

Disadvantages of boundary spanning products are that having an unclear and ambiguous identity decreases the chances of receiving attention as well as not being perceived as legitimate and trustworthy [72].

This chapter starts off by exploring the definitions and characteristics of the pharmanutrition industry in the European health and life science sector. At present, unstandardized terminology describing pharmanutritional products is often perceived as confusing [23]. The focus will be on defining the industry boundaries and illustrating industry convergence. By taking conventional foods at one end of the spectrum, and pharmaceutical products at the other, the pharmanutrition industry can be split further into two categories falling within this spectrum: functional foods and medical nutrition.


Fig. 3.1 Industries situated in the health and life science sector

Section two exemplifies the developmental trends of the medical nutrition industry. Here, various industry life cycle scenarios are defined in order to forecast the direction in which this pharmanutrition industry could mature. Concepts such as the innovation cliff and jumping the S -curve are described, and strategies to overcome these common bottlenecks are proposed.

The final section describes patenting behaviour in the medical nutrition industry, offering a patent decision framework for intellectual property protection strategies. The chapter rounds-off with a general discussion as to the successful development of the medical nutrition industry.

### 1.1 Setting the Scene

In order to understand the convergence of the conventional foods, functional foods, medical nutrition and pharmaceutical industries, it is useful to review each industry.

### 1.1.1 Conventional Foods

The conventional foods industry (Table 3.1) encompasses a broad range of nutritional products for consumption, ranging from natural sources to genetically,
Table 3.1 Pharmanutrition industries' characteristics

| Product term | Product type | Target population | Function | Purchasing | Reimbursement | Clinical trials | Estimated market size at present | Example |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Conventional foods | Regular food | Healthy individuals/general population | Basic nutrition | Supermarket | No | No | $\begin{aligned} & \text { €900-- } \\ & 1,000 \\ & \text { billion } \end{aligned}$ | - Regular margarine <br> - Yoghurt |
| Functional foods | Addition of nutrient to regular food | Healthy individuals/general population | Health promo-tion/reduction of risk of disease | Supermarket | No | Required in case of a health claim | €7-9 <br> billion | - Butter fortified with omega 3 fatty acids <br> - Yoghurt with probiotic cultures |
| Medical nutrition | Specific combination of nutrients | Patients | To manage a disease state/alleviate symptoms | Pharmacy | Country dependent | Required in case of a product claim | $\begin{aligned} & € 1.5-2.5 \\ & \quad \text { billion } \end{aligned}$ | - Oral liquid supplement enriched with extra proteins for patients with sarcopenia <br> - Oral liquid supplement enriched with proteins, reduced mineral (phosphate) and liquid content for kidney patients |
| Pharmaceuticals | Chemical entity | Patients | Disease prevention/ curing | Pharmacy | In most cases | Required | $\begin{array}{r} € 110-130 \\ \text { billion } \end{array}$ | - Penicillin <br> - Aspirin |

biologically and/or chemically modified food substances. It is considered to be at the opposite end of the industry spectrum, unrelated to the pharmaceutical industry. This product category is defined according to EU legislation as "any substance or product, whether processed, partially processed or unprocessed, intended to be, or reasonably expected to be ingested by humans. Foods include drinks, chewing gum and any substance, including water, intentionally incorporated into the food during its manufacture, preparation or treatment." Food is consumed to provide nutritional support for the body. It is usually of plant or animal origin and contains essential nutrients, such as carbohydrates, fats proteins, vitamins or minerals [21]. Foods do not include: live animals unless they are prepared for placing on the market for human consumption, plants prior to harvesting, medicinal products, tobacco and tobacco products, narcotic or psychotropic substances, and residues and contaminants. Both international trade and technological developments have contributed to a significant increase in the available foods and other edible ingredients.

With the increasing pace of developments in the food industry, EU regulatory bodies realised the need for a formalised safety assessment of new foods In the EU [28], the general policy on food safety has been laid down in the EU White Paper on Food Safety [8]. This document outlines a comprehensive range of actions required to complement and modernise existing European food legislation, which in turn led to the introduction of the General Food Law (Regulation (EC) 178/2001). This regulation formed the basis for the establishment of the independent European Food Safety Authority (EFSA) in 2002. In summary, these regulations are necessary to ensure EU unified food safety standards.

Conventional foods are inherently linked to an individual's health. As a result, conscious consumers seek out the health properties of natural food substances.

### 1.1.2 Functional Foods

The term "functional food" was first introduced in Japan in the 1980s as FOSHU (Food for Specified Health Uses) and has since developed into a successful and lucrative industry [51, 60]. The Japanese interest in functional foods spread towards the Western world in the early 1990s. As a result, the Western functional food industry has evolved at the intersection of the pharmaceutical and food industries $[10,60]$. This product category consists of food products with added health benefits when compared to the regular nutritional value of the traditional food product [30]. To date, most countries do not have a formal and legislative definition of the term functional food. Even for experts, delineating the boundaries between food and functional foods is challenging [60]. According to the EU-project Functional Food Science in Europe (FUFOSE), functional foods are defined as: "A food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects. .." [12]. The functional food industry is seen to have evolved from the convergence of the food industry and health and life science sector. Comparable to the pharmaceutical
industry, which is based on traditional healing experiences such as willow bark (aspirin), functional foods are based on traditional nutrition folklore such as fatty fish, at present often supplemented as a refined omega fatty acid.

As awareness and trust concerning food related health benefits is growing among the general public, consumer demands are changing. They collectively seek foods with added health benefits, which include functional foods. Most early developments of functional foods were food products enriched with vitamins and/or minerals. Soon, foods fortified with various essential micronutrients-such as phytosterol and soluble fibre-became more popular [61, 62].

Through functional foods, consumers aim to prevent diseases and improve their physical and mental well-being [39]. The majority of functional food products are aimed at optimising health by increasing energy-levels, by boosting the immune system or through the prevention of chronic illnesses (including cancer, cardiovascular disease, Alzheimer's disease and osteoporosis) [30]. Especially in the Western world, any innovation targeting those disease areas are considered valuable. This is due to a combination of the following reasons: health-care costs are increasing; people demand an improved quality of life; and there is a steady increase in life expectancy. Combined with the general saying that "prevention is better than the cure," consumers are more actively pursuing healthy lifestyle and dietary choices.

Consumer perception of functional foods is strengthened by means of nutrition and/or health claims. A claim is defined by the Codex Alimentarius, as "any representation which states, suggests or implies that a food has particular characteristics relating to its origin, nutritional properties, nature, production, processing, composition or any other quality" [26]. To ensure that claims on foods and food constituents are scientifically justified, the European Union published Regulation No 1924/2006 on nutrition and health claims made on foods [3]. This regulation distinguishes two categories of claims on foods: health claims and nutritional claims. Nevertheless, functional foods are not regulated in the same way as pharma (EMA or FDA). According to functional food legislation, health claims state, suggest or imply a relationship between food and health. Such claims include reduction of risk of disease claims, function claims or claims referring to the growth and development of a child.

Nutrition claims state, suggest or imply that a food has particular beneficial nutritional properties due to the energy it provides or the nutrients it contains. Examples hereof are content claims or comparative claims, e.g. "this product contains calcium" or "this product is low in sugar." Explicit conditions are provided in EU legislation for claims such as "source of", "rich in", "reduced", "fat-free" [26]. The European Food Safety Authority (EFSA) carries out the scientific assessments of these claims in Europe. The final approval is provided by the European Commission and member states but is strongly based on the scientific opinions of EFSA as to whether the claim is sufficiently substantiated [16,50].

Since many applicants have encountered difficulties in submitting EFSA acceptable scientific evidence to be granted a health claim, they published in July 2007 its "scientific and technical guidance for the preparation and presentation of the
application for authorization of a health claim." This publication is pursuant to Article 14 of Regulation 1924/2006 in order to assist the applicant with submitting health and nutritional claims. As may be expected, the reactions from various stakeholders regarding this EFSA document differ considerably [67]. Some stakeholders have brought up the issue that the current EFSA approach may hamper innovation. Others state that on the long term, Regulation 1924/2006 will improve the reliability and credibility of health claims on foods. According to yet other experts, this regulation will not empty the functional food shelves but solely change the look of those shelves.

### 1.1.3 Medical Nutrition

Medical nutrition is perhaps the most confusing category, subject to different interpretations between, as well as within, geographical regions. Terms include medical nutrition, clinical nutrition, medical foods, enteral nutrition, foods for special medical purposes, and dietary supplements [20, 23, 34, 57].

In Europe, medical nutrition is not regulated by the EMA (European Medicines Agency) but is governed by the Foods for Special Medical Purposes (FSMP) Directive. The design and production of medical nutrition is predominantly based on scientific knowledge. In this Directive, medical nutrition is defined as: "foods that meet the particular nutritional requirements of persons affected by or are malnourished because of a specific disease, disorder or medical condition" [7]. This category includes oral nutritional supplements as well as tube feeding, of which the latter is administered via nasogastric, nasoenteric or percutaneous tubes. There are three categories in medical nutrition:

1. Nutritionally complete foods that can serve as the sole source of nutrition for a patient;
2. Nutritionally complete foods with an adapted nutrient formulation which can also serve as the sole source of nutrition for a patient;
3. Nutritionally incomplete foods which are not suitable as the sole source of a patient's nourishment [7].

As a result of the patient specific needs, medical nutrition is often personalised in order to optimise the health-benefit effect. The European Union provided manufacturers with basic rules concerning the vitamin and mineral substances that are needed for covering particular requirements of intended users [7]. The legislation of medical nutrition is harmonised on EU level, but in case of the Directives it is implemented in the individual Member States.

Medical nutrition spans both conventional food and pharmaceutical categories. Nevertheless, medical nutritional products are intended for patients suffering from a disease and are predominantly prescribed by a medical professional. Therefore, medical nutrition products are perceived to be more related to the latter category. As a consequence of this industry convergence, it is confusing for the regulatory authorities, medical nutrition companies and market, how to validate the safety,
efficacy and quality of a medical nutritional product for example. In the pharmaceutical industry, clinical trials are an essential aspect in new product development. For the medical nutrition industry in Europe, clinical trials are optional. Companies may choose to carry out clinical trials to obtain sufficient evidence on the efficacy of a product to be able to substantiate a product claim. These product claims are often important in the process of applying for reimbursement. The requirements for reimbursement are dependent on the health-care system of the particular country and the reimbursement decision rests with the respective countries' advisory committees [25, 48].

Already in the last few decades, pharma-like clinical evidence concerning the effectiveness of medical nutrition has significantly enhanced its credibility [35]. Medical nutrition is becoming a well-accepted form of nutritional support for patients suffering from disease-related malnutrition. Disease-related malnutrition is a highly underestimated condition, prevalent throughout hospitals, community health-care centres (outpatients, care homes, general practice) and other community settings [64]. Malnutrition is defined as "a state of nutrition in which a deficiency, excess (or imbalance) of energy, protein and other essential nutrients causes measurable adverse effects on tissuelbody form (body shape, size and composition) and function, and clinical outcome" [17]. There are many causal factors leading to disease-related malnutrition, yet in general, it is the underlying medical condition that affects the intake of essential micronutrients.

### 1.1.4 Pharmaceuticals

The pharmaceutical industry represents the other end of the health and life science sector spectrum, and in turn is highly unrelated to the conventional foods industry. During the 1980s, the pharmaceutical industry experienced an exponential growth spurt, leading to the highest product turnover and market approvals of new chemical entities (NCEs) known to history. Nevertheless, this growth has significantly slowed down at the start of the millennium, due to a number of reasons including, but not limited to rising development costs, enhanced best-standard of care, blockbusters patent expiry and intensified global competition [31, 40]. Pharmaceutical new chemical entities are defined by the EU legislation as: "any substance or combination of substances presented for treating or preventing disease in human beings. Any substance or combination of substances which may be administered to human beings with a view to making a medical diagnosis or to restoring, correcting or modifying physiological functions in human beings is likewise considered as a pharmaceutical product" [9].

Pharmaceutical new chemical entities are considered prescription medicines, used for therapeutic treatment or prophylaxis of a chronic or acute disease. They can only be obtained at a pharmacy with a prescription from a physician. The drug development value chain is considered as one of the most (inter-) nationally regulated processes, whereby the NCE has to demonstrate specific safety, efficacy, quality and ethical standards throughout the discovery, preclinical, clinical and
market phases. As a result, the average clinical development phase of the value chain takes over 12 years and requires an investment close to $€ 0.6$ Billion [55]. Since 1995, the European medicines Agency (EMA) is responsible for the scientific evaluation and monitoring of the safety and efficacy of pharmaceutical products in Europe.

## 2 Trends in the Medical Nutrition Industry

From the four above-mentioned industries, the medical nutrition industry offers the most potential for innovation (Fig. 3.1). This industry has experienced vigorous growth in the last few decades [69]. This growth can be attributed to several factors, such as the increase in ageing population and the growing body of scientific evidence concerning the effectiveness of medical nutrition. At present, various companies have entered the industry domain and are intensely competing with each other. To assess today's and tomorrow's medical nutrition innovation opportunities, it is crucial to monitor industry development.

### 2.1 Medical Nutrition Industry Development

The industry output of the European medical nutrition market is dominated by five companies. The key participants owning of the total EU medical nutrition market share include Abbott Nutrition, B Braun, Danone, Fresenius Kabi and Nestle.

As an industry emerges its innovation activities correspondingly develop. It is therefore crucial for companies within emerging industries to manage innovation using appropriate strategies and business models. There is an extensive body of business oriented literature demonstrating that effective management of innovation works best when matched with the distinct stages of industry development [38]. Examining these patterns is a crucial prerequisite for adopting the appropriate innovation strategies and business models for improving product development and enhancing value creating activities [38].

First, we identify the current stage of development in which the industry is in. In short, industry development is represented by an S-curve, delineating four key stages: emerging, growth, maturity and saturation [2, 19]. The main method for evaluating the industry development phase is by analysing the technological state-of-the-art via patent applications. These are a primary measure reflecting an industries' technological development, which in turn illustrates on the industry's development phase. We visualised a cumulative patent application timeline for the development of medical nutrition from 1995 to 2009 [69]. Here we update, including 2012 (Fig. 3.2). Since 2002, a steep increase in cumulative patenting activity is observed, which is considered to indicate that the technological development of the industry is currently in the growth stage.


Fig. 3.2 Medical nutrition industry development 1995-2012. Updated from [69]


Fig. 3.3 Medical nutrition industry development scenarios. Black line: Classic S-curved technology life cycle [69]. Red line: Steep S-curved technology life cycle. Blue line: Innovation cliff. Green line: Jumping the S-curve. Adapted from: [1, 19, 22, 37, 71]

Nevertheless, it is of importance to forecast the industry development curve, in order to infer future performance (Fig. 3.3). From a macro-perspective, four different future scenarios may exist for medical nutrition industry development, namely, classic S-curve, steep S-curve, innovation cliff and jumping the S-curve. Here, we
will argue the possibility of each of the four scenarios, based on literature review and interviews with key opinion leaders in the field of medical nutrition [70].

### 2.1.1 Classic S-Curved Technology Life Cycle

The classic technology development $S$-curve was introduced in economics by Mansfield [37] in a publication concerning the diffusion of new technologies. The S-curve has since been widely used in management and economic theory [22]. The classical S-curve starts off with the emerging stage, which is characterised by a relatively low technological growth, followed by the growth stage, in which the technological progress rises steeply, and then by the maturity phase, where the growth slows and when it has reached saturation, and reveals a plateau. During the saturation stage, the technology approaches its underlying natural limitation.

Based on the development stage an industry finds itself in, strategic R \& D decisions can be made [19]. The classic S-curve of technology development is worth keeping in mind when considering the current status of the medical nutrition industry and where this industry may be headed. At present, the medical nutrition industry finds itself in the growth phase of the technology life cycle. Based on our data, if the industry performance continues to follow the classic curve, saturation could be reached by mid 2024.

### 2.1.2 Accelerated S-Curved Technology Life Cycle

For a very successful and fast-growing industry, the angle of the upward inflection in the emerging and growth phases may be less than $120^{\circ}$ [71]. The curve follows a similar pattern to the classical S-curve, and eventually levels off at a sustainable high level. One aspect that contributes to the steepness of the curve during the emerging and growth phases is the length of the product development timelines: the shorter this timeline, the steeper the curve. The product development timelines for medical nutrition are significantly shorter when compared to pharmaceutical new product development, yet longer when compared to other fast-moving consumer goods (e.g. conventional foods). Therefore, we predict that the emerging and growth curve for medical nutritional products will fall in between the two other industry categories. Based on this knowledge, it is assumed to be highly unlikely that the medical nutrition industry performance will follow the steep S-curve.

### 2.1.3 The Innovation Cliff

An industry is, more often than not, perceived as durable and stable, capable of surviving many decades. Nevertheless, industries are fragile and prone to collapsing [14]. This is represented by the green curve in Fig. 3.3, which illustrates the so-called 'innovation cliff' scenario.


Fig. 3.4 The lemming effect

During this scenario a technology initially follows the performance characteristics of the classic $S$-curve in the emerging and growth phases, and all seems well. However, the curve is suddenly truncated [71] while the industry plunges off the metaphorical innovation cliff, and seizes to exist any longer (Fig. 3.4). Many different factors can lead to the sudden demise of a technology. Two key factors contributing to this phenomenon include innovation barriers and/or reduced innovation adoption. Surprisingly, the majority of interviewed medical nutrition key opinion leaders predict that the medical nutrition industry is heading towards an innovation cliff within the coming 2-3 years. Based on theoretical models adopted from literature and results from our previous research [70], we propose two different explanations as to why the medical nutrition industry might be headed towards this innovation cliff: (1) Technology/innovation development and (2) technology adoption.

### 2.1.4 Abernathy-Utterback Technology Development Life Cycle

The technology development life cycle explores the roles of the manufacturing companies, as they respond to the forecasted unmet needs within the market. It describes a scissor-curve technology life cycle describing the evolutionary phases of technology development. Abernathy and Utterback's technology life cycle (Fig. 3.5) consists of three phases: fluid, transitional and mature [66]. The fluid phase is characterised by extreme diversities in new product designs. It is in this phase where competitors attempt to meet the various needs of the emerging customer, resulting in a high throughput of innovative product designs in order to grab the attention of the first-mover consumers.

The fluid phase then gives way to a transitional phase, where product innovation decreases and process innovation is on the rise. During this phase a dominant design typically emerges, which has been accepted either by the market or selected as such by the regulatory authorities. Some technologies eventually transition to the mature phase, where product and process innovation lose momentum and the primary focus of the company is mainly set on reducing the manufacturing cost.


Fig. 3.5 Abernathy-Utterback technology development life cycle and the industry life cycleadapted from [29, 66]

When including the classical S-curve describing the industry life cycle to the Abernathy-Utterback model, the mid-emerging phase of the industry life cycle is manifested slightly before the crossing of product and process innovation in the fluid phase. This implies that not all of the customers' needs have been fulfilled and the dominant design has not yet been adopted.

This description typifies the current EU medical nutrition industry situation, which is supported by previous research into innovation barriers within the medical nutrition industry [70]. As a result, the main obstacles include the regulatory ambiguity at both the clinical research as well as at the reimbursement level. Clinical research is perceived by the surveyed KOLs as the main innovation barrier, and it is intricately linked to other financial barriers. This includes the consideration of the chances for being granted reimbursement, which would ultimately stimulate the decision to perform clinical studies. This lack of clarity and standardisation may prevent the adoption of a dominant design. Therefore, a slippery slope is assumed, linking the clinical research barrier with the absence of establishing a dominant design, which in turn reduces the capacity for process innovation. All in all, this scenario would result in medical nutrition industry heading towards the innovation cliff.

### 2.1.5 The Chasm of Technology Adoption

The technology adoption life cycle is a model developed to understand the acceptance of innovation by the consumer market over time. Geoffrey Moore discovered that companies often fail to make the transition from the growth phase to maturity in the technology adoption life cycle (Fig. 3.6) [43]. This gap is known as the chasm, during which product sales drop [43]. Crossing this chasm is often nearly


Fig. 3.6 The medical nutrition chasm of technology adoption adapted from Moore [43]
impossible but progressing beyond it is considered crucial for the ability of an innovative industry to reach the stage of maturity and saturation.

In the medical nutrition industry, innovation adoption is influenced by both health-care professionals and patients. Generally, health-care professionals prescribe medical nutrition and assess which type/nutrient content of medical nutrition is best. However, medical nutrition product characteristics such as taste, smell and tolerance are assessed by the patient. In the view that the medical nutrition industry is a relatively young industry [69], innovation adoption is still at an early stage. The early adopters, in this case mainly the nutrition-oriented health-care professionals, have realised the potential of medical nutrition. Nevertheless, the awareness of available products is low [70] which may cause the medical nutrition industry to fall victim to the chasm.

The challenge of crossing the chasm in this case is to raise awareness among all health-care professionals concerning nutritional interventions through medical nutrition. Subsequently, if awareness among the medical professionals is heightened, they will be able to educate their patients which in turn will stimulate innovation adoption.

Based on Sects. 2.1.4 and 2.1.5, the synergistic effects of technological development and market adoption pose a serious risk for the medical nutrition industry to face the innovation cliff. It is, therefore, in any scenario, of utmost importance to address innovation barriers and increase general awareness on effectiveness of medical nutrition in order to prevent this negative scenario from happening in reality.

### 2.1.6 Jumping the S-Curve

As dark and gloomy as the previous scenario might seem, this scenario provides a more optimistic future for the medical nutrition industry. Generally, once the growth phase has been surpassed, the natural evolution of the industry is to reach the stage of saturation, where technological growth reaches homeostasis. Successful industries are those with companies that manage to jump the classical S-curve halfway through its growth phase to the next technology S-curve. Such a feat can only be accomplished when companies understand the dynamics of the S-curve, which implies the anticipation of market decline. One way of jumping the S-curve and taking advantage of this knowledge, is to radically innovate their way to a new S-curve [46].

Generally there are two types of innovations: incremental and radical. Incremental innovations consist of minor improvements or adjustments to existing inventions or technologies. Radical inventions exhibit key characteristics that are inherently different from existing inventions or technologies. The latter type is considered to form a crucial basis from which subsequent incremental development may evolve [42,58]. Most organisations are familiar with leveraging core products through incremental innovation. This approach is perceived as less risky. It assures positive revenue growth as opposed to the discontinuous and radical approach of breakthrough innovation. In prior research, we demonstrated that even though radical innovation is crucial for industry and company performance, only a few medical nutrition companies innovate radically [69].

For an industry to jump the S-curve, companies are to strategically innovate towards the next S-Curve and jump at the optimal moment. Generally, the optimal time to start building the next S-curve is during the growth phase of the technology life cycle. Whilst in the technological growth phase, companies are still able to maximise their returns while starting to invest in a new radical technology [1, 46]. One way for the medical nutrition companies to jump the S-curve is by identifying new opportunities, such as unmet needs. This can be in the form of addressing unmet patient needs, related to product characteristics, but also by responding to unmet medical needs.

As a rule of thumb, if one company successfully jumps the S-curve through radical innovation, the (incremental) others may follow. The radical innovator will always benefit from first-mover advantages and has a chance of establishing a dominant technology design. Furthermore, radical technology innovation is a strategy to overcome the innovation barriers as described in Weenen et al. [70].

### 2.2 Intellectual Property Strategies in Medical Nutrition

The first and a crucial step for successful innovation within a competitive industry is to protect your inventions through intellectual property (IP) rights [33, 65]. Especially in the early stages of industry development in the emerging and growth phases, the choice of appropriate IP protection methods is fundamental. Different IP methods include: trade secrets, copyright, trademark, defensive publishing and patents [24]. In the view that patents are most valuable to the health and life sciences, we will focus on this particular IP right.

The 5 major players in the European medical nutrition industry exhibit four different patent strategies. This leads to the assumption that patents are perceived with different value for protecting medical nutrition inventions [69]. The origin of the parent-industry could partially account for the difference in patenting cultures. While two of the European medical nutrition companies originate from the food industry, the others stem from the pharmaceutical industry. Generally, the food industry relies heavily on trademarks and trade secrets, and is less familiar with patenting. This is in sharp contrast to the pharmaceutical companies, who are notorious patenting machines.

Patent strategies consist of blocking the competition from commercially exploiting the invention, protective patent thickets/walls, or using a patent application solely for the purpose of a marketing tool [4, 24, 56]. Research shows that the first listed motive to patent is considered to be the most important to medical nutrition industry [68]. Although patents are considered valuable instruments for protecting innovations, companies should always look beyond the boundary of the patent and carefully assess if any other relevant IP methods are applicable [68]. By means of a 7-step medical nutrition IP decision framework, companies and academic R \& D department can assess for each individual invention which IP method-or combinations of IP methods-is most appropriate (Fig. 3.7).

Most medical nutrition companies apply the strategy of patenting to their incremental inventions. This appears to be a similar trend as what has been observed in the pharmaceutical industry where on average more than half ( $51 \%$ ) of all FDA approved drugs are incremental innovations [13]. There are two reasons explaining this pattern. First of all, it is easier to develop an incremental innovation, to stabilise market presence and enhance existing product life cycles, based on an existing product in contrast to developing a radical innovation from scratch. Secondly, incremental inventions have generally proven to be of higher value than radical inventions [69]. Rationally, radical inventions are often high risk and only a few may result in a marketed product that yield a profit.
Fig. 3.7 Intellectual property decision framework tailored to the medical nutrition market

## 3 Discussion: Bridging the Medical Nutrition Innovation Cliff

The health and life sciences are moving towards pharmanutrition oriented product development such as medical nutrition. Faced by innovation declines, pharma and nutrition industries are converging in order to fill the gap. On the one end the conventional food industries are converging with more health-oriented industries, while on the other hand the pharmaceutical industry is moving into the (pharma) nutrition space. It is estimated that in approximately 20 years, $50 \%$ of the pharmanutrition industry will be pharma owned [63]. Enabled by a growing body of evidence, technology development and plenty of unmet needs to fulfil, the medical nutrition industry offers ample future potential. The industry development forecast analysis shows four possible future scenarios. These scenarios include both pessimistic as well as optimistic possible outcomes. Currently the newly emerging medical nutrition industry is within the growth phase of the industry life cycle yet all signs currently point in the pessimistic direction that the medical nutrition industry is heading towards an innovation cliff. In view of this diagnostic observation, the industry has the chance to pre-emptively jump the cliff by starting a new S-curve. The optimal time to start building the next S-curve is during the growth phase of the technological life cycle. Although the medical nutrition industry is currently encountering rapid growth in the growth phase of the technological life cycle, it is time to start thinking ahead. To prevent the dreaded industry saturation plateau, or even worse, the innovation cliff that may lie ahead, companies must realise that incremental innovation alone is insufficient. The solution for future success lies in the radical innovations. These radical innovations allow for jumping the S-curve, gain competitive advantage and start building the medical nutrition industry's future.

An illustrative case-in-point of a more mature industry which has been facing innovation decline since the early 1990s is the pharmaceutical industry. In its early history, the productivity of the pharmaceutical industry and market approval of innovative therapies were relatively easy, which is explained by some critics due to the selection of low-hanging fruits [49]. Currently, the pharmaceutical pipeline is drying up as patents on blockbuster products are expiring and the realisation is kicking in that incremental innovation is insufficient for sustaining business models [27, 31, 44]. The perception of this innovation deficit has motivated large firms to exploit various other strategic options for capturing radical innovations. Since the early 1990s the pharmaceutical industry has been going through significant strategic consolidation of large pharma firms as well as the acquisition of small biotech (Appendix A). Solving this innovation deficit required that firms successfully combined or coordinated merger and acquisition (M \& A) activities, strategic alliances, and licensing deals alongside conventional in-house R \& D [11, 36, 41].

Learning from the pharmaceutical industry, staying ahead of the medical nutrition innovation cliff requires radical innovation. Although the adoption of a clear generic competitive corporate strategy such as described by Porter [53] is essential,
we focus on the implementation of internal development versus acquisition strategies. We propose two development strategies for the medical nutrition industry to achieve this and jump to the next S-curve: first by incorporating radical innovation strategies into their own corporate DNA (organic growth) and second through capturing radical innovation by acquiring smaller innovative medical nutrition start-ups (inorganic growth). The first can only be accomplished if companies adopt systematic processes for initiating, supporting and rewarding radical innovation in-house activities [32, 47]. The challenge in this organic growth strategy lies within the fact that it is easier for existing companies to innovate incrementally since this only requires the leveraging of existing knowledge and resources. On the contrary, new entrants will have a considerable advantage in radical innovation since they do not have to change their knowledge background. Furthermore, large companies, such as the medical nutrition market leaders, may have a difficult time implementing radical innovation because they operate under a "managerial mindset/constraint."

The second strategy of inorganic growth through radical innovation acquisition only offers potential if medical nutrition start-ups continue to emerge and invest in the development of radical innovation. Entrepreneurial start-ups are a valuable source of knowledge necessary to develop radical innovation [15]. Research has shown that active acquisition industries encourage radical innovation, particularly at the SME level [52]. This is in line with the theory of contestable markets, which states that the entry of new business only stimulates industry development and additionally also offers benefit for existing companies [45].

The medical nutrition industry, at present in the growth stage of the industry life cycle (Fig. 3.2), may be considered as especially attractive to start-ups. When demand is growing in an industry, firms can achieve initial success without the intense competitive threat that firms face in mature and overregulated markets. In other words, there is more than sufficient market opportunity available for multiple entrants to achieve commercial successes [54].

Since development and production costs are relatively high in the medical nutrition industry, it is highly unlikely that medical nutrition start-ups will develop into fully integrated nutrition companies [FINCOs]. Most likely, medical nutrition market leaders will view these small innovative firms as prey as opposed to competitors, and will incorporate them into their companies. Even if the SMEs are the source of new ideas, commercialization and wide product diffusion will usually happen only after acquisition by the incumbent. Generally, being acquired is an attractive exit strategy for small firms.

In a similar profile as the pharmaceutical industry but 15 years later, M \& A activity within the medical nutrition industry has increased since 2004 (Fig. 3.8). A total of 11 mergers and acquisitions and 3 joint ventures/partnerships have occurred within this industry. In particular since 2010, acquisition has become more frequent. More start-ups may be realising the potential of the medical nutrition industry in the last few years and are entering the playing field. In addition, large medical nutrition companies may already encounter difficulties in developing


Fig. 3.8 Medical nutrition industry M \& A
radical innovations and are shifting from organic to inorganic growth through acquisition.

However, companies cannot solely rely on insourcing innovation since this is often only a quick-fix. Additionally, if entry barriers prove to be unscalable for medical nutrition start-ups, the flow of innovation will come to a halt, and the acquisition opportunities for large medical nutrition companies will decline accordingly. The optimal innovation strategy is a balanced integration of both organic and inorganic growth. Such a strategy will enable medical nutrition companies to jump the S-curve themselves when acquisition opportunity is low and stock up on radically innovative start-ups when it is an active acquisition industry.

## Appendix A: Pharmaceutical M \& A



Pharmaceutical M \& A from 1990 to 2013. Blue: industry incumbent; Dark blue: Large-scale M7A (above $\$ 10$ billion); Grey: Medium M \& A between $\$ 1$ and $\$ 10$ billion

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Part II
Inflammation and Immunity

# Chapter 4 <br> Modulation of the Gut Ecosystem in Irritable Bowel Syndrome 

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## 1 The Aberration of the Gut Ecosystem in IBS

### 1.1 Gut Microbiota in Healthy Individuals

From birth on, our gastrointestinal tract is colonized by a complex ecosystem consisting of several hundred bacterial species. The bacterial cells in the human body outnumber the mammalian cells by a factor of 10 , and the amount of bacteria increases gradually from very low numbers in the stomach to concentrations of approximately $10^{12}$ bacterial cells per gram of luminal content in the colon, with an especially steep gradient at the ileocecal valve [93].

These organisms normally live in a well-balanced symbiotic state with their host and have an important impact on our health. They form a crucial barrier against pathogens and are involved in the development and maturation of our immune system. In addition, they play a vital role in the metabolism of nondigestible compounds and in the supply of essential vitamins and short chain fatty acids.

Before birth, our intestinal tract is basically sterile. Colonization with microbiota starts during the process of delivery by exposure to the extrauterine environment such as maternal vaginal, fecal, and skin microbiota. During the first months of life, the composition becomes more and more complex, and varies widely between individuals. It takes about 1 year until this rather coincidental, chaotic intestinal microbiota is transformed into a more adult-like, stable community [72]. Different factors such as delivery mode (vaginal or caesarean), infant diet (breast or formula feeding), and the use of antibiotics are likely to influence the early microbiota composition and maturation of the immune system, which in turn might have potential long-term effects on adult microbiota and health later in life [91]. The

[^3]altered microbiota in children delivered by caesarean section might for instance contribute to their higher risk of developing allergic diseases [7].

The individual intestinal microbiota in healthy adults is composed of up to 1,000 microbial species [115], and remains remarkably stable even after a 10-year period [84]. Each adult has its own subject-specific intestinal microbiota composition; however, we all share a common microbial core [79]. It has recently been suggested that all humans can be classified into one of three so-called enterotypes, which are characterized by relatively high levels of one of the three genera Bacteroides, Prevotella, or Ruminococcus [6]. With increasing age, the core microbiota changes and shows a high interindividual variability that is strongly influenced by diet and living situation [19, 20].

It needs to be highlighted that the true extent and diversity of the human microbiota are still unknown. Culture-independent techniques, which enable an unbiased detection of all bacteria present in the human gut based on 16S rRNA (small subunit ribosomal RNA) sequences, have recently been developed. Assays such as phylogenetic microarrays [83] or barcoded pyrosequencing [2] have revealed that the number of intestinal bacteria is much greater than previously thought, and new bacterial species are being discovered continually.

### 1.2 Gut Microbiota in IBS

Alterations in the normal composition of the intestinal microbiota are associated with a variety of disorders, including inflammatory bowel diseases and obesity [32, 54]. It is now widely accepted that a dysbalanced gut microbiota composition also plays an important role in the pathophysiology of irritable bowel syndrome (IBS) [88].

IBS is a very common disorder with a worldwide prevalence of $10-20 \%$. Even though it is not life-threatening or associated with higher mortality, it profoundly affects the patients' quality of life and causes substantial economic costs due to the need for medical consultation and work absenteeism [29, 89]. Symptoms vary between patients and include constipation and/or diarrhea, abdominal pain and cramps, flatulence, fecal urgency, a sense of incomplete evacuation, and relief of pain or discomfort upon defecation [57].

Even though the etiology and pathophysiology of IBS are complex and not well understood, it is well accepted that a dysregulation of the microbe-gut-brain axis plays a very important role. Associated aberrations include visceral hypersensitivity, abnormal gut motility, and autonomic nervous system dysfunction [47]. In addition, there is a growing amount of data revealing a contributing role of an aberrant immune system in the pathogenesis of IBS. Mild immune activation has been found both locally in the gut and systemically [9], and mucosal biopsies from IBS patients are characterized by an increased quantity of various immune cells [18, 24]. Own preliminary data show that mucosal biopsies from IBS patients display an altered composition of immune cells, including immune fingerprinting of

## Microbiota in IBS



Fig. 4.1 Putative mechanisms behind modulating the gut microbiota in IBS. In IBS, the intestinal microbiota shows an aberrant diversity and is characterized by low numbers of beneficial bacteria (shown in green, e.g., bifidobacteria, lactobacilli, and butyrate producers). In addition, abundance of specific harmful bacteria (shown in red, e.g., $R$. torques) has been reported. Probiotics and prebiotics might act by increasing the numbers of beneficial bacteria, while antibiotics predominantly deplete harmful ones. Fecal microbiota transplantation (FMT) introduces a healthy, diverse microbiota
intraepithelial and lamina propria lymphocytes. Furthermore, psychological and environmental factors like anxiety, depression and significant negative life events are believed to contribute to IBS development [30].

A considerable body of evidence points to the presence of a disturbed intestinal microbiota composition in IBS. A specific subtype, the so-called post-infectious IBS, develops after an episode of acute infectious gastroenteritis and is causally linked to aberrations in the gut ecosystem [99]. Moreover, IBS symptoms can be improved by treatments targeting the microbiota such as antibiotics, probiotics and prebiotics (Fig. 4.1) [46, 87, 98]. Importantly, several studies have demonstrated
that the composition of the microbiota in IBS patients is distinct from that of healthy controls [97]. Numerous bacterial species have been found to be differentially abundant in IBS. However, the results are rather inconsistent and no species could be specifically linked to IBS so far.

On the one hand this is probably due to the various techniques that have been applied. Among them are both high-throughput methods such as 16 S rRNA sequencing and phylogenetic microarrays aiming to analyze the immense diversity of the intestinal microbiota population, as well as qPCR and FISH (fluorescence in situ hybridization) assays evaluating only a certain defined set of bacteria. On the other hand, the pathology of IBS has a very heterogeneous character and shows a large interindividual variation of aberrations along the microbe-gut-brain axis without a distinct link between the pathophysiologic mechanism and symptom generation. In addition, different classifications (or none at all) of patients according to symptoms criteria have been used, and it is difficult to account for exogenous factors. Especially diet has a strong influence on the microbiota composition [111].

Earlier studies investigating fecal microbiota in IBS using culture-based analyses detected decreased amounts of bifidobacteria and lactobacilli compared to controls [8]. The first study to apply a specifically designed qPCR assay covering 300 species was published in 2005 [62]. The authors found lower amounts of Bifidobacterium catenulatum and Clostridium coccoides in fecal IBS samples. In addition, amounts of lactobacilli were significantly decreased in the diarrheapredominant group compared to the constipation-dominant subgroup. The same group was also the first to apply high-throughput 16 S rRNA gene cloning and sequencing (after fractioning the community DNA according to the $\% \mathrm{G}+\mathrm{C}$ content), and found changes especially in the phyla Firmicutes and Actinobacteria [49]. A follow-up study analyzing the entire microbiota (without fractioning) of only the diarrhea-predominant IBS subtype $(n=10)$ detected high numbers of Proteobacteria and Firmicutes and low numbers of Actinobacteria and Bacteroidetes compared to controls [51].

Rajilic-Stojanovic et al. analyzed the microbial composition of fecal samples of 62 IBS patients and 46 controls using a phylogenic microarray (HITChip) that enables the unbiased detection of over 1,000 phylotypes [82]. In accordance with other studies, they found that the intestinal microbiota of IBS patients differed significantly from controls, and detected an increased Firmicutes to Bacteroidetes ratio. In addition, they could show that the abundance of several members of Firmicutes and Proteobacteria correlated with IBS symptom scores. For instance, pain scores correlated negatively with Bifidobacterium spp. The lower abundance of bifidobacteria has been reported in several studies [49, 50, 62] but was not found in others [42].

A recent study examined the relationship between fecal microbiota composition and clinical and physiological parameters using pyrosequencing of 16 S rRNA [42]. In this study, the IBS patients clustered into three different groups based on their microbiota composition. Two groups clustered very differently from the healthy controls, whereas the so-called "normal-like IBS group" was
indistinguishable from the controls, and consisted of 15 of the 37 included patients. In the first two groups, the clearest difference in microbiota composition was a higher Firmicutes to Bacteroidetes ratio. Several bacterial taxa were found to correlate with specific clinical symptoms, some in the group comprising all IBS patients and some in the subgroups. For example, the abundance of Cyanobacteria was associated with satiety, bloating and gastrointestinal symptom scores, whereas Proteobacteria correlated with an increased mental component and pain threshold. A study using qPCR found that the amount of Ruminococcus torques was positively correlated with the severity of self-reported IBS symptoms [61]. Interestingly, an increased number of this species in IBS patients has been reported in other studies [49, 82]. Parkes et al. investigated the mucosa-associated microbiota of IBS patients using in situ hybridization of five bacterial-group specific oligonucleotide probe sequences on rectal biopsies. They found that IBS patients in general had a significantly higher number of mucosa-associated bacteria, comprising predominantly bacteroides and clostridia, and found correlations between several species and IBS symptoms, including a negative correlation of bifidobacteria and lactobacilli with stool frequency [73].

The association of specific bacteria with specific IBS symptoms is a promising tool to provide insight into factors contributing to IBS. However, it needs to be taken into account that identical symptoms are not necessarily related to the same pathophysiology.

Unlike the aforementioned study, most studies focus on investigating fecal microbiota. Not many results on mucosa-associated bacteria can be found, even though it is known that the compositions of these can differ [116]. Kerckhoff et al. found lower amounts of bifidobacteria in diarrhea-predominant IBS compared to constipation-predominant IBS and controls using FISH [50]. Carroll et al. used a molecular fingerprinting technique to investigate fecal and unprepared colon mucosal samples of patients with diarrhea-predominant IBS and healthy controls. They found distinct microbial communities between fecal and mucosa samples and between IBS and controls in both types, with a diminished microbial biodiversity only in fecal samples [16]. The possible effects of bowel preparation on the mucosal microbiota composition are discussed later in this chapter.

Own preliminary data showed that IBS patients have a lower proportion of butyrate-producing microbiota compared to healthy controls. Butyrate is the dominant short-chain fatty acid produced by microbial fermentation of undigested dietary carbohydrates in the gut. It is an important energy source for epithelial cells and has many beneficial effects on colonic mucosal function including inhibition of inflammation and carcinogenesis and promotes the colonic defense barrier [35-37].

An additional aberration in IBS seems to be an abnormal microbial gene variety, also referred to as microbial diversity or heterogeneity. However, results described in literature are inconsistent, and both a loss of diversity [17, 21] and an increased heterogeneity have been reported [81]. Both conditions might be indicative of the inability of the IBS ecosystem to maintain its normal composition. A loss of diversity is usually associated with the outgrowth of certain species, while a high
degree of variability could refer to a disturbed community trying to recover and reestablish its previous state [88].

## 2 Antibiotics as Cause or Treatment in IBS

The administration of antibiotics is well known to have both short-term and longterm effects on the composition of the gut ecosystem. In most individuals, the microbiota appears to recover within days or weeks after cessation of antibiotic treatment [25,56]. However, in some cases these alterations can result in a persistent depletion of beneficial bacteria and/or overgrowth of harmful ones [25, 26, 43], as is the case in for example in Clostridium difficile infection. Attention should be paid to the fact that the sensitivity of the methods used for detection of microbial changes is limited by the techniques applied, and deeper analyses might be necessary to detect more subtle alterations.

Antibiotic treatment early in life may be related to the development of certain diseases later on, and has been especially associated with immunological disorders such as asthma and allergies [28]. There is some evidence that the use of antibiotics is connected to an increased risk to develop IBS. A retrospective survey including 421 subjects ( 48 with IBS) found a correlation of antibiotic use with IBS symptoms [68]. Another retrospective review of 26,107 medical records of patients exposed to broad-spectrum antibiotics showed a higher prevalence of IBS development among patients receiving macrolide or tetracycline [107]. In a prospective case-control study by Maxwell et al., subjects receiving antibiotic treatment were more likely to suffer from bowel symptoms than controls during a 4-month follow-up period [64]. Even though further studies are necessary to investigate this in detail, it is possible that a change in the intestinal microbiota caused by antibiotics could contribute to the development of IBS. However, it needs to be considered that also the infection leading to the prescription of antibiotics could be the underlying cause of the higher risk to suffer from IBS.

Nevertheless, antibiotics might not only be a possible trigger of IBS, but on the contrary, also show potential as a successful treatment option. The first antibiotic investigated in a clinical study was neomycin, an antibiotic that is not absorbed in the gastrointestinal tract. It was demonstrated to be effective in improving IBS symptoms ( $35.0 \%$ improvement in a composite score compared to $11.4 \%$ improvement in placebo treatment) [74]. However, its rather severe adverse effects and the fact that it provokes a rapid clinical resistance limit its clinical use [87].

Nowadays the antibiotic of choice in treating IBS is rifaximin. It is a semisynthetic derivative of rifamycin with a low side effect profile and no demonstrable systemic absorption [10]. It is approved by the US Food and Drug Administration for the treatment of traveler's diarrhea and hepatic encephalopathy, but still lacks approval in several other countries. Its efficacy for IBS treatment has been tested in various clinical trials, the largest being the TARGET 1 and TARGET 2 studies [75]. Here, a total of 1,258 subjects with nonconstipated IBS were included.

Treatment with rifaximin ( $550 \mathrm{mg} /$ day ) for 14 days resulted in a significantly higher percentage of patients reporting relief of global IBS symptoms compared to the placebo-treated group ( $41 \%$ vs. $31 \%$ ), and effects were sustained for at least 10 weeks. In addition, bloating, abdominal pain, and loose or watery stools were improved. These results are supported by a meta-analysis that included five articles and found a modest therapeutic improvement of IBS symptoms using rifaximin with a therapeutic gain similar to other currently available IBS therapies [69]. The American College of Gastroenterology Task Force rated rifaximin as a strong drug with moderate evidence for the treatment of IBS with diarrhea [1]. As mentioned before, the reported side effects of rifaximin are very low. Its so-called "number to harm" was evaluated to be 846, meaning that 846 patients would benefit from it before 1 harm event would occur [95]. In addition, rifaximin was effective in retreating patients that presented with a relapse after the first antibiotic treatment, and it does not seem to provoke clinical resistance [76, 112].

Hardly any studies have examined the modes of action of antibiotics with regard to IBS treatment. The aforementioned study from Pimentel et al. investigating the effect of neomycin on IBS found that subjects with IBS often presented with abnormal values in the lactulose breath test (LBT), and antibiotic treatment resulted in normalization of the LBT along with symptom improvement [74]. The authors suggested that the excessive gas production might be caused by the presence of small intestinal bacterial overgrowth (SIBO), a condition where an abnormal number of bacteria is present in the small bowel, and that antibiotics were able to reverse this aberrant colonization. However, the use of LBT to diagnose SIBO is controversial, and several other studies did not find an association between IBS and SIBO [31, 77]. Instead it might be possible that rifaximin reduces the total number of bacteria, especially in the large intestine, which could lead to a decreased amount of gas produced by bacteria, resulting in less flatulence and bloating.

In conclusion, it has been demonstrated that nonabsorbable antibiotics are able to-at least-partially improve IBS symptoms, confirming that alterations in the gut microflora play an important role in the pathophysiology of IBS. However, even though rifaximin seems to be safe, it does not have a very high efficacy and its longterm effects have not been investigated. It is still unclear which bacteria are specifically targeted by the antibiotics and whether this or a decrease in the total bacterial number is responsible for their beneficial effects. In addition, a possible harmful effect of antibiotics on the intestinal microbiota composition also needs to be considered.

## 3 Probiotic Therapy in IBS

Probiotics are defined as living microorganisms, often consumed as food products, which upon ingestion survive the passage through the stomach and have beneficial effects on human health. In order to reach the intestine intact, they need to be resistant to gastric acid and digestive enzymes. However, recent research has shown
that nonviable probiotic bacteria may possess strong bioactivity in the small bowel [104].

The most commonly administered kinds belong to the genera Lactobacillus or Bifidobacterium, and they can be used alone (monospecies) or in combination with several other species (multispecies).

Strong evidence for an effective clinical use of probiotics has been demonstrated for the treatment of antibiotic-associated, traveler's and pediatric diarrhea [65, 66, 100]. In infants with infantile colic, Lactobacillus reuteri significantly improved symptoms, which was demonstrated by a clearly reduced daily crying time [90].

The mechanisms behind the beneficial effects of probiotics are still not completely understood. One mode of action is their antagonistic activity against pathogenic species. By adhering to the intestinal mucosa, probiotics replace existing pathogens or inhibit their adherence, thus providing a healthy microflora [94]. Probiotics can also act against harmful bacteria via the secretion of antimicrobial substances, so-called bacteriocins [23]. In addition, probiotics are able to enhance epithelial barrier function by activating signaling pathways resulting in an increased expression of tight junction proteins or in enhanced mucus production [48, 98]. Another important function of probiotics is their ability to induce beneficial immune responses. These can take effect by direct interaction with immune or epithelial cells, or via secreted molecules [71, 78, 104]. By inducing the expression of opioid and cannabinoid receptors, some probiotics might be able to modulate the perception of visceral pain [86].

All these mechanisms suggest that probiotics could be a promising treatment option in IBS, and numerous controlled clinical trials testing the effect of a wide selection of probiotic strains on IBS have been performed [97]. In general, most of the higher-quality clinical trials so far yielded positive results. Some, however, showed no beneficial effects in IBS and one study even reported symptom deterioration using Lactobacillus plantarum MF1298 [55]. Several meta-analyses came to the conclusion that probiotic use improves IBS symptoms and might be a promising treatment option [40,67,70]. Meta-analyses combining the results of studies using different probiotic strains carry the risk of masking the success, or failure, of a specific strain. Accordingly, the authors agreed that it still needs to be further investigated which strains and which doses are most effective.

Probiotics that demonstrated IBS symptom improvement in more than one controlled clinical trial with a substantial number of patients include Bifidobacterium infantis 35624 [71, 110] and the so-called "Finnish combination" consisting of Lactobacillus rhamnosus GG, L. rhamnosus Lc705, Propionibacterium freudenreichii ssp. shermanii JS and Bifidobacterium breve Bb99 or Bifidobacterium animalis ssp. lactis Bb12, respectively [44, 46].

It is known that different probiotics have distinct functional effects in the human intestine [103]. Some strains have been shown to improve total symptom scores in IBS patients, while others primarily seem to affect bloating and flatulence or stool frequency [97]. Several studies did not distinguish between the different subtypes of IBS such as diarrhea or constipation-predominant IBS, discounting the fact that most strains are probably more effective in treating one kind than the other. It would
of course be ideal to administer a probiotic that specifically targets the respective predominant symptoms of an individual IBS patient; however, as mentioned earlier, one needs to be aware that identical symptoms are not necessarily related to the same pathophysiology. An additional factor to be considered is that clinical trials are often conducted in a hospital setting, which may give rise to an inclusion bias in comparison to subjects suffering from IBS in the general population. These groups may differ in the proportion of the various pathophysiologic mechanisms contributing to IBS symptoms.

Only a few probiotic intervention studies looked deeper into the pathophysiologic mechanisms and evaluated for instance the impact of the tested probiotics on the microbiota composition in IBS.

Kajander et al. investigated the effect of the multispecies "Finnish combination" (see above) on the intestinal microbiota composition of IBS patients. They applied 16 S rRNA gene targeted qPCR assays and assessed the presence of short-chain fatty acids and bacterial enzymes in fecal samples [45]. They did not detect any differences, apart from an increase in Bifidobacterium spp. in the placebo and a decrease in the treatment group. They suggested that other mechanisms besides an increased colonization with the administered bacteria must have been responsible for the beneficial effects on IBS symptoms, probably involving a direct interaction with the intestinal epithelium. Another explanation could be a more dominant effect of some probiotics in the small bowel rather than in the colon. Probiotics may provoke a direct metabolic or immunologic effect in the small bowel [101, 103, 104]. In addition, the applied methods were probably not sufficient to detect the underlying microbial changes. In a subsequent study, the same group applied a similar qPCR assay with a broader target of phylotypes to evaluate the effect of the same probiotic combination on the fecal microbiota in 42 IBS patients and reported that a phylotype with $94 \%$ similarity to Ruminococcus torques was decreased and Clostridium thermosuccinogenes $85 \%$ increased in the probiotic compared to the placebo group [58].

So far it is still not known if probiotics have a higher efficacy if administered as monospecies or multispecies. As several pathophysiologic mechanisms are involved in IBS and in addition, patients present with different aberrations along the microbe-gut-brain axis, a probiotic multistrain combination could provide a broader treatment comprising a variety of needs. In a multispecies mixture, one strain could deliver a beneficial immune effect while another strain improves intestinal barrier function. A multispecies probiotic could also potentially be more effective in the various segments of the intestine. Furthermore, it was shown in an in vitro human intestinal mucus model that individual strains may strongly enhance each other's adherence if combined with other strains, with some combinations being more effective than others [22]. However, besides a potential synergistic effect, probiotics could also exert antagonistic effects against each other if administered in combination.

Even though further research is necessary, some probiotic strains seem to be beneficial in the treatment of IBS. According to the current knowledge, their efficacy is similar to antibiotics. One of the clear advantages of probiotics over
conventional pharmacological medication is their favorable adverse effect profile, which enables chronic administration and preventive treatment.

## 4 Prebiotics in IBS

Prebiotics are nondigestible food compounds that serve as substrates for specific desirable bacteria in the intestine and stimulate their growth and/or metabolism, resulting in beneficial effects on the host's well-being and health [85].

Most prebiotics specifically increase the number of health-promoting bifidobacteria and lactobacilli. Many compounds with prebiotic effects belong to the group of nondigestible carbohydrates, more precisely oligosaccharides or polysaccharides, and include oligofructose (inulin) and trans-galactooligosaccharides. They naturally occur in many edible cereals, fruits, and vegetables such as wheat, bananas, onion, garlic, chicory, and artichokes, where they function as carbohydrate stores [80, 92]. In addition, oligosaccharides with prebiotic effects are found in human mother's milk and are thought to contribute to the high amount of bifidobacteria and lactobacilli detected in the feces of breast-fed compared to formula-fed babies. Supplementation of infant formulas with human milk-like prebiotics appears to have a beneficial immunological effect resulting in lower incidence of allergies and infections [5]. In adults, suggested health benefits of prebiotics include protection against traveler's and antibiotic-associated diarrhea [27, 53].

Few studies have investigated the effect of prebiotics on IBS symptoms. In a double-blind crossover trial with 21 IBS patients no effect of oligofructose (Raftilose 95) administration over a 4-week time course could be observed, even after separate analysis of the diarrhea and constipation-predominant subgroups [41]. The authors speculated that the administered dose of $6 \mathrm{~g} /$ day might have been too low to show demonstrable effects. Another clinical trial tested the effect of a novel trans-galactooligosaccharide in a 12-week parallel crossover design [96]. 44 IBS patients were included and two different doses of the prebiotic were used (3.5 and $7 \mathrm{~g} /$ day). Both doses significantly increased bifidobacteria and lactobacilli numbers. The lower dose was able to improve stool consistency, flatulence and bloating as well as total symptom score and subjective global assessment values of the IBS patients, whereas the higher dose only improved subjective global assessment and anxiety scores. It needs to be pointed out, however, that also the placebo showed positive effects regarding flatulence, abdominal pain and total symptoms.

An important readout of these studies could be the absence of reported side effects, which is not necessarily expected. Even though bifidobacteria and lactobacilli themselves do not produce gases as part of their metabolism, the rapid fermentation of the prebiotics in the proximal bowel often causes increased intestinal gas production. This can lead to enhanced flatulence and bloating even in healthy subjects, and would be an especially unfavorable feature in IBS, where patients already suffer from these symptoms and often experience visceral
hypersensitivity [59]. An ideal prebiotic to be used in IBS should therefore ferment slowly throughout the entire colon, so that the produced gases are evenly distributed, thus causing fewer complaints.

Prebiotics have mostly been investigated regarding their effect on bifidobacteria and lactobacilli. As described before, IBS patients tend to have a lower proportion of butyrate-producing microbiota. The administration of butyrate via enemas resulted in a substantial decrease of visceral perception in healthy volunteers, suggesting a possible beneficial effect in disorders associated with visceral hypersensitivity such as IBS [105, 106]. The interaction of butyrate with the receptor GPR43 expressed on immune cells seems to play an important role in the regulation of immune response in the gut [52,63]. Thus prebiotic compounds that specifically serve as substrates for butyrate-producing bacteria could be especially beneficial in improving IBS symptoms.

The modes of actions of prebiotics are not only limited to the stimulation of growth of beneficial bacteria and their metabolic products. Even though there are only few high-quality human studies testing the effect of prebiotics alone on the immune system, it has been suggested that prebiotic compounds are able to directly interact with carbohydrate receptors on intestinal epithelial and immune cells [92]. This has already been clearly demonstrated for other members of the group of nondigestible carbohydrates, the $\beta$-glucans. These compounds can be found in various grains, mushrooms, and yeast. Per definition they do not qualify as prebiotics, as they have not been shown to specifically affect certain beneficial bacteria, but they have been widely studied with regard to their direct, receptormediated effects on various immune cells [108].

Prebiotics and probiotics can also be administered in combination, and are denoted synbiotics. The presence of the prebiotic aims at enhancing the viability and activity of the administered probiotic and of resident beneficial bacteria, at best resulting in a synergistic effect. So far, there is only one placebo-controlled trial evaluating the effect of synbiotics on IBS symptoms. It included 68 IBS patients and reported improvement of abdominal pain and bowel habits using a novel synbiotic known as SCM-III that successfully increased lactobacilli, eubacteria, and bifididobacteria [102]. Further beneficial effects have been described in several open-label studies; however, those results need to be assessed with caution as the placebo response in IBS is high [80].

## 5 Colonic Cleansing and the Effects on the Ecosystem

As many IBS patients, although taking prescribed medication, still suffer from symptoms, it is not surprising that they often resort to self-treatment options offered by complementary and alternative medicines [113]. A commonly used selftreatment is the use of oral laxatives or self-administered enemas with the aim to clean or even detoxify the colon and thereby improve symptoms such as diarrhea, constipation or flatulence.

A recent study has shown that a standard bowel cleansing procedure using a polyethylene glycol-based preparation, as it is routinely performed to prepare the colon for colonoscopy, leads to changes in the composition of the mucosaassociated intestinal microbiota in healthy individuals [38]. These changes might be due to a loss of mucosa-associated bacteria and the associated biofilm. A similar effect was observed with regard to the fecal microbiota composition [60].

The impact of such a bowel cleansing on IBS symptoms has never been investigated, and similar to antibiotic use, both beneficial and harmful effects seem possible. In IBS patients, it could be that the bowel cleansing leads to a reduction in the overall amount of bacteria and gives the intestinal microbiota an opportunity to reestablish a healthy balance. This effect could for instance be promoted by a parallel administration of probiotics. However, at least to our knowledge, there are no reports in the literature about a relief of symptoms after performing colonic cleansing in IBS patients, indicating that there is probably no long-term effect.

In healthy subjects, colonic cleansing could also lead to a dysbalance in the normal microbiota. Especially in individuals whose microbial balance has been challenged before, for instance by recent infections or the use of antibiotics, such a bowel cleansing could be a final trigger leading to sustained aberrant intestinal ecosystem.

## 6 Fecal Microbiota Transplantation in IBS

Fecal microbiota transplantation (FMT) consists of the infusion of suspended stool from a healthy donor into the intestine of a patient with the aim to restore a disturbed intestinal microbiota towards a normal ecosystem. Its use in Chinese medicine goes back to the fourth century where it was applied to treat food poisoning and severe diarrhea [114]. Nowadays, FMT is established as a highly efficient treatment for recurrent Clostridium difficile infection, where perturbations of the intestinal microbiota seem to be responsible for the overgrowth of pathogenic Clostridium difficile strains [39]. In this disorder, FMT has a cure rate of over $90 \%$ [33], and has been proven to be a durable and safe method according to a recent multicenter long-term follow-up study [15]. In addition, FMT treatment in this study was highly acceptable to patients: $97 \%$ of the treated patients would be willing to undergo another FMT in case of recurrent Clostridium difficile infection, and $53 \%$ would choose it as their first treatment option before antibiotics.

FMT might be a promising treatment for other diseases that are causally linked to alterations in the gut microbiota. Vrieze et al. demonstrated that the transfer of fecal microbiota from healthy lean donors into patients with metabolic syndrome increased their insulin sensitivity and reduced triglyceride levels [109]. FMT positively changed the gut microbiota of the recipients, resulting in a higher proportion of health-promoting butyrate-producing bacteria.

The successful application of FMT has also been reported in other disorders including inflammatory bowel disease, multiple sclerosis, autism, and chronic fatigue syndrome, mainly reflecting case studies [4, 11, 13, 14].

No randomized clinical trials investigating the impact of FMT on IBS have been published so far. Only one study reported possible positive effects in patients with IBS symptoms [12]. The same group applied a mixture of cultured, nonpathogenic bacteria resembling normal gut microbiota into the cecum of IBS patients and reported improved symptoms in 25 out of 33 [3].

The successful improvement of IBS symptoms using antibiotics, prebiotics, and probiotics suggests that alterations in the gut microbiota are involved in the mechanisms behind. However, these treatments are usually not very efficient and tend to provide only moderate and transient effects, probably due to the fact that only a small part of the complex microbial ecosystem is affected. FMT, however, results in durable changes of the colonic microbiota that can still be detected 6 months after the treatment [34]. Exchanging the microbiota of an IBS patient with the microbiota of a healthy donor holds the potential to be a lot more efficient in reestablishing a normal, healthy microbiota, and FMT could therefore be a promising novel treatment option for IBS.

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# Chapter 5 <br> Role of Omega-6 and Omega-3 Fatty Acids in Inflammatory Bowel Disease 

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## 1 Introduction

The intestinal immune system maintains both a state of tolerance toward intestinal luminal antigens and the ability to eliminate enteric pathogens [1]. This balance is achieved through several mechanisms including reciprocal regulation of pro-inflammatory, effector $\mathrm{CD} 4^{+} \mathrm{T}$ cells and tolerising, suppressive Tregulatory (Treg) cells. Inflammatory bowel disease (IBD), which includes ulcerative colitis and Crohn's disease, is a chronic relapsing intestinal inflammatory disorder of the gastrointestinal (GI) tract. In IBD, the balance between the pro-inflammatory effector T cells and the tolerising suppressive Treg cells is altered. Consequently, the deleterious effects of pro-inflammatory T cells outweigh the tolerising, antiinflammatory effects of Treg cells resulting in uncontrolled active intestinal inflammation resulting in disease.

[^4]
## 2 Inflammatory Bowel Disease Subtypes

Ulcerative colitis is characterized by inflammation limited to the intestinal mucosa with extension from the rectum in a uniform manner to involve part of, or the entire colon [2]. In contrast, Crohn's disease can affect any part of the GI tract, but most commonly affects the terminal ileum and proximal colon, with patchy transmural, granulomatous inflammation that results in inflammatory, stricturing, or penetrating/fistulising disease [2].

## 3 Inflammatory Profiles, Th1 and Th17 Responses, and Eicosanoids

Crohn's disease is associated with a predominant T helper (Th) $1 / \mathrm{Th} 17$ cellmediated response induced by interleukin (IL)-12, IL-23, and IL-27 with a concomitant increase in production of IL-2, IL-17, IL-18, IL-26, interferon (IFN)- $\gamma$, and tumor necrosis factor (TNF)- $\alpha$ [3-11]. The importance of Th17 cells, which express the IL-23 receptor (IL23R) on their surface, is further supported by genome-wide association studies, which have demonstrated the IL23R and other genes involved in the differentiation of Th17 cells as IBD susceptibility genes [1214]. Furthermore, in transgenic mice that overexpress the IL-23 subunit p19, severe systemic inflammation, involving both the small and large intestine, has been observed [15], highlighting this pathway in promoting strong activation of effector T cells and perpetuation of organ-specific inflammatory responses. While IL-23 stabilizes the Th17 phenotype [3, 7-10, 16-18], IL-12 and IL-27 promote Th1 responses [19-22], and suppress the development of Th17 effectors [23-25]. Eicosanoids produced from the omega-6 (n-6) polyunsaturated fatty acid (PUFA) arachidonic acid (ARA; 20:4n-6) include the 2-series prostaglandin (PG) $\mathrm{PGE}_{2}$, which promotes IL-23 and inhibits IL-12 and IL-27, and the 4 -series leukotriene (LT) $\mathrm{LTB}_{4}$, which is elevated in the mucosa of Crohn's disease and ulcerative colitis patients and in experimental models of IBD [26-29]. These same mediators have been shown in experimental models to promote the accumulation of Th17 cells in inflamed tissue [26-28], leading to further neutrophil accumulation, and activation of fibroblasts, epithelial cells, and macrophages to release pro-inflammatory cytokines and chemokines and metalloproteinases [30]. Furthermore, IL-17synergizes with lipopolysaccharide (LPS) to induce cyclooxygenase (COX) 2 expression in colonic sub-epithelial myofibroblasts [31] maintaining a pro-inflammatory environment. In contrast, ulcerative colitis is associated with a predominant Th2-mediated response characterized by natural killer (NK) T cell secretion of IL-13 and increased production of IL-4 and IL-5 [32-34]. However, there is now clear evidence that there is considerable overlap in inflammatory profiles between Crohn's disease and ulcerative colitis and Th1 and Th17 responses are involved in both diseases [25, 35-37].

## 4 Changing Incidence of IBD

The rising incidence of IBD has been highlighted by two recent comprehensive reviews of temporal trends in worldwide incidence rates of pediatric and adult IBD [38, 39]. These studies reaffirmed rising global rates in both children (due primarily to a rising incidence of Crohn's disease) and adults (with $56 \%$ of Crohn's disease and $29 \%$ of ulcerative colitis studies having shown a statistically significant increased incidence since the 1980s) in both developed and developing nations [38, 39]. Moreover, studies have shown that individuals migrating from low prevalent regions (e.g., South Asia) to higher prevalent countries (e.g., England and Canada) are at increased risk for developing IBD, particularly among first- and second-generation immigrants, highlighting the importance of environmental influences [40-42].

## 5 Dietary Fat and IBD

Among the various environmental influences that potentially contribute to the pathogenesis of IBD, dietary factors are decidedly plausible, potentially through effects on the intestinal epithelial barrier, the mucosal and systemic immune systems and mucosal inflammatory response, and through modulation of intestinal microflora, each of which has been implicated in IBD. In Western countries, the association between plasma LDL cholesterol and atherosclerotic heart disease has led to the replacement of dietary sources of saturated animal fats with vegetable oils high in the n-6 PUFA, linoleic acid (LA, 18:2n-6), as well as in margarines and shortenings. Technological advances of the twentieth century associated with increased availability and reduced cost has further augmented consumption of liquid vegetable oils. This strategy associated with increased accessibility has lead to increased consumption of LA from around $3 \%$ dietary energy in the1930s to about $7 \%$ in the 1980s. Currently, n-6 PUFA intake represents approximately $7 \%$ of dietary energy in the USA, about $5 \%$ in Canada [43-48], and in most European countries intake of PUFA ranges from 4 to $6 \%$ of energy [49, 50]. Linoleic acid can be converted to ARA by the pathway shown in Fig. 5.1. The intake of the bioactive n-3 PUFAs eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), found mainly in seafood, has either remained the same or decreased resulting in a marked increase in the balance of n-6 to n-3 fatty acid intake in the diet, with consequential changes in abundance of ARA, EPA, and DHA in plasma membrane phospholipids and alterations in inflammatory tone towards a pro-inflammatory environment [51]. Indeed, a considerable body of experimental and clinical data has accumulated in recent years to show the availability of unesterified ARA released from cell membrane phospholipid is fundamentally linked to inflammation through mechanisms that involve further metabolism to pro-inflammatory eicosanoids (Figs. 5.1 and 5.2), including


Arachidonic acid (20:4n-6)


Fig. 5.1 Metabolic pathway of conversion of the precursor $n-6$ fatty acid linoleic acid to the eicosanoid precursor arachidonic acid. COX cyclooxygenase, LOX lipoxygenase


Fig. 5.2 The pathways of eicosanoid synthesis for arachidonic acid. COX cyclooxygenase, $L O X$ lipoxygenase, HPETE hydroperoxyeicosatetraenoic acid, HETE hydroxyeicosatetraenoic acid, $L T$ leukotriene, $P G$ prostaglandin, $T X$ thromboxane. Note that not all metabolites are shown
$\mathrm{PGE}_{2}$ and $\mathrm{LTB}_{4}$, with an enhanced Th1/Th17 response [26-28, 52-54]. EPA and DHA can act to counter the effect of ARA [51].

That the dietary changes described here coincide with the rising incidence of IBD was first observed in studies in adults conducted in the US in Olmstead County and in the UK in Cardiff [39]. Conversely a favorable n-6 to n-3 PUFA balance should promote an environment more tolerant to immunological challenge. In Japanese and Inuit societies with traditionally high EPA and DHA intakes from fish or marine mammals, and low intakes of n-6 PUFA, the rates of IBD have been low. However, as the Japanese diet has become increasingly westernized, the incidence of IBD, and specifically Crohn's disease, has increased and this was linked to rising intake of n-6 PUFA [55].

## 6 Timing of Dietary Exposure

The variable age of onset of IBD and duration of subclinical disease makes it exceedingly difficult to prove a causal relationship with diet. Moreover the timing of dietary exposure is likely exceedingly important and probably precedes the onset of clinical disease possibly by many years. Epidemiological studies suggest that the early dietary experience wherein nutrient deficiencies or imbalances occur has the potential to alter the normal developmental trajectory and lead to long lasting effects on cell function and disease susceptibility [56, 57]. In a recent experimental model, maternal dietary fat intake during gestation and lactation was associated with altered colonic membrane fatty acids in the newborn and nursing rat, altered colonic epithelial barrier integrity with long-lasting effects and perturbed mucosal response to chemical-induced (dinitrobenzene sulfonic acid; DNBS) colitis in later life, in the absence of long-lasting effects on colonic lipids [52]. This study supports the double hit theory wherein changes in colonic membrane $n-6$ and $n-3$ fatty acids in early life altered the developmental trajectory and predisposed or primed the host to an exaggerated immunological response to an inflammatory insult in later life.

As PUFA are essential nutrients, the developing fetus and young breast-fed infant depend solely on the mother for an adequate supply of both $n-6$ and $n-3$ fatty acids. Placental transfer and secretion into breast milk of $n-6$ and $n-3$ fatty acids is variable and dependent on maternal dietary intake of n-6 and n-3 fatty acids, which in turn influences plasma membrane phospholipids and inflammatory tone of the offspring [52,58-60]. Consequently, a marked increase in the balance of n-6 to $\mathrm{n}-3$ fatty acid intake in pregnant and lactating women associated with the changing trends in dietary lipid consumption over the later half of the twentieth century has likely been accompanying by marked changes in abundance of ARA, EPA and DHA in intestinal plasma membrane phospholipids of offspring. This dietary change has potential consequential effects on epithelial barrier integrity and intestinal mucosal inflammatory tone. In this regard, 15-day-old neonatal rats in a high LA maternal dietary group accumulated colonic membrane ARA and manifested with a severe inflammatory response and tissue damage to dinitrobenzene sulfonic
acid (DNBS)-induced colitis [60]. Alternatively, a more balanced maternal dietary n-6 to n-3 fatty acid with high $\alpha$-linolenic acid (ALA; 18:3n-3) was associated with ALA, EPA and DHA accumulation in colon phospholipids and a shift in intestinal mucosal inflammatory tone toward an anti-inflammatory milieu with a resultant reduction in the intestinal inflammatory response to an inflammatory insult. Additionally, the changes in maternal diet in all likelihood influence the developing intestinal microbiome of the offspring.

## 7 Dietary Intake of n-3 PUFAs in Healthy Human Volunteers

Studies in healthy human volunteers have helped increase our understanding of the anti-inflammatory potential of fish oil [51]. Supplementing the diet of healthy volunteers with fish oil containing between 3 and 15 g of EPA and DHA per day has shown a decrease in neutrophil and monocyte chemotaxis towards various chemoattractants including bacterial peptides, $\mathrm{LTB}_{4}$, and human serum [61-63], though a dose response study suggests that near maximum inhibition occurs at an EPA and DHA intake of 1.3 g per day [63]. Similarly dietary supplementation with 1.5 g of EPA and DHA per day has been shown to decrease expression of major histocompatibility complex class molecules and intercellular adhesion molecule-1 on the surface of human monocytes stimulated ex vivo with IFN- $\gamma$ [64]. While some studies have shown that fish oil supplementation providing least 2 g EPA and DHA per day decreases production of TNF- $\alpha$, IL-1, IL-6, or $\mathrm{PGE}_{2}$ by mononuclear cells [65-70], several others have failed to show an anti-inflammatory benefit. Though the reasons for these discrepancies remain unclear, technical factors, and the relative contributions of EPA and DHA in combination with patient heterogeneity and polymorphisms in genes affecting cytokine production are likely important determinants [71, 72]. Studies suggest that the effect of dietary fish oil on TNF- $\alpha$ production is dependent on the TNF- $\alpha-308$, and the TNF $\beta+252$ alleles [71, 72].

## 8 Dietary PUFAs and Adult Models of Experimental Colitis

The pro-inflammatory consequences of high dietary n-6 fatty acid intake are evident in adult models of experimental colitis. High dietary intake of LA prior to and during the course of TNBS and Citrobacter rodentium-induced colitis enriched colon phospholipids with LA and ARA and exacerbated host mucosal Th1/Th17 response with increased severity of tissue damage [73, 74]. Conversely, high intake of EPA and DHA often given as fish oil prior to and during the course of an infectious insult with Citrobacter rodentium [74], or an inflammatory insult with

TNBS [73, 75, 76], DSS [77, 78], or acetic acid [79] ameliorated the inflammatory response and colon tissue damage. Additionally, high intake of fish oil in IL-10 knockout mice that spontaneously develop colitis was associated with a significant reduction in colonic inflammation [80]. Similarly, a study in Fat-1 transgenic mice, which expresses the Fat-1 gene encoding ann-3 fatty acid desaturase enzyme, that converts n-6 PUFA to n-3 PUFA, showed an attenuated inflammatory response to DSS-induced colitis and the presence of resolvin E1, resolvin D3, and neuroprotectin D1 in colon tissue [81]. Pretreatment of mice with resolvin E1 (a pro-resolving EPA metabolite) in TNBS-induced colitis significantly attenuated the inflammatory response, colonic damage, and mortality, suggesting that the generation of resolvins could be a potentially important anti-inflammatory mechanism of action of n-3 PUFA [82]. Collectively, the experimental studies show beneficial effects of n-3 PUFAs through influences on the local mucosal microenvironment to support a reduction in synthesis of pro-inflammatory mediators (e.g., MCP1, MIP2, KC, IFN- $\gamma$, TNF- $\alpha$, IL-6, IL-12, IL-17, IL-21, IL-23, inducible nitric oxide) [73, 74, 81, 82] and eicosanoids [75], resulting in a reduction in mucosal Th17 cell accumulation and inflammatory damage.

## 9 Dietary n-3 PUFAs and Patients with IBD

Whilst the beneficial effects of EPA and DHA, often given as fish oil, have been shown in a spontaneous model of intestinal inflammation [80] and in experimental models of colitis [74-76, 78, 79], therapeutic benefit has not been consistently observed in clinical trials in patients with IBD [83-87]. Nevertheless, in several studies in patients with IBD who supplemented their diets with fish oil, EPA and DHA was incorporated into intestinal mucosal tissues creating a mucosal microenvironment with the potential to reduce mucosal inflammation [88-94]. Some randomized controlled trials in IBD patient treated with fish oil have reported therapeutic benefit, including improved clinical scores, improved sigmoidoscopy scores, and an attenuation in histological damage associated a lower relapse rate and reduction in corticosteroid use [51]. The dose of EPA plus DHA used in these clinical trials has typically been between 2.5 and 6 g per day, with an average intake of about 4 g per day [51]. In a 1-year double-blind, placebo-controlled trial in Crohn's disease patients, a $33 \%$ absolute reduction in the 1-year risk of relapse was observed in 39 patients treated with $2.7 \mathrm{~g} / \mathrm{d}$ of enteric-coated $\mathrm{n}-3$ PUFA compared to 39 patients receiving placebo [85]. In contrast, no benefit was observed in a second 1-year risk of relapse trial in Crohn's disease patients in remission on corticosteroids and supplemented with $5 \mathrm{~g} / \mathrm{d}$ of $\mathrm{n}-3$ PUFAs [95] or in two largescale multicenter, randomized, double-blind, placebo-controlled studies (EPIC 1 and EPIC 2) with $3 \mathrm{~g} /$ day n-3 PUFAs as maintenance therapy in patients with quiescent Crohn's disease conducted in Canada, Europe, Israel, and the USA between January 2003 and February 2007 [87]. For EPIC-1, 188 patients were assigned to receive n-3 PUFA and 186 patients to receive placebo and
corresponding numbers for EPIC-2 were 189 and 190 patients, respectively. The rate of relapse at 1 year in EPIC-1 was 31.6 \% in patients who received n-3 PUFA and 35.7 \% in those who received placebo and corresponding values for EPIC-2 were 47.8 and $48.8 \%$, respectively. In a meta-analysis of 13 IBD studies with fish oil supplementation that reported clinical outcomes, variable effects of n-3 PUFAs were reported on clinical score, sigmoidoscopic score, histologic score, induced remission, and relapse [96, 97]. However, there was only sufficient data to perform meta-analysis on the relative risk of relapse and for ulcerative colitis with no benefit reported [96, 97]. Nevertheless, there was a statistically nonsignificant reduction in the requirement for corticosteroids for n-3 PUFAs relative to placebo in 2 studies and no studies evaluated the effect of n-3 PUFAs on the requirement for other immunosuppressive agents. Additional meta-analyses evaluating maintenance of remission in ulcerative colitis and Crohn's disease identified limited, if any, benefit [86, 98-100]. Thus, the studies to date suggest only weak evidence that n-3 PUFAs have clinical benefit in patients with IBD. It is important to emphasize that differences in study design, patient selection, patient heterogeneity and differences in formulation, dose, and duration of administration of n-3 fatty acids have confounded our ability to adequately assess the clinical benefit of $n-3$ PUFAs intake. Moreover, in experimental models of IBD, most often n-3 PUFAs are administered prior to and during the course of infectious/inflammatory insult in contrast to the administration to patients with established intestinal inflammatory disease. Hence, n-3 PUFA supplementation is not currently supported for clinical use in patients with IBD, but further well-designed clinical trials with appropriate dosing and duration of EPA and DHA should still be considered.

## 10 Conclusion

There is considerable evidence that EPA and DHA, the major $n-3$ PUFAs found in seafood and in marine oils, are important regulators of the inflammatory response with actions that are in part mediated through replacement of ARA in cell membranes. However, they are also metabolized to weak eicosanoids and perhaps more importantly to potent pro-resolving mediators, and they can attenuate T cell reactivity, production of pro-inflammatory cytokines and chemokines, leukocyte chemotaxis, and leukocyte-endothelial cell interactions [51]. Animal models of n-3 PUFA and intestinal inflammation and pathology consistently demonstrate a benefit. Some trials of fish oil supplementation in IBD have shown clinical and endoscopic benefit, but unfortunately the findings have been inconsistent and meta-analyses have concluded that there is currently no clear evidence of benefit. Dose-dependent actions of marine n-3 PUFAs on inflammatory responses have not been well described, but it appears that a dose of at least 2 g of EPA and DHA per day is necessary to achieve an anti-inflammatory effect [51]. Perhaps even a higher dose is required in persons with active inflammatory disease. A better understanding of the dose-response relationship is needed in patients with IBD. Conversely,
an increased intake of n-6 PUFAs is associated with increased synthesis and membrane incorporation of ARA with accompanying production of pro-inflammatory mediators, and increased oxidative stress in n-6 fatty-acid-rich membranes. Epidemiological, clinical, and experimental data suggest that high dietary n-6 fatty acid intake as is typical in a Western diet has the potential to exaggerate the inflammatory response suggesting a plausible link to increased dietary consumption and increasing incidence of IBD. Accordingly, implementing a diet appropriately high in n-3 fatty acids and lower in n-6 fatty acids with a more balanced $n-6-n-3$ fatty acid ratio could provide a means to protect against the development of aberrant inflammatory disease or to limit the inflammatory process in established disease. Such an approach might be one environmental strategy employed to limit the rising incidence in IBD, but will likely need to be implemented prior to or early in the development of the disease.

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# Chapter 6 <br> N-3 Polyunsaturated Fatty Acid and Neuroinflammation in Aging: Role in Cognition 

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## 1 Introduction

The central nervous system (CNS) has long been considered as a privileged organ from the point of view of immunity, as the blood-brain barrier (BBB), thanks to its tight junctions, limits the entry of immune cells, notably lymphocytes, into the brain. Research in neuroimmunology has shown that the brain possesses its own system of defense, which, in addition to being activated by immune stimuli, is closely linked to the immune system. Inflammatory cytokines, which are important mediators of communication within the immune system, also act in the brain, in particular by activating the innate immune cells of the brain that in turn, produce inflammatory cytokines [30,31]. The synthesis of brain cytokines is finely regulated, allowing them to return to basal levels without leading either to a rupture of the BBB or to cerebral lesion. On the other hand, when these factors are synthesized in large quantities or in a chronic manner by the brain, they have toxic effects on neurons, resulting in substantial neuronal dysfunction that can lead to cell death. The alteration of neuronal functions induced by cytokines action is also seen during aging, where microneuroinflammation, characterized by microglial reactivity and the chronic production of low levels of inflammatory cytokines, occurs [75]. This microneuroinflammation, which increases the vulnerability of the aging brain to immune stimuli, is characterized by the increased production of brain cytokines and the risk of developing delirium and/or neurodegenerative disorders with an inflammatory component, such as Alzheimer's disease [97]. Accordingly, clinical and epidemiological studies have shown a correlation between the systemic expression levels of inflammatory cytokines and the incidence of functional/behavioral

[^5]alterations (cognitive or mood disorders) in elderly subjects. In this context, limiting the development of chronic neuroinflammation represents a key element in the protection of the brain against neurodegenerative disorders.

Diet constitutes a strategy of choice for such an approach, since it represents an environmental factor to which individuals are exposed throughout their life. Increasing attention has been paid to omega-3 ( $\mathrm{n}-3$ ) and omega- $6(\mathrm{n}-6)$ polyunsaturated fatty acids (PUFAs), micronutrients that are essential since they cannot be synthesized de novo by the organism. An increasing database attests of the powerful immunomodulatory effects of PUFAs [20]. Thus, n-3 PUFAs form the basis of lipid derivatives (neuroprotectins and resolvins) with anti-inflammatory properties, whereas n-6 PUFAs are the precursors of the proinflammatory prostacyclins and stimulate the production and activity of inflammatory cytokines. The brain is extremely rich in PUFAs and the accumulation of PUFAs in brain tissues takes place during the perinatal period in proportions which are dependent on maternal dietary levels. Conversely, their levels diminish as the individual ages, but can be corrected by appropriate nutritional strategies. During the last few decades, the lifestyle of Western societies has evolved towards a decrease in energy expenditure mainly related to sedenterization and a change in our dietary habits towards the consumption of energy-rich foods with high levels of saturated fats, n-6 PUFAs, and sugar, and poor in vitamins and micronutrients [112]. This dramatic reduction in the dietary supply of n-3 PUFAs and the corresponding increase in n-6 PUFAs, leading to an imbalanced $n-6-n-3$ ratio currently estimated at 12-20 in developed countries (of note, the current recommended ratio is 5), could therefore contribute to the sensitization of the brain to inflammatory cytokines, and thus to the development of neurodegenerative and/or neurobehavioral disorders.

## 2 The Innate Immune System of the Brain (BIIS)

At the periphery, tissue injuries caused by trauma or pathogens induce an immediate local inflammatory response involving local cells and characterized by the synthesis and release of proinflammatory factors, among which cytokines and chemokines, followed by systemic recruitment of immune cells. The purpose of this local response is to eliminate pathogens and to promote tissue repair. However, the failure of resolving the insult and deregulated injury result in chronic inflammation, which is toxic for the tissue, and result in cytodestruction. In addition, inflammation involving the peripheral innate immune system can affect the brain [30,31]. However, coming to the inflammation in the brain it appears that the very well recognized principles of peripheral inflammation cannot be strictly applied to the brain. The term neuroinflammation is broadly used then to discriminate brain inflammatory response from peripheral inflammatory response. However, the definition of neuroinflammation is still a matter of debate. Indeed, some authors claim that neuroinflammation corresponds to the elaboration of neuroinflammatory responses linked to the influx of peripheral innate immune cells (macrophages
etc.) in the brain [53]. Other authors use the term neuroinflammation to describe the brain inflammatory response involving not only peripheral immune cells influx in the brain but also the discrete response of brain innate immune cells (BIIS) so called microglia [49]. In this case, microglia response is not limited to sterile stimuli but also to pathogens (virus, bacteria, etc.). In the case of microglial activation in the brain associated to aging, obesity, neurodegenerative diseases such as Alzheimer disease (AD) or Parkinson disease, the term pseudo-inflammation has been proposed. Interestingly, the use of the term neuroinflammation suggests that the reaction occurring in the brain is distinct from peripheral inflammation, since it is mediated by microglia. In this review, as we focused of BIIS and in particular microglia, the term neuroinflammation will be used.

### 2.1 Resident Microglial Cells and Blood Derived Monocytes

Microglial cells are the most important part of the innate immune system of the brain (BIIS). These cells are the parenchymal resident macrophages of the brain and constitute the first line of immune defense of the brain (phagocytosis, antigen presentation and secretion of proinflammatory cytokines) [15]. They account for $5-20 \%$ of the non neuronal glial cells. Microglia are distinct of brain macrophages that are found in the meninges, choroid plexus, and perivascular space thanks to their different developmental origin. Indeed, very recent data highlight that microglia derives from macrophages produced by primitive hematopoiesis in the yolk sac [47, 64] while brain macrophages derive from precursor blood monocytes that are formed in the bone marrow from hematopoietic stem cells [105]. Microglia precursors colonize the CNS during the embryonic and fetal phases of development [102]. Interestingly, an increase of CD11b+/F4/80+ microglia occurs in the postnatal brain of rodents [2]; however, whether this is due to the proliferation of microglia precursors or the recruitment of monocyte-derived microglia precursors is still unknown.

Despite a huge amount of data on microglia, little is known about the phenotype and function of microglia in the brain under physiological conditions. It is anyway largely accepted now that microglia has a surveillance and maintenance role in normal brain function. Such an activity is supported by recent evidence that microglia processes are highly motile in the uninjured brain and contact synapses quite frequently [33, 95, 124]. Such an interaction is believed to be involved in synapse maturation and elimination in the adult brain [121] and synaptic pruning (phagocytosis of synapses) during brain development [96]. Synaptic pruning is necessary for normal brain development [96, 107] and requires cellular contact and involves the phagocytic receptor CR3 and CX3CR1 [107, 125].

Microglia are particularly sensitive to changes in their microenvironment and readily become activated in response to infection, trauma, or disease [55]. Microglia phagocytes apoptotic neurons and reduces debris and neuroinflammation which, in turn is beneficial to alive neurons [94]. In inflammatory situation, the phagocytic
adaptor protein MFG-E8 is released by microglia and binds to phosphatidylserine (PS) exposed on apoptotic neurons. This activates neuronal phagocytosis by microglia via the vitronectin receptor [93]. AnnexinA1, an eat-me signal released by microglia serves as a bridge between PS and dying neuron, help the microglia to discriminate between apoptotic neurons and healthy neurons [83]. Activated microglia in inflammatory state appears to lose their ability to discriminate between apoptotic and viable neurons, resulting in phagocytosis of healthy neurons.

In the adult brain, microglial cells have a ramified morphology when quiescent (Fig. 6.1) and an amoeboid morphology when activated. Ramified microglia cells generally do not display phagocytic activity and weakly express ligands and receptors involved in macrophage function. Disseminated throughout the brain parenchyma, they use their processes to receive signals such as nonself and danger-self ligands from their microenvironment, which reveal the existence of the presence of a pathogen or a lesion respectively. In order to do this, microglial cells express a set of pattern recognition receptors (PRRs) including the Toll-like receptors (TLRs) that allow the recognition of PAMPs (pathogen-associated molecular patterns), such as the bacterial endotoxin [14, 81]. Besides pathogens, dangerassociated molecular pattern (DAMP) molecules, the endogenous PRR danger-self ligands, activate the brain inflammatory reaction [132]. The activation of PRRs by PAMPS and DAMPs induces the secretion of cytokines and chemokines by microglia which thereby coordinate the inflammatory reaction, thanks to the expression of membrane receptors for the inflammatory cytokines interleukin (IL) $-1 \beta$, tumor necrosis factor (TNF) $\alpha$, and IL- 6 and several chemokines. In vivo, IL- $1 \beta$, TNF $\alpha$, and IL- 6 are produced by microglia in response to peripheral immune stimuli like the bacterial endotoxin lipopolysaccharide (LPS) [32].

### 2.2 Microglial Cells Plasticity

The BIIS response promotes the clearance of pathogens, toxic cellular debris, and apoptotic cells and therefore protects the brain. Indeed, a complete blockade of microglial activity exacerbates brain damage in adult and neonatal hypoxic ischemic injury models [71]. However, the sustained expression of inflammatory factors such as cytokines can lead to neurodegeneration. The BIIS response is therefore a double-edged sword representing a fine balance between protective and detrimental effects and therefore need to be tightly controlled. Microglia phenotypes, so called polarization, could be crucial in the protective or detrimental role of PRR-activated BIIS response toward neurons. According to what was described for macrophages, authors have suggested that activated M1 cells have cytotoxic properties, M2a are involved in repair and regeneration, M2b have an immunoregulatory phenotype while M2c have an acquired-deactivating phenotype [98] (Fig. 6.2). In vivo, microglia express proinflammatory cytokines associated with an M1 response (IL-1, IL-6, IL-12, and TNF $\alpha$ ) in response to an immune stimulus [98]. Microglia polarization into a M1 phenotype is transient. Microglia returns to a surveying M2


Fig. 6.1 Photomicrograph of microglial cells (green, CX3CR1-GFP $+/-$ mouse) and neurons (Violet, nucleus labeled with anti-NeuN) in the dentate gyrus of mouse hippocampus. Left corner: magnification of unitary microglial cell (maximum projection of Z-stack)


Fig. 6.2 Microglia phenotype plasticity. Microglia can adopt different phenotypes: M1 (classical activation), M2a (alternative activation), M2b (immunoregulatory), and M2c (acquireddeactivation). According to their phenotype, microglia cells express different clusters of differentiation (CD) such as CD86 or CD206, or type-II proteins of major histocompatibility complex (MHC) and secrete different cytokines and chemokines. $C C L$ chemokine (C-C motif) ligand, IFN interferon, $I L$ interleukin, $L P S$ lipopolysaccharide, $T G F$ transforming growth factor, TNF tumor necrosis factor, Yml chitinase 3-like 3
state when the immune stimulus is resolved thanks to anti-inflammatory mediators [15]. In particular, IL-10 and IL-4 are important mediators of M1 microglia deactivation [42]. Importantly, M1/2b induces increasing and M2a-induced decreasing neuronal loss [26]. Recently Girard et al. have nicely demonstrated in organotypic hippocampal slices that infiltrating macrophages are cytotoxic while microglia are protective, independently of its M1/M2 phenotype [48]. Some molecules emerge as potent regulators of the balance between M1 and M2 microglia, opening new avenues for the treatment of neuroinflammation. As an example, lipocalin-2 has very recently been shown as playing a key role in the M1 polarization of microglia through the inhibition of IL-10, an anti-inflammatory cytokine M2-related [60].

### 2.3 Neuron-Microglia Interactions

The extent of neuroinflammation depends on the bidirectional interactions between neurons and microglia. Recruitment and activation of microglial cells require well organized reciprocal communication between neurons and microglia. Recent evidence indicates that neurons control microglia activity. As a result, neurons release ON or OFF signals to regulate the activation of microglia. OFF signals (CD200, CX3CL1, CD47, CD55, and HMGB1) are produced by healthy neurons to keep microglia in their surveillance mode. On the opposite, damaged neurons express inducible ON signals (chemokines, purine, and glutamate) to activate microglia and phagocytosis [15]. Interestingly, such neuronal-glial interactions are impaired in the aged brain leading to amplified and prolonged microglial activation and production of proinflammatory cytokines [118]. Recent data highlighted the importance of CX3CL1, a 73-amino acid protein with a chemokine domain, in the communication between microglia and neurons and in the control of neuroinflammation [12]. In the brain, CX3CL1 is expressed by healthy neurons and binds to the fractalkine receptor (CX3CR1) which is exclusively expressed by microglia [56]. CX3CL1 has anti-inflammatory and neuroprotective activities as it reduces neuronal apoptosis [120]. In addition, CX3CL1 contributes to maintaining a resting phenotype in microglia and controls the overproduction of nitric oxide synthase (iNOS), IL-1 $\beta$, IL-6, and TNF $\alpha$ in response to an insult. CX3CR1 knockout mice display increased IL-1 $\beta$ expression in microglia both in basal level and after a LPS treatment [100, 103]. The increased IL-1 $\beta$ expression is associated to the impairment of cognitive function, neurogenesis, and synaptic activity [5, 103] and increased neuronal death [100]. These results reinforced the idea that CX3CL1 is a key factor to regulate microglial activity in both physiological and pathological conditions.

To end, the protective effects of microglia towards neurons have been suggested to involve neurotrophic factors as demonstrated in vitro. Indeed, IL-4 conditioned microglia release IGF-1 (insulin-like growth factor 1) which exerts neuroprotective, survival, and pro-regenerative activities [19]. BDNF (Brain-derived neurotrophic factor) is also thought to be released by microglia to stimulate axonal sprouting toward wound edge [10]. However, whether microglia release neurotrophic factors in healthy condition is poorly demonstrated.

### 2.4 BIIS Facilitates Cognition in Physiological Conditions

The idea that BIIS is involved in normal cognitive processes came from studies more than a decade ago. As (1) cytokines, chemokines, and their receptors are expressed in the brain, (2) neurons and microglia communicates, and (3) microglia contacts both presynaptic and postsynaptic elements, it was suggested that the BIIS is a neuromodulator in the healthy brain [130]. Transgenic mice for cytokines (IL-1 $\beta$, IL-6, and TNF $\alpha$ ) or chemokines (CX3CR1) have memory impairment [52, 68, 103], suggesting that these factors promote learning and memory. Mice deficient in TNF $\alpha$ exhibit marked reduction in neuronal arborization in the hippocampus together with hippocampal-dependent memory test impairment [51]. IL-1 receptor deficient mice and brain overexpressing IL-1 receptor antagonist (IL-1ra) mice displayed a slower rate of learning in the spatial memory paradigm [4, 52]. Interestingly, mice with genetic deletion of P2X7-ATP receptors, which are critical for IL- $1 \beta$ production in microglia, displayed altered spatial memory and no IL- $1 \beta$ expression and abrogation of hippocampal neural activation following exposure to this memory test [68]. Icv administration of low dose of IL- $1 \beta$ results in better memory [131]. All together, these data strongly support the idea that a low level of IL- $1 \beta$ in the hippocampus plays an important role in learning and memory processes. In addition, a huge amount of data clearly shows that cytokines are involved in physiological synaptic plasticity, in particular long-term potentiation (LTP), known to underlie memory storage in the hippocampus [117]. In the healthy hippocampus, microglia also actively participates in adult neurogenesis [111]. As at least half of the new cells produced die, microglia actively remove the apoptotic cells through phagocytosis without being activated [111]. To end, chemokines produced by microglia and neurons have been reported to facilitate memory, together with neurobiological processes thought to be involved in memory [126].

## 3 BIIS in the Aging Brain, Effect on Cognition

### 3.1 Microglia Are Primed in the Healthy Aged Brain

Aging is associated with senescence of microglia, impaired microglia phagocytic activity, low-grade neuroinflammation, and cognitive impairment. Chronic low grade inflammatory state in the aged brain is characterized by a higher expression of proinflammatory cytokines IL-1 $\beta$, IL-6, and TNF $\alpha$ to the detriment of antiinflammatory factors such as IL-10 and IL-4. This state is called inflammaging at the periphery and in the brain. The overproduction of proinflammatory cytokines in the absence of infection or injury in the aged brain could be linked to the impairment of microglial activity. Indeed, microglia number and activity increase in normal aging [35]. These cells, in addition to producing proinflammatory cytokines, display the presence of lipofuscin granules, and decreased processes complexity, a morphological change found in activated microglia [55, 121]. In addition, microglia in the aged brain expresses higher levels of CD86, major histocompatibility complex II (MHC II), TLR, and CR3/CD11b which are markers of activated microglia [98]. Senescent microglia has reduced phagocytic activities of betaamyloid in aged transgenic mice which could be due to its M1 phenotype [57]. The mechanisms involved in increased microglia activation in the aged brain is not fully understood; however, as CD200 and CX3CR1 expression are impaired it could be possible that neuron-glia interactions are disturbed [35]. Impaired interplay between neurons and glia may be responsible for derangements from normal brain aging to neurodegenerative processes. When challenged with immune stimuli or a stress, aged animals clearly mount an exaggerated neuroinflammatory response, characterized by the overproduction of proinflammatory cytokines (IL-1 $\beta$, IL-6, TNF $\alpha$, iNOS) compared to young congeners $[6,50,116]$. In addition, microglia from aged animals is activated for a longer duration when challenged, suggesting an alteration of the shut-off system. Proinflammatory cytokine overexpression to insult or infection is linked to the microglia priming or sensitization, which was first defined by Cunningham et al. [29]. In adult, microglia shifted to a M2 phenotype under IL-4 treatment while aged microglia retained a M1 phenotype [42]. Aged mice display a prolonged downregulation of CX3CR1 together with decreased CX3CL1 in the brain after a LPS treatment [128]. As a result, M1 polarization is associated with the initiation and perpetuation of neuroinflammation, while M2 polarization of microglia is involved in the resolution of neuroinflammation in the aged brain. The failure of aged microglia to polarize from a proinflammatory to an anti-inflammatory phenotype supports the detrimental role of primed microglia in neurodegenerative diseases with a self-sustaining and self-amplifying cycle of neurotoxicity. As an example, the inhibition of lipocalin-2 expression reduces M1 polarization, microglial M1 gene expression, and neuroinflammation-associated impairment in motor behavior and cognition [60]. These new knowledge stimulate research
aiming at developing drugs targeting the M 1 polarization to promote M 2 when appropriate [73].

### 3.2 Inflammaging and Cognition

Recent clinical and experimental data have shown a strong association between blood proinflammatory cytokines levels, especially IL-6, quality of life, and neuropsychiatric symptoms in a cohort of elderly subjects [23, 24] and in aged laboratory mice $[69,91]$. Significantly elevated levels of pro-inflammatory cytokines, such as IL- $1 \beta$, in key brain regions responsible for mediating memory, such as the hippocampus, have been shown to impair a wide range of memory processes [7-9, 130].

The mechanisms underlying the effect of proinflammatory cytokine on mood and cognitive disorders have been intensively studied in rodents [130]. Studies using minocycline, a tetracycline derivative that inhibits microglial activation and cytokine production, show a link between brain cytokine production and depressive-like symptoms as well as spatial memory impairment [32]. In addition to impairing the metabolism of serotoninergic and glutamatergic neurotransmission systems, which are well known players in mood and cognition respectively, brain proinflammatory cytokines alter hippocampal synaptic plasticity in adult and aged rodents [79]. Importantly, we have recently showed in a population of elderly subjects that age-related low-grade systemic inflammation was associated with alterations in the activity of two enzymatic pathways, the indoleamine 2,3 dioxygenase (IDO) and guanosine-triphosphate-cyclohydrolase-1 (GTP-CH1) pathways, which are involved in the metabolism of key monoamines [24]. Interestingly, increased IDO activity was associated with the depressive symptoms of lassitude, reduced motivation, anorexia, and pessimism in the same population. In contrast, decreased GTP-CH1 activity correlated more with neurovegetative symptoms, including sleep disturbance, digestive symptoms, fatigue, sickness, and motor symptoms.

Age-induced IL-1 overproduction in the brain, and more particularly in the hippocampus, is associated with a decrease in synaptic plasticity measured by LTP in the dentate gyrus, which could explain the cognitive impairment observed in the elderly [9, 79]. Receptors for IL-1 are distributed with a high density in the hippocampus, where IL-1 exerts inhibitory effects on memory [9]. There is also evidence for a role of endogenous brain IL-1 in the normal physiological regulation of hippocampal plasticity and learning processes [79]. Low levels of IL-1 are essential for memory and plasticity, whereas higher levels of IL-1, similar to those achieved during aging and neurodegeneration, can be detrimental [130]. Several inflammatory factors, among IL-1b, induce neuronal hyper excitability through the NMDA receptor 2B phosphorylation and Ca2+ influx (reviewed in [130]. In susceptible rodents with synaptic loss (aging, prion disease), hyper-excitability induced by inflammatory stimuli results in delirium, which further impairs
cognitive functioning [92] and excitotoxicity, apoptosis, and neurodegeneration [109]. IL-1 $\beta$ impairs the release of glutamate from synaptosome in vitro [122]; however, whether its overexpression in the aging brain has the same effect remains to be determined. IL-1-induced BDNF decrease in the hippocampus could account for LTP impairment and memory deficit including in the aged rodents [9, 28].

Normal aging is associated to a decline in neurogenesis that could support the cognitive decline [17, 36]. Among other factors, chronic neuroinflammation is supposed to be an important contributor to such impairment. Indeed, long-term administration of LPS suppresses hippocampal neurogenesis through decreasing new neuron survival and the recruitment of new neurons in hippocampal networks [66]. Such an effect could be mediated by high level of proinflammatory cytokines, in particular IL-6, IL-1 $\beta$, and $\mathrm{TNF} \alpha$ [90, 108, 127]. On the opposite, antiinflammatory cytokines such as TGF $\beta$, IL-10, and IL-4 enhance neurogenesis through their effect on neuronal progenitor differentiation [3, 11]. Interestingly, the administration of minocycline (an inhibitor of microglia activation) or IL-10 to transgenic mice model of AD attenuates the reduction of neurogenesis observed in these animals $[16,65]$. In the aged hippocampus, decreased neurogenesis is correlated with microglia activation and cytokines production [45, 67]. CX3CL1 administration and IL-1 $\beta$ blockade both restore neurogenesis in the hippocampus of aged rodents [5, 45]. Whether chemokines and cytokines effect on neurogenesis is involved in age-related memory impairment remains to be determined. However, a very elegant study recently highlighted aged-related chemokines CCL2, CCL11, and CCL12 as systemic factors of neurogenesis and memory impairment further reinforcing this idea [123].

BIIS is considered to be important in AD. High levels of pro and antiinflammatory factors, increased PRR expression and chemokines are found in the brain of AD patients [73]. However, whether these factors are a cause or a consequence of AD is still a matter of debate as $\mathrm{A} \beta$ is known to activate microglia cells, which in turn produce proinflammatory factors. Very recent data highlighted that microglia, because of its impaired activity in the AD brain, cannot phagocyte $\mathrm{A} \beta$ that therefore accumulate. In turn, $\mathrm{A} \beta$ accumulation activates microglia in a chronic proinflammatory state that contributes to the disease progression and, ultimately cognitive decline [73]. IL-1 overexpression has been implicated in both the initiation and progression of neuropathological changes [104]. Accordingly, overexpression of IL-1 in the Alzheimer brain has been linked to an increased microglial activity, frequently associated with amyloid plaques. In addition, brain from Tg 2576 mice (a model of Alzheimer disease) exhibited significant increases in IL-1 expression in comparison to healthy animals. Moreover, aged Tg2576 showed mounted and exacerbated cytokine response to LPS, a process that may be responsible for the amplification of degenerative processes. Recent data suggest however that in transgenic mice overexpressing IL-1 $\beta$, amyloid and tau pathology are differentially regulated, with a reduction in amyloid deposit and an exacerbation of Tau hyperphosphorylation [46]. Such effects could involve CX3CL1 as its lack of activity has been reported to either decrease [27] or activate $A \beta$ clearance
[76]. Further studies are needed to precise the role of CX3CL/CX3CR in these processes.

In the last decades, several trials aiming at reducing brain inflammation in AD patients show poor positive results [1, 18]. Because the role of BIIS in the development of AD is complex, other strategies aiming at optimizing microglia activity, rather than just blocking inflammatory factors synthesis in the brain could be more beneficial [73].

## 4 Polyunsaturated Fatty Acids and BIIS

PUFAs of the n-3 or n-6 families are essential nutrients, as they cannot be generated de novo in mammals. In plants, they exist as precursors (linoleic acid (18:2 n-6, LA) and $\alpha$-linolenic acid (18:3 n-3, ALA)) that are metabolized by a series of elongation and desaturation steps into arachidonic acid ( $20: 4 n-6, A A$ ) in the first case and eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) in the second (Fig. 6.3). These PUFAs are incorporated into cell membranes as phospholipids. The liver is the principal site of conversion of the precursors LA and ALA into long-chain PUFAs, although other organs such as the brain also express the necessary elongases and desaturases. Since the two series of PUFAs compete for the use of the enzymes necessary for their biosynthesis, and because they have distinct physiological properties, the $n-6-n-3$ ratio in the diet is of particular importance. Foods that were previously consumed by humans were rich in n-3 PUFAs (products of hunting), while those consumed today are poor in these nutrients. Since the industrial revolution, the ratio of n-6-n-3 PUFAs in the diet has increased from 1 to almost 20 in industrialized countries like the United States, leading to a significant deficiency in n-3 PUFAs [113].

The dietary deficiency in n-3 PUFAs is associated with significant decreases in DHA in the brain, and could thus promote neuroinflammatory processes and the subsequent development of inflammatory-based CNS disorders [75]. Supporting this notion is the very low incidence of inflammatory disorders (e.g., psoriasis, asthma, multiple sclerosis) in populations, such as Greenland Inuits, with a high n-3 PUFAs dietary intake due to elevated fish consumption. The effect of $n-3$ supplementation is currently subject to debate. While some clinical studies have reported anti-inflammatory effects of n-3 PUFAs administered in the context of chronic and autoimmune inflammatory disorders, other reports fail to reproduce these findings. Conversely, dietary supplementation with fish oil rich in long-chain n-3 derivatives, including EPA and DHA, leads to an improvement in symptoms in patients with rheumatoid arthritis, chronic inflammatory intestinal disorders, or multiple sclerosis [21].



Eicosanoids (series 2 and 4)


22:6n-3


Eicosanoids (series 3 and 5)
Resolvins (series D and E) / Neuroprotectins

Fig. 6.3 $\mathrm{N}-6$ and $\mathrm{n}-3$ polyunsaturated fatty acids (PUFAs). $\mathrm{n}-6$ and $\mathrm{n}-3$ essential fatty acids precursors are linoleic acid (LA) and $\alpha$-linolenic acid (ALA). These precursors are metabolized into arachidonic acid (AA) and eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), respectively. AA is metabolized into derivatives that belong to the eicosanoid family, series 2 and 4. EPA and DHA metabolic derivatives belong to the eicosanoid family, series 3 and 5, resolving family (series D and E) and neuroprotectins

### 4.1 N-3 PUFAs and Age-Related Neuroinflammation

Experiments conducted in animals have highlighted brain DHA as a potent mediator of the protective effects of dietary n-3 PUFAs. Because it cannot be synthesized de novo in mammalian cells, brain DHA must be provided in the diet, either in the form of its precursor $\alpha$-linolenic acid ( $\alpha$-LNA, 18:3n-3) or in the form of DHA. Low dietary intake of n-3 PUFA decreases DHA levels in the animal brain. As a result, emotional behavior (depressive-like symptoms and anxiety) as well as learning and memory are impaired as shown by us and others [41, 70, 74, 91]. On the opposite, positive effects of diet enriched in DHA on learning and memory have been demonstrated in laboratory animals [25, 44, 129]. During aging, the levels and the turn-over of brain PUFAs decrease, particularly in the hippocampus, cortex, striatum, and hypothalamus. Brain levels of DHA and AA diminish in aging rats with alterations in cognition and in LTP in the hippocampus [38]. In transgenic SAMP8 mice, in which aging is accelerated, DHA decreases with age whereas lipid peroxidation increases [99]. In addition, the conversion of the precursors LA and ALA into their long-chain derivatives becomes less efficient. In fact, the activity of
the desaturases responsible for the conversion of LA and ALA into their respective long-chain derivatives, and the activity of the $\Delta 6$ desaturase in particular, decreases with age in the liver and the brain. Phospholipid synthesis pathways are also altered with age, thus reducing the incorporation of PUFAs into membranes. The combination and interaction of these different alterations associated with aging contributes to a reduction in the level of DHA, i.e., a reduction in the index of membrane fluidity, in the brain of elderly people. In animals, aging was found to be associated with a decrease in the membrane content of AA in the hippocampus together with an attenuation of LTP that can be reestablished by a diet containing AA [84]. These data support the idea of the importance of DHA dietary supply in aged subjects.

As mentioned above, PUFAs represent potent immunomodulatory agents. Increased levels of n-6 PUFAs, in particular AA is associated to increased inflammatory signaling and decreased DHA levels in the brain. Indeed, DHA decreases the expression of brain inflammatory markers following systemic LPS administration [87], brain ischemia-reperfusion [72, 80], and spinal cord injury [59]; however, the direct effect of DHA on BIIS is hard to conclude since primary injury was also improved. We have recently demonstrated in vitro that the production of IL- $1 \beta$ and TNF $\alpha$ by murine microglia induced by LPS was strongly inhibited by DHA through its effect on LPS signaling pathway Nuclear Factor $\kappa$ B [34]. In vivo, chronic dietary n-3 PUFA deficiency significantly increased the production and release of IL-6 and TNF $\alpha$ in the blood [85]. In addition, mice exposed throughout life to a diet devoid of n-3 PUFAs displayed lower brain DHA level and higher LPS-induced IL-6 in the plasma and in the hippocampus [87]. With aging, IL-6 expression was increased in the cortex of both $\mathrm{n}-3$ deficient and $\mathrm{n}-3$ adequate mice while IL-10 expression was decreased with no effect of long-term $\alpha$-LNA-deficient or -enriched diet [91]. On the opposite, short-term exposure to dietary EPA reduced IL-1induced spatial memory deficit and anxiolytic behavior [114, 115] and improved LPS and A $\beta$-induced inhibition of LTP in both adult and aged rats [88]. The expression of markers of microglial activation (CD68, MHCII, and CD11b) increases with age in animals, as does the number of microglia in the brain of humans, attesting of the occurrence of age-related neuroinflammation [50]. Microglial cell reactivity is involved in the age-dependent increase in the production of inflammatory cytokines, as demonstrated by the inhibition of inflammatory cytokine overexpression by minocycline in aged rats [54]. In epidemiological and observational studies, a higher level of blood n-3 PUFAs is associated with lower proinflammatory cytokine production [37, 43, 61, 62]. In a cohort of elderly subjects, depressive individuals with an elevated plasma $n-6-n-3$ ratio were found to exhibit higher levels of TNF $\alpha$ and of IL-6 [62]. F2-isoprostane, used as an oxidative marker and telomere length, used as an indicator of immune cell aging, are decreased in the blood of subjects supplemented with EPA/DHA [63]. Additionally, n-3 PUFA supplementation in elderly subjects reduced the levels of inflammatory cytokines produced by blood leukocytes stimulated in vitro [86]. The production of PGE2 by monocytes is inversely correlated to the EPA content of leukocytes obtained from aged subjects after the consumption of dietary complements containing different doses of EPA [101]. In rats, microglial
Quiescent or M2
microglia


Fig. 6.4 Potential role of n-3 PUFA in inflammaging. In the aged brain, microglia are primed and polarized into a M1 phenotype and secrete pro-inflammatory cytokines that could play a role in cognitive impairment. The protective effect of n-3 PUFAs toward cognitive deficit in aging could be linked to the promotion of an anti-inflammatory M2 phenotype
activation, production of IL- $1 \beta$ and alterations in hippocampal LTP with age were attenuated by EPA [78, 82]. Importantly, a 2-month fish-oil dietary supply increases DHA in the brain, prevented proinflammatory cytokines expression and astrocytes morphology changes in the hippocampus, and restored spatial memory deficits and Fos-associated activation in the hippocampus of aged mice [69]. To the extent that the level of peripheral cytokines reflects that of cytokines in the brain, these results suggest that dietary n-3 PUFAs modulate neuroinflammation and associated neurobehavioral effects in elderly individuals [75] (Fig. 6.4).
$\mathrm{N}-3$ and n-6 PUFAs are substrates for phospholipase (PL), cyclooxygenase (COX), or lipoxygenase (LOX) which are enzymes involved in inflammatory signaling cascades. As a result the inflammatory cascade involves PLA2 that cleaves the PUFAs esterified to the sn-2 position of phospholipids and release free n-3 and n-6 PUFAs. AA is then converted by COX-1/2 into derivates such as prostaglandins, thromboxanes, orprostacyclins with proinflammatory activities, while EPA is the precursor of series 3 prostaglandins with anti-inflammatory properties. The mechanisms underlying the anti-inflammatory effect of DHA in the brain are still poorly understood. Interestingly, DHA is metabolized by acety-lated-COX-2 in the presence of aspirin in resolvins. The recent discovery of a novel family of endogenously generated autacoids, namely, resolvins and protectins, with potent anti-inflammatory and proresolving activities offer to better understand the protective effect of DHA in the brain [110]. In particular, resolvin D1 (RvD1), which originates from DHA via lipoxygenase, promotes the resolution of inflammation and is found in the brain [58]. Very interestingly, DHA and resolvinD1 promotes macrophage polarization toward a M2 state in obese mice adipose tissue [119]. In vitro, RvD1 promotes Abeta phagocytosis [89]. Neuroprotectin D1 (NPD1), aDHA derived docosanoid is expressed in the brain and have antiinflammatory and protective activities [13]. Chronic infusions of DHA or NPD1 in the brain significantly decreased neuroinflammatory processes triggered by a middle cerebral artery occlusion [80]. NPD1 had a more potent effect than DHA [77, 80]. However, it remains to demonstrate that NPD1 is the intermediary of the anti-inflammatory effect of DHA in the brain.

Epidemiological studies reveal the importance of n-3 PUFA levels in the development of age-linked neurodegenerative disorders. Thus, decreases in plasma and brain DHA levels have been shown in patients with Alzheimer's disease. These results, however, remain controversial, since other studies have demonstrated an increase or an absence of variation in brain DHA levels in similar populations. Nonetheless, the risk of dementia was found to be augmented in elderly subjects presenting low levels of circulating EPA [106]. In addition, regular consumption of diets rich in n-3 PUFA, such as the Mediterranean diet, appears to contribute to a decrease in the risk of depression and/or dementia in the elderly [39, 40]. The use of a mouse model of Alzheimer's disease, the Tg2576 mouse, has demonstrated that a dietary supply of DHA leads to a reduction in the formation of amyloid plaques. However, the administration of dietary supplements containing DHA to patients with Alzheimer's disease or mild cognitive impairment has not yielded conclusive results [22].

## 5 Conclusion

There is growing evidence that the expression and action of proinflammatory cytokines in the brain are responsible not only for the development and maintenance of mood and cognitive disorders during the host response to infection, but also during chronic inflammatory states and aging. In addition, neuroinflammation can have detrimental consequences on neuronal viability, especially when maintained over long periods of time and transiently amplified by peripheral infectious episodes. All of this points to the interest of finding new ways of controlling inflammation in the brain. Because of their abundance in the brain and their modulatory effects on inflammation and cell functions, PUFAs definitely play a role in this process. However, this role needs to be better characterized by multidisciplinary studies aimed at assessing the effects of these molecules at different levels, from the molecular level to that of the organism as a whole.

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# Chapter 7 <br> Nutritional Programming of Immune Defense Against Infections in Early Life 

Alma J. Nauta and Johan Garssen

## Abbreviations

| LCPUFAs | Long-chain Polyunsaturated fatty acids |
| :--- | :--- |
| DOHaD | Developmental Origins of Health and Disease |
| HMOS | Human milk oligosaccharides |
| Ig | Immunoglobulin |
| lcFOS | Long-chain Fructo-oligosaccharides |
| NK | Natural killer |
| scGOS | Short-chain Galacto-oligosaccharides |

## 1 Introduction

The critical role of environmental factors during early-life development with potential long-term effects on health has been addressed in the "developmental origins of health and disease" ( DOHaD ) paradigm and is supported by accumulating evidence obtained from epidemiological and animal studies [31]. Early-life programming is becoming an accepted scientific concept and leads some to suggest that the genetic impact is perhaps overestimated. The concept of programming during early life was proposed by studies of Barker et al., who revealed that events in early life could influence longer-term disease risk and that under conditions of

[^6]suboptimal in utero nutrition, the fetus must adapt to its environment to ensure survival of the organism [9, 37]. Evidence is accumulating that early-life factors play an important role in the development of the immune system and that events or specific exposure during pregnancy can modify gene expression through epigenetic mechanisms and thereby determine the functionality of the immune system. In particular, the importance of nutrition and the effects of nutritional imbalances, both deficiency and excess, on morbidity and mortality from infectious diseases in infants and young children have been addressed [45, 49, 64, 65, 76, 80]. Early alterations in the immune system are also relevant for other immune-mediated diseases, such as autoimmune and allergic disorders; however, the focus of this book chapter is on the consequences for the defense against infections.

## 2 Neonatal Immune System

Although the immune system at birth is competent and able to defend against infections and to respond to immunization, it invariably differs to the immune mechanisms present in adults. Exposures after birth are essential to further develop certain aspects of the adaptive and innate immune system that are not fully functional at birth. The relative immaturity of the immune system at birth is the consequence of the low antigen exposure up to birth as well as the natural biased status of the immune system against the production of pro-inflammatory cytokines to prevent adverse immunological reactions between mother and fetus during pregnancy [50]. Moreover, the delayed maturation may provide a window of opportunity for the development of tolerance.

In newborns, the immune system is still adapted to antenatal life and thus a few of its components are not yet adapted to postnatal challenges (reviewed by [55]). In brief, the mucosal and epithelial layers are less developed and show a higher permeability, indicating that the integrity of the physical immune barrier is not complete. The secretion of proteases and antimicrobial peptides which are involved in the chemical barrier are not fully developed [50]. Circulating levels of complement proteins are low and granulocytes including neutrophils and eosinophils and tissue macrophages are reduced in number and functionality. Furthermore, natural killer (NK) cell activity is low at birth, even though NK cells are predominant during early infancy and early childhood and the high level of NK cell cytotoxicity may make up for part of the immaturity of the adaptive immune system [90].

Neonatal T cells are able to respond to environmental antigens and in general antigen-specific immune responses can be generated in infants. However, the neonatal T cells exhibit a tolerogenic bias as to avoid inappropriate reactivity to common and harmless antigens during the postnatal maturation process and the acquisition of antigen-specific memory. As a consequence, during the neonatal period, cell-mediated immune responses to infectious pathogens and vaccines are inefficient [27, 79]. Recently, evidence has been provided that fetal hematopoietic stem cells give rise to T cells that differ both genotypically and phenotypically from
adult T cells [63]. These data support the hypothesis of "layered" immune development as postulated by Herzenberg et al. [38]. In this hypothesis, different layers of increasingly complex mechanisms arise during different stages of development that recognize and control specific pathogens. Most data has been derived from murine studies, describing an initial burst of $\gamma \delta \mathrm{T}$ cells playing an important role in defense against infections [13]. Although relatively little is known about this population of $\gamma \delta \mathrm{T}$ cells in humans, evidence is emerging and describing the position of these cells to contribute to immune protection at birth [29].

Although the number of B cells in the neonate is very high, the maturation of plasma $B$ cells is not yet completed at birth, leading to an impaired antibody isotype switching. As a consequence, the immunoglobulin (Ig) levels in the circulation of the newborn infant are low, apart from IgG of maternal origin, and several studies suggest that adult-equivalent levels of immunoglobulins are achieved by approximately the age of 10 years [2]. T cell-dependent B cell responses can be detected shortly after birth to most protein antigens and the earliest layer of humoral defense is represented by so-called unconventional B1a cells that are produced in fetus and undergo self-renewal in the periphery instead of bone marrow [25].

The cells of the gut immune system develop in proximity to large communities of microorganisms in the intestinal lumen. Available evidence indicated that intestinal bacteria play a crucial role in establishing and in the maturation of the immune system [55]. The first postnatal year of life seems to be a key period for programming the immune system. The feeding (i.e., breast milk) and other factors to which newborn is subjected (i.e., antibiotics) may have an influence on indigenous gut microbiota and subsequent immune development.

## 3 Role of Nutrition in Immune System Development

Different epidemiological studies support the hypothesis that modifications in environmental factors, including dietary changes represent a critical factor underlying the rise in the immune disorders. Especially the early-life nutritional exposure has been suggested to have a key impact on the developing immune system probably via epigenetic mechanisms like DNA methylation, histone modifications, and/or RNA silencing.

Human milk is the first dietary exposure in infancy. It is considered the best nutritional option to stimulate the development of the neonatal immune system, as it contains a wide range of immune modulatory factors including human milk oligosaccharides (HMOS), nucleotides, fatty acids, immunoglobulins, cytokines, immune cells, lysozyme, and lactoferrin[12, 32, 72, 87]. Moreover, human milk has been shown to be a consistent continuous source of bacteria to the infant gut, including staphylococci, streptococci, bifidobacteria, and lactic acid bacteria [21, $35,56,57,73$ ] that may play a significant role in the postnatal development of the immune system.

The composition of human milk can be influenced by maternal diet, and therefore illustrating the importance of the maternal nutritional status. Though maternal deficiencies have been demonstrated to contribute to deficiencies in their infants [3], the association between maternal nutritional status and human milk immune factors is less clear [71].

Nutritional factors prenatally may also influence the developing immune system. Differences in the immune response are already detectable at birth, suggesting that in utero environmental exposures have the capacity to modify the set-point of the immune system at birth [58]. A range of other maternal dietary factors in pregnancy have been implicated in immune development, including polyunsaturated fatty acids (PUFA), and a range of specific vitamins and micronutrients [17]. One of the most notable examples is the observation in mice that dietary folate intake in pregnancy can specifically alter the allergic predisposition in offspring via epigenetic mechanisms [40]. In line with this is an observation in a human study showed that the folate intake during pregnancy was associated with risk of childhood wheezing [36].

## 4 Early-Life Nutrition: Malnutrition and Infection

It is widely accepted that malnutrition leads to impaired immune status and consequently influence the body's immune defense against infections [18]. Malnutrition impairs immune responsiveness by different mechanisms, including impaired mucosal barrier function, reduced complement activation, deficient antibody production, and reduced numbers of circulating T cells and dendritic cells. Malnutrition and infections exists in a vicious circle whereby infectious episodes contributes to nutrient deficiencies via decreased absorption, altered nutrient transport, direct nutrient loss and increased energy requirements, impaired gut function and microbiota, further impairing the immune defense and increasing susceptibility to infections [81].

Most studies have dealt with the impact of undernutrition on immune status, whereas the impact of overnutrition is less studied. The latter has recently gained attention due to the obesity pandemic in both developed and developing countries.

### 4.1 Undernutrition and Infection

Studies have shown that undernutrition in critical periods of gestation and neonatal maturation impairs the development and differentiation of the immune system (reviewed by [24]). Permanent structural and functional changes in the developing immune system may occur in malnourished infants. Undernutrition may also delay the maturation of the infant gut microbiota or skew it toward a different and
persistent configuration, and maybe more relevant, their metabolic activities, thereby impairing the development of the immune system [33].

Cohort studies in Gambia demonstrated increased risk of death from infection in adults born in the hungry season [64] that was associated with reduced thymic size and function [23]. A recent study has offered a possible influence of prenatal nutritional status on epigenetics regulation: different methylation patterns observed depending the season of conception in Gambia whether it is dry or rainy season [88]. Similar observations have been derived from other cohort studies, showing association between low birth weight as an indicator of maternal malnutrition and impaired functional response to vaccines [60, 67], although not confirmed by other studies [66]. Limited evidence from murine and epidemiological studies suggests that nutritional impairments can exert transgenerational effects.

Murine studies demonstrated immune abnormalities in first and second generation offspring of maternal malnutrition [10, 19]. Observations from the Dutch famine also suggested that the effects of maternal intrauterine undernutrition may be extending to the next generation [52]. These observations strengthen the concept of programming by epigenetic mechanisms that can be transmitted to the next generation.

Prenatal and perinatal deficiencies in micronutrients, particularly zinc, copper, selenium, iron, and antioxidant vitamins A, C, and E have overall impact on both cellular and humoral responses. Furthermore, oxidative stress during infections is worsened if micronutrients are deficient (reviewed by [24, 46]). Results from maternal supplementation trials strongly suggest the immune programming effect of micronutrients [46, 71], although the relative clinical importance of different micronutrients on immunity to infection is difficult to establish.

### 4.2 Overnutrition and Infection

Epidemiological and experimental data show that overnutrition during pregnancy and/or lactation predisposes offspring to develop a metabolic syndrome-like phenotype. Recent studies provided evidence for the epigenetic regulation of a specific gene in mice, JmjC-domain-containing histone demethylase 2A (JHDM2a) plays a key regulator role in the development of obesity and metabolic syndrome [43]. There is strong evidence indicating that obesity negatively impacts immune responses against infections. Obesity is characterized by a state of low-grade, chronic inflammation and altered immune cell function [61]. The fatty acid composition of immune cells is known to have a major regulatory effect on immune responses [16] and also evidence is accumulating on the role of leptin in host defense [61]. For example, greater susceptibility to respiratory infections is observed in obese leptin-deficient humans and leptin-receptor deficient mice [54] and increased amoebic colitis in congenital deficiency of leptin or its receptor [53] suggesting a requirement for leptin in innate and adaptive immune response to
infection. However, future research is required to understand the exact mechanisms underlying the reduced immunocompetence in the obese.

Recent studies indicated the role of the microbiota in harvesting and storage of energy, and described the alterations in the gut microbiota patterns in obese subjects [8, 84]. A new paradigm has been postulated by a recent study that specific diets, including a fat-enriched diet, induce a modification in the intestinal microbiota [15]. Translocation of bacteria and bacterial antigens into the host, towards metabolically active tissues, can trigger a chronic inflammatory status and consequently impaired metabolic functions such as insulin resistance and excessive adipose development via direct communication between inflammatory cells and metabolic cells as described in a recent review on the underlying cellular and molecular mechanisms [51]. In the case of pregnant overweight women, the aberrant microbiota related to overweight or excessive weight gain may be transferred to the infant, thereby predisposing the infants to unfavorable metabolic development with consequences on the immunological programming and susceptibility to infections. Interestingly, Collado et al. reported that maternal overweight and excessive weight gain also influenced the composition of the human milk microbiota and the immunomodulatory potential of human milk [22], providing an additional mechanism that explains the nutritional programming of the immune system.

In the context of nutritional programming, the development of vaccine response has been determined as a broad indicator of the functionality of the immune system respond to infections. As described in the previous paragraph, there is a lack of consistency across the studies that examined the link between early-life nutritional status and vaccine responsiveness [60,66,67], although the lack of an effect in the study by Moore et al. may be due to the younger age of the individuals in which the differences are not yet apparent. A recent review that critically analyzed the available literature by Savy et al. indicated that there is little evidence to indicate that current nutritional status or nutrient supplementation has clinically important effects on vaccine efficacy [77], although different limitations have to be considered in this review, including the methodology to access vaccination efficacy and the difficulty in separating effects of malnutrition from those of infection, that mostly co-exist.

## 5 Nutritional Supplementation in Early Life and Infection

The concept of early-life nutritional programming and the inherent plasticity conferred by epigenetic mechanisms also provides opportunities for nutritional intervention strategies. The timing of nutritional interventions is critical, as earlylife events occurring during critical windows of immune vulnerability can have long-term impact on immune development (Fig. 7.1). The strong association of the gut microbiota composition with the development of the immune system has prompted several studies to examine the effect of probiotic supplementation, both prenatally and postnatally.


Fig. 7.1 The immune defense against infections in the offspring is influenced by maternal and infant gut microbiota, by epigenetic regulation of gene expression, and by prenatal/neonatal nutrition, in direct and indirect ways

### 5.1 Probiotics

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [1]. Animal models and clinical trials of supplementation with probiotics have demonstrated diverse effects on immune function against infections. Different studies have been conducted in order to determine its efficacy in managing and preventing infections, including infectious diarrhea, traveler's diarrhea, necrotizing enterocolitis, Helicobacter pylori infections, and respiratory tract infections (reviewed by [89]). Sufficient and consistent data exist for the management of infectious diarrhea in infants and traveler's diarrhea, antibiotic-associated diarrhea, and necrotizing enterocolitis for which it could be concluded that certain probiotics, under certain conditions, and in certain target populations, are beneficial in reducing the risk of infection. Certain probiotics may also reduce the risk of various symptoms of respiratory tract infections in adults and children, including ear, nose, and throat infections, although data are currently far too limited to distill any clinical recommendations in this area [39, 75]. However, no general conclusions can be drawn due to the different types of infections that have been examined. The lack of consistency among studies focusing on specific infections, in study design, applied probiotic strains, outcome parameters, and study population, along with the still limited number of studies, complicated the analysis. For future studies it is recommended that researchers provide adequate power, identify pathogens, and report both clinical outcomes and immune biomarkers relating to putative underlying mechanisms.

The exact mechanisms of action of probiotics and/or their metabolites has not been fully elucidated yet, but it has been suggested that next to the known and well described interaction with the immune system, the functional effects of probiotics may also be the result of epigenetic modifications [17]. In addition, the effect of probiotics supplemented during pregnancy has been reported to decrease vaginal infections that may provide benefits for the infants [91]. Probiotics also have been reported to provide an effective treatment in mastitis [5]. As mastitis can influence the composition of human milk [42], and thereby the immune system of infants, suppressing mastitis may provide a relevant strategy in strengthening the infant immune system.

### 5.2 Prebiotics

Beneficial effects on both the gut microbiota and immune system have been described for prebiotics. Prebiotics are nondigestible oligosaccharides that reach the colon intact and are known for their ability to selectively stimulate the growth and activity of bacteria that exert positive health effects [30]. A plethora of data is available that indicate the effect of supplementation with prebiotic oligosaccharides on the composition and activity of the gut microbiome (including [41, 47, 68]). Studies in pregnancy are still limited, but in animal models prebiotics had been shown to alter colonization in offspring [59]. To our knowledge there is only one clinical study that demonstrated favorable effects on the maternal gut microbiota by supplementation with prebiotics. However, the number of the subjects in the study was too small $(n=48)$ to reliably assess any clinical effects [78]. Both clinical studies and experimental animal models have shown encouraging results that supplementation with specific mixtures of prebiotics may impact the immune response to infections. Administration of a specific mixture of short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS; 9:1 ratio) early in life has been shown to reduce the number of infections [6, 14]. In the follow-up study, the protective effect against infections was still evident at the age of 2 years [7], suggesting a longer-term effect of the specific mixture of prebiotics, even beyond the intervention period. Although available data report that there is no effect of prebiotics supplementation on the prevention of infections [82], the effect may be specific for the examined prebiotic mixture. The risk of using the definition of prebiotics is that all mixtures are defined as one category, whereas the effects may be very specific. Therefore, careful interpretation of metaanalysis on the existing data is warranted.

Modulation of the intestinal microbiota that affects early-life immune development has been suggested to be one of the putative mechanisms for the protective effect of prebiotics [44]. It has been hypothesized that prebiotics may also directly interact with cells of the immune system in a microbiota-independent mechanism [86]. Available evidence suggests that human milk oligosaccharides may have systemic effects in infants as these oligosaccharides have been found in urine [20,

70]. At present, there is no evidence for the direct effect of prebiotics on epigenetic regulation of gene expression. It would be interesting to explore whether supplementation with prebiotics can alter the composition of the human milk and thereby exert their functional effects.

More trials are clearly needed, despite the promising early results that support the importance of prebiotics in achieving a beneficial gut microbiota composition and the host-microbe interactions at critical periods for the potential prevention of human disease.

### 5.3 LCPUFAs

Essential fatty acids must be acquired from the diet and are the precursors for longchain polyunsaturated fatty acids (LCPUFAs) that have been implicated as being important for the development of the immune system [34]. There is strong evidence from experimental studies showing that supplementation with LCPUFAs influences the immune system in the offspring. Supplementation with omega-3 PUFAs during pregnancy and lactation have demonstrated higher provision to the offspring and that early supplementation was associated with immunological changes, such as increased cytokine production in cord blood [26, 28, 48, 74]. Epidemiological studies suggest that dietary exposure to omega- 3 LCPUFAs during pregnancy and early in life may improve the immune defense against infections. However, in contrast to the evidence from epidemiological studies, there are only a few intervention studies reporting the effects of LCPUFAs on infections [11, 62, 83] and the available literature seems to be limited to infections related to respiratory disease. A recent study in animals demonstrated a programming effect of maternal diet supplemented with LCPUFAs on the offspring's immune response, and the lactation period appeared to be the period that conferred most susceptibility to immune programming [85]. The immunomodulatory effects of LCPUFAs may program the infant's immune system development via epigenetic mechanisms, although no specific epigenetic markers have been defined yet which are associated with the reported immune programming effects. Besides that, the knowledge on the association of LCPUFA with the modulation of the gut microbiota is lacking and is only addressed by a few scarce studies [4, 69].

## 6 Conclusion

The immune system of infants is not fully functional at birth, rendering them highly susceptible to infections. It is now widely accepted that environmental exposures during early life, in particular nutrition, is an important determinant of the efficiency of neonatal and potential adult's immune responses to infection. Emerging understanding of the protective and predisposing effects of early nutrition on
healthy development of the immune system provides opportunities to improve health and reduce the risk of diseases later in life. A better understanding of the biological mechanisms involved, including the important role of the (maternal) intestinal microbiota, the relative contributions of individual components of the diet as well as the time constraints (window of opportunity), is important to enable design of effective intervention strategies and to combat the burden of infectious diseases.

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# Chapter 8 <br> Impact of Nondigestible Oligosaccharides on Gut-Associated Lymphoid Tissue and Oral Tolerance Induction 

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## Abbreviations

AD Atopic dermatitis

ALDH Aldehyde dehydrogenase
AOM Azoxymethane
CCR Chemokine (C-C motif) receptor
CD Crohn's disease
conA Concanavalin A
DC Dendritic cell
DTH Delayed type hypersensitivity
Foxp3 Forkhead box P3
GALT Gut-associated lymphoid tissue
HMOs Human milk oligosaccharides
IBD Inflammatory bowel disease
IEC Intestinal epithelial cells
IFN Interferon
Ig Immunoglobulin
IgfLC Immunoglobulin free light chains
IL Interleukin
lcFOS Long-chain fructo-oligosaccharides
MDP Macrophage-DC precursors
MLN Mesenteric lymph nodes
NOD Nucleotide oligomerization domain
PRR Pattern recognition receptors
PBMC Peripheral blood mononuclear cells (PBMC)

[^7]| pDC | Plasmacytoid DC |
| :--- | :--- |
| PP | Peyer's patches |
| RA | Retinoic acid |
| scGOS | Short-chain galacto oligosaccharides |
| SCFA | Short-chain fatty acids |
| sIgA | Secretory immunoglobulin A |
| TLR | Toll-like receptors |
| TNF | Tumor necrosis factor |
| Th | Thelper cell |
| Tc | Cytotoxic T-cell |
| TGF- $\beta$ | Transforming growth factor beta |
| Treg | Regulatory T-cells |
| UC | Ulcerative colitis |

## 1 General Effects of Dietary Nondigestible Oligosaccharides

The incidence of allergic and inflammatory disease is increasing rapidly in the western societies. A main factor responsible for this may be dietary alterations. These changes include enhanced lipid intake and altered quality of the lipids (increasing ratio of n-6 polyunsaturated fatty acids (PUFA) over n-3 PUFA, increasing intake of saturated versus non-saturated fatty acids) and reduced vitamin and fiber intake. Neonates form a sensitive group in which these dietary alterations may have impact. During gestation and up until weaning (approximately at 6 month of age) their nutrient supply is provided by the mother via the placenta and after birth via breast milk. The immune system is readily developed during gestation; however in the neonatal period there is a critical window in which maturation of the gastrointestinal (gut-associated lymphoid tissue) and systemic immune system occurs. At birth the immune response is Th2 polarized, the Th1 counterpart is weak and needs to be primed during immune maturation in order to reduce the susceptibility to develop atopic disease. This occurs in the same time frame as gut maturation. Increasing evidence indicates that maternal intake of n-3 PUFA during pregnancy protects the child from developing atopic disease while the amount of n-6 PUFA intake correlates with increased allergy risk [1-3]. These PUFA together with numerous additional nutrients can pass the placenta barrier and hereby directly affect health and growth of the child. After birth until weaning breast milk is the main nutrient source of the newborn. Breast milk contains a whole range of components that may enhance gut and immune maturation. In comparison to bottle-fed children breast-feeding may prevent against the development of allergic disease and reduces the incidence of infections (reviewed by Boehm and Moro [4]). Although PUFA may well play a role in shaping the immune response after birth, in this respect the most evidence of components in breast milk that protect neonates from developing allergies is built around nondigestible oligosaccharides, after
lactose and lipids the major component in human milk ( $7-15 \mathrm{~g} / \mathrm{L}$ ) [5]. The mechanistic immunologic basis by which human milk oligosaccharides (HMOs) may affect the mucosal immune system and reduce the sensitivity to develop allergic disease will be the topic of this book chapter.

### 1.1 Structure and Function of Nondigestible Oligosaccharides

Breast milk contains $7-15 \mathrm{~g} / \mathrm{L}$ HMOs and human colostrum even $20-23 \mathrm{~g} / \mathrm{L}$ [5, 6]. After lactose and lipids HMOs are the third major solid component in breast milk and extremely complex comprising more than 1,000 different components [5]. Only 85 of these have been isolated and identified [5]. HMOs start with lactose to which galactose (Gal) and N -acetylglucosamine (GlcNAc) are repetitively attached via a $\beta$-glycosidic linkage ([Gal( $\left.\beta 1-3 / 4) \mathrm{GlcNAc}(\beta 1-]_{\mathrm{DP}} n=0-25-3 / 6\right) \mathrm{Gal}$ ( $\beta 1-4$ )Glc) [6]. Galactose is coupled to its lactose backbone and generates by $3^{\prime}, 4^{\prime}$, or $6^{\prime}$ galactosyl-lactoses, compelling of a total concentration of $1 \mathrm{~g} / \mathrm{L}$ in human milk [7]. These core structures are further coupled to fucose by fucosyltransferases in mammary epithelial cells to form neutral oligosaccharides and/or sialic acid via $\alpha$-glycosidic linkage produced by sialyltransferases to form acidic oligosaccharides which differs between individuals due to genetic factors [5, 6]. Alternatively sulfate instead of sialyl groups is added to oligosaccharides that are categorized to the acidic group [5]. This makes sialic acid, $N$-acetylglucosamine, l-fucose, D-glucose, and galactose the most important sugars present in HMOs [7]. HMOs consist of 80$85 \%$ neutral oligosaccharides while $15-20 \%$ are acidic. Depending on the time postpartum and Lewis type, human milk contains different oligosaccharide profiles; for example $\alpha 1,2$-fucosylated oligosaccharides decrease steadily during the first 3 month of lactation [5]. The Lewis type is important since this determines the type of $\alpha$-fucosyltransferase present [5]. Breast milk from woman with Lewis ${ }^{\text {a }}$ ( $\sim 17 \%$ of European population) nonsecretor blood group for example produces lower levels of neutral oligosaccharides than woman with Lewis ${ }^{\text {b }}(\sim 73 \%)$ secretor blood group and lacks $\alpha 1,2$-fucosylated oligosaccharides [5]. Authors suggested that newborns from this group of individuals would be less protected against stable toxin-E. coli infection since this oligosaccharide can compete with the pathogen for the host surface receptor [5]. Apart from lactose human cannot digest $\beta$-glycosidic galactose linkages nor $\alpha$-glycosidic fucose and sialic acid linkages, making these oligosaccharides surviving intestinal transfer; however they can be selective fermented by commensal microflora that do express these glycosidases [4, 6]. HMOs not only survive intestinal transfer, they are also taken up and come available systemically. ${ }^{13} \mathrm{C}$ labeled HMOs are detected in the urine of infants fed with breast milk of lactating women who ingested a ${ }^{13} \mathrm{C}$-galactose load [8]. The ${ }^{13} \mathrm{C}$-galactose was
retrieved in breast milk 8 h after ingestion. In the infant urine ${ }^{13} \mathrm{C}$ was mainly found back in fucosyl-lactose, -lacto-N-tetraose (LNT), -LNT, and difucosyl LNT; hence lactose and neutral oligosaccharides but also N -acetylneuraminyl containing acidic HMOs were detected [8, 9]. It was determined that 14 h after intake $1 \%$ of the ${ }^{13} \mathrm{C}$ was excreted in the urine of the lactating woman. Twenty hours after intake by the mother $0.6 \%$ of the ${ }^{13} \mathrm{C}$ reached the urine of the infant [8]. The intake of HMOs was calculated on $50-160 \mathrm{mg}$ per suckling of which $1-3 \mathrm{mg}$ would reach the urine per day [9]. Meaning that HMOs would reach blood concentrations of $100-200 \mathrm{mg} / \mathrm{L}$ [10]. Based on in vitro results, the uptake of neutral HMOs by intestinal epithelial cells was suggested to be mediated via receptor-mediated transcytosis while acidic HMOs diffuse via the paracellular route [11].

Over 1,000 different oligosaccharides compose the HMO fraction, while that of animal milk is much less complex since for example this is low in fucose linkages. In addition, the oligosaccharide content is much lower (10-100 times) than in human [4, 6, 7]. Other alternative sources for oligosaccharides are for example plants. Although the HMOs composition is complex and functional aspects remain to be elucidated, synthetic oligosaccharides have been marketed that are able to mimic some of the functional aspects of HMOs. Short-chain galacto-oligosaccharides $(\operatorname{scGOS})\left(\left[\operatorname{Gal}(\beta 1-4)_{\mathrm{DP} n=3-8}\right](\beta 1-4) \mathrm{Glc}\right)$ are generated via enzymatic elongation of lactose, derived from the whey fraction of cow's milk, using $\beta$-galactosidase. Long-chain fructo-oligosaccharides (lcFOS) $\left(\left[\operatorname{Frc}(\beta 2-1)_{\mathrm{DP}} n>21\right]\right.$ $(\beta 2-1) \mathrm{Glc})$ are composed of fructose moieties derived from chicory inulin. The 9:1 scGOS/lcFOS mixture (Immunofortis ${ }^{\mathrm{T}}$ ) resembles the molecular size distribution of neutral HMOs (Fig. 8.1). Although the composition of acidic HMOs is different from citrus pectin derived acidic oligosaccharides [galacturonic acid $(\alpha 1-4)_{\mathrm{DP}} n=1-20$ ], it may be added to the scGOS/lcFOS mixture to provide a 9:1:2 ratio, since HMOs are composed of neutral and acidic oligosaccharides. Besides inulin-derived lcFOS also inulin-derived scFOS (Raftilose P95 dp 2-8) enhances Bifidobacteria growth and is used as prebiotic [7, 12]. scGOS products contain about 24-55 \% oligosaccharides; further components are lactose, glucose, and galactose [7]. Although it has been established that HMOs are taken up in the intestine, since they can be retrieved in urine, it is not known for synthesized oligosaccharides like scGOS/lcFOS whether they come available systemically. Many plant-derived oligosaccharides have been identified such as manno-, pectic-, soybean-, isomalto-, and xylo-oligosaccharides and lactulose in a majority of studies inulin/FOS (Synergy ${ }^{\text {T }}$ ) or GOS/transgalactosylated oligosaccharides (TOS) is used [7]. Randomized clinical trials performed with artificially prepared oligosaccharides include scGOS, lcFOS, pAOS, scGOS/lcFOS/pAOS, oligofructose plus inulin, polydextrose plus scGOS either or not with lactulose; however inulin/FOS or the 9:1 mixture of scGOS/lcFOS is used most often [13].
a

b


Fig. 8.1 Structure of most used nondigestible oligosaccharides. (a) Short-chain galacto-oligosaccharides (scGOS) derived from galactose, $\beta(1-4)$ coupled galactose, and a terminal glucose (dp 3-8). (b) Fructo-oligosaccharides (FOS) are derived from chicory inulin (dp 7-60, mean > 23), scFOS (mean dp $<20$ ), and long-chain lcFOS (mean dp $>20$ ), $\beta(1-2)$ coupled fructose and a terminal glucose. Figures from thesis Bastiaan Schouten, ISBN 978-90-393-5210-6, with permission

### 1.2 Effect of Nondigestible Oligosaccharides on Bowel Function and Microflora

In breast milk-fed infants as well as infants fed formula milk supplemented with scGOS/lcFOS, and not in infants fed non-supplemented formula milk, the bacterial flora is rich in Bifidobacteria and Lactobacilli resulting in the same short-chain fatty acid (SCFA) profile upon fermentation of the oligosaccharides by the microflora (reviewed by Boehm and Moro [4]). Both in preterm and term infants 28 days of scGOS/lcFOS supplementation ( $4-10 \mathrm{~g} / \mathrm{L}$ ) was found to enhance the number of fecal Lactobacilli and/or Bifidobacteria and improved stool consistency (softer stools) and defecation [14-16]. Indeed prebiotic fibers scGOS/lcFOS supported selective growth of Bifidobacteria (breve, infantis, bifidum) and Lactobacilli at the cost of Bifidobacterium adolescents (found in adults) while pathogenic bacteria such as Escherichia coli, Klebsiella, and Clostridium remained unaltered. This may not only be caused by selective fermentation of oligosaccharides by Bifidogenic flora but as well due to the drop in pH as a consequence of fermentation [14, 15]. The low pH inhibits the growth of pathogens while commensal bacteria are unaffected [4]. The SCFAs which are generated upon bacterial fermentation of fibers such as scGOS/lcFOS are known to affect immune function and target all cells of the immune system [17]. They can bind the G-protein-coupled receptor 43 (GPR43) (acetate and propionate) and GPR41 (butyrate) [18]. SCFA patterns
and concentration alter due to bacterial fermentation of nondigestible oligosaccharides. Typical SCFA concentrations in the intestine are around $70-120 \mathrm{mM}$ depending on the location and type of fiber, while this is $1-100 \mu \mathrm{M}$ in the blood. Not only the bacteria or bacterial products but also nondigestible oligosaccharides themselves may protect against gastrointestinal infections since they may adhere to intestinal epithelial cells thereby inhibiting the adhesion of pathogens [6]. Early colonization upon use of scGOS/lcFOS may last for 6 months after scGOS/lcFOS supplementation had stopped; Lactobacilli and Bifidobacteria counts were still increased in children provided with scGOS/lcFOS for a period of 6 months starting directly after birth [16]. Besides scGOS/lcFOS also an intervention trial with healthy term infants comparing scGOS/lcFOS/pAOS ( $8 \mathrm{~g} / \mathrm{L}$ ) supplementation with a control formula showed improved stool consistency in the supplemented group [19]. In a placebo-controlled multicentre study (open label) supplementation of scGOS/lcFOS ( $4 \mathrm{~g} / \mathrm{L}$ ) for 12 month resulted in $50 \%$ reduction in acute diarrhea and gastroenteritis. In addition, the use of antibiotics for treatment of upper respiratory tract infections was reduced by almost $50 \%$ [20]. Also supplementation of scGOS/lcFOS ( $8 \mathrm{~g} / \mathrm{L}$ ) for 6 months to a group of 102 healthy term infants was found to reduce the episodes of infections and the cumulative incidence of recurrent upper respiratory tract infections by over $50 \%$ compared to the placebo group [21]. In a large trial in which mothers carrying high-risk children were provided with a mixture of four different beneficial bacteria and scGOS ( $8 \mathrm{~g} / \mathrm{L}$ ) during pregnancy, while their offspring was supplemented up until 6 months after birth. It was observed that the supplementation was safe. At the age of 2 years it was established that the cumulative incidence of respiratory infections was reduced in the supplemented group [22]. A recent ESPGHAN report concludes that scGOS/ lcFOS has no adverse effects on growth in healthy infants and reduces risk of some allergic reactions and some types of infection, although it was stated that there is a need to confirm these results in other studies. It was concluded that specific oligosaccharides are able to enhance fecal Bifidobacteria counts, lower pH , and soften stool; an overview of typical studies is provided by Macfarlane et al. [7, 13]. Hence via modulation of the intestinal microflora nondigestible oligosaccharides may support gastrointestinal and immune maturation and preserve immune homeostasis.

## 2 Effects of Dietary Nondigestible Oligosaccharides on the Systemic Immune Response

The incidence of chronic inflammatory diseases such as allergies, inflammatory bowel disease (IBD), and autoimmune diseases is steadily rising in the western society. The hygiene hypothesis was installed to be able to explain this development. It was suggested that altered composition of the gut microbiota and reduced exposure of infants to pathogens affect postnatal maturation of the immune system
resulting in increased disease susceptibility [23]. Effects of HMOs on the immune system are largely unknown since not many studies are performed using isolated HMOs. However synthesized nondigestible oligosaccharides have been shown to modulate the bacterial flora and in addition affect the immune system.

### 2.1 Studies in Mice

Allergic sensitization is characterized by T helper 2 cell (Th2) polarization of the immune response towards specific antigens which can be counteracted by Th1 immunity or suppressed by regulatory T-cells. In neonates the immune system is Th2 oriented and HMOs in breast milk may help in immune maturation by supporting the development of oral tolerance, a regulatory T-cell response (Treg) and/or Th1 immunity capable of counteracting a Th2 response. scGOS/lcFOS have been shown to dose dependently enhance the Th1-dependent antigen-specific delayed-type hypersensitivity (DTH) response in a murine vaccination model in association with enhanced Bifidobacteria and Lactobacilli counts when provided prior to and during vaccination [24]. Inulin, short chain FOS, or a combination of lcFOS/inulin ( $2 \mathrm{w} / \mathrm{w} \%$ ) did not enhance the DTH. lcFOS/inulin however did enhance Bifidobacteria and Lactobacilli counts similar to the scGOS/lcFOS mixture [24]. Hence enhanced Th1 immunity induced by scGOS/lcFOS may include mechanisms beyond the impact of these oligosaccharides on the commensal microflora. Addition of acidic pAOS to the scGOS/lcFOS mixture in a 1:1 or 1.8:0.2 ratio further enhanced the vaccination DTH response in association with additional increase in Lactobacilli counts [24, 25]. However $2 \%$ pAOS alone also successfully increased the DTH response, this effect was in the absence of prebiotic activity (no increase in Bifidobacteria nor Lactobacilli), and was associated with a reduction in Th2 cytokines as measured in a splenocyte restimulation assay [24]. scGOS/ lcFOS/pAOS only was able to support the vaccination response when provided prior to the first encounter with the influenza vaccine [25]. Prior to or after the second vaccination it did not enhance the DTH indicating the relevance of the presence of this type of immune priming oligosaccharides at first encounter of a new antigen. Hence specific oligosaccharides may enhance Th1 polarization in a murine vaccination model. Furthermore the oligosaccharides have been shown to prevent allergic symptoms in a murine asthma model and a mouse model for orally induced cow's milk allergy [26, 27]. Dietary intervention with scGOS/lcFOS/ pAOS as well as scGOS/lcFOS was also able to reduce metacholine-induced airway hyperresponsiveness in ovalbumin-sensitized mice (acute asthma model) which was associated with reduced inflammatory cell counts in the broncho-alveolar fluid [27].

### 2.2 Studies in Human

Some studies have determined the effect of isolated HMOs on human immune cells. In a study of Eiwegger et al. HMOs were analyzed for their capacity to induce immune responses in cord blood mononuclear cells [28]. Acidic and neutral oligosaccharides were isolated from human milk. Upon 20 days of culture with the acidic oligosaccharide fraction ( $1 \mu \mathrm{~g} / \mathrm{mL}$ ) intracellular IFN- $\gamma$ and surface expression of CD25 were enhanced in CD4+ Th cells and IFN- $\gamma$ and IL-13 in CD8+ cytotoxic T-cells (Tc). Neutral HMOs ( $10 \mu \mathrm{~g} / \mathrm{mL}$ ) reduced the percentage of IL-4 positive Tc cells. Hence HMOs may have the intrinsic capacity to modify the adaptive T-cell response. In another study of Eiwegger et al., cord blood mononuclear cells were exposed to several HMOs or scGOS/lcFOS or pAOS [29]. In this study 72 h of exposure to $100 \mu \mathrm{~g} / \mathrm{mL}$ acidic HMOs resulted in increased secretion of IFN- $\gamma$ and a similar tendency for IL-10. Acidic HMOs also reduced Th2 type cytokine expression in PBMC from allergic persons while increasing Th1 type IFN $-\gamma$, hence skewing away from the allergic phenotype. In addition to affecting the T-cell response acidic HMOs have been shown to reduce neutrophil activation by $20 \%$ via suppression of platelet neutrophil complex formation [30]. These effects were similar to sialyl-Lewis x and authors suggested that HMOs may be able to bind P-selectin that facilitates complex formation thereby reducing interaction between the platelets and neutrophils. Via similar mechanisms acidic HMOs may be able to reduce monocyte, lymphocyte, and neutrophil rolling and adhesion to endothelial cells [31,32] which may also imply an anti-inflammatory role for HMOs. In a large clinical trial the immune phenotype of peripheral blood mononuclear cells (PBMC) was studied. Healthy term infants received either breast milk, formula milk, or formula milk supplemented with scGOS/lcFOS ( $6 \mathrm{~g} / \mathrm{L}$ ) during a 6 -month period [33]. After 6 months of supplementation in the breast milk group the percentage of white blood cells was increased and the percentage of activated Tc cells was decreased compared to the control group as well as the scGOS/lcFOS group. The same tendency was shown for activated Th cells and serum IL- 5 levels. Hence in these healthy infants the oligosaccharides did not modify the T-cell phenotype similar to values in the breast-fed group. However, serum tumor necrosis factor (TNF) $-\alpha$ was increased compared to the breast-fed group and IL-10 showed the same tendency implying some effect by the oligosaccharides. IFN- $\gamma$, IL-2, and IL-4 serum concentrations were equal between groups. Effects of HMOs on the B-cell response are largely unknown. In a study of Eiwegger et al. however, HMOs did not affect total IgE and IgG1 production by cord blood mononuclear cells nor CD22 and HLA-DR surface expression. These studies were performed in presence or absence of anti-CD3 activated T-cells and contained a positive control group using IL-4 and anti-CD40 in which these immunoglobulins were successfully induced [28]. Oligosaccharides did also not affect the humoral response in healthy human infants. Healthy infants receiving formula milk supplemented with scGOS/ lcFOS had comparable concentrations of serum immunoglobulin (Ig) E, IgG, IgA, and $\operatorname{IgM}$ after 6 months of supplementation compared to breast-fed infants
[33]. However, in the group of infants exclusively fed formula milk, the addition of scGOS/lcFOS resulted in enhanced serum IgG levels compared to the control formula milk group after 6 months of supplementation. In a dietary intervention study in which 215 healthy infants were randomized to receive either a control formula milk or formula milk supplemented with scGOS/lcFOS ( $6 \mathrm{~g} / \mathrm{L}$ ) for 6 months, fecal secretory $\operatorname{Ig} A$ ( $\operatorname{sIgA}$ ) was almost three times higher than the placebo group and comparable to breast-fed infants [34]. In addition, Bifidobacteria counts were increased while clostridium counts were reduced.

## 3 Impact of Dietary Nondigestible Oligosaccharides on the Gut-Associated Lymphoid Tissue (GALT)

The intestine not only functions as a digestive organ but contributes extensively in the orchestration of the local and systemic immune response while containing $60 \%$ of all immune cells present in the body [35]. Dietary components such as nondigestible oligosaccharides may be able to modulate immune responses generated in the gut-associated lymphoid tissue (GALT) which enables them to contribute to optimized immune function in health and disease. The sugar moieties in nondigestible oligosaccharides may have a direct effect on the mucosal immune system and in particular the epithelial layer or have indirect effects via adaptation of the intestinal microflora and the fermentation pattern. In addition to sugar moieties, bacterial cell components are able to modulate mucosal immune responsiveness via activation of pattern recognition receptors present on intestinal epithelial cells and underlying cells of the innate and adaptive immune system. The following paragraph elaborates on how the mucosal immune response is regulated and the impact that dietary nondigestible oligosaccharides and microbial components may have on the mucosal immune function. It will be highlighted how the immune response in the GALT is regulated and which cell types interact for its optimal function. Until now little is known about the specific effects of oligosaccharides on the GALT; therefore the effects on general immune cells which may translate to features that take place in the GALT are described.

### 3.1 Innate and Adaptive Immune System of the GALT and Oral Tolerance

The GALT is organized in inductive and effector compartments and has a key decisive function in regulating immunity or tolerance towards intestinal contents [36]. The luminal mass consists of residual dietary components such as food antigens, bacteria, and digestive fluids. Within the GALT it is determined whether an antigen or microbe should be regarded as harmful or harmless. Consequently


Fig. 8.2 Organization of the gut-associated lymphoid tissue. A monolayer of epithelial cells separates the luminal contents from the underlying immune tissues. Antigens are taken up by DC and presented to naïve T-cells in the PP or MLN. Here either immunity or tolerance is induced and via the MLN T-cells traffic through the blood stream and home back into the lamina propria were they exert their effector (a.o. Th1, Th2, Th17) or suppressive (Treg) function. Figure from thesis Bastiaan Schouten, ISBN 978-90-393-5210-6, with permission
regulated immunity or tolerance is generated. Local intestinal immune responses can be transferred to systemic immunity or tolerance. An example of this is oral tolerance induction, a food antigen that has been provided via the oral route prior to systemic exposure will not elicit a systemic immune response for example when applied to damaged skin (skin prick test) [36, 37]. When mucosally induced immune tolerance (oral tolerance) is not fully established, allergic disease can develop in neonates and autoimmune diseases (rheumatoid arthritis, type 1 diabetes) or IBD is prone to develop later in life [23]. Environmental factors such as dietary constituents and the colonization of commensal microorganisms at the mucosal surface are crucial for the generation of mucosal tolerance [38] (Fig. 8.2).

The intestinal mucosa consists of a monolayer of epithelial cells (IEC) that separates the luminal contents from the underlying immune cells. These IEC not only provide a barrier function but also control the mucosal immune system via cell contact-mediated signals as well as by the secretion of soluble mediators [23, 39]. In the inductive compartments of the Peyer's patches (PP) and mesenteric lymph nodes (MLN), naïve lymphocytes are gathered in an organized fashion ready to be exposed to antigen-bearing dendritic cells (DC). These secondary lymphoid
tissues develop after birth depending on the presence of microflora [23]. The PP have residence directly under the epithelial lining and are covered with M-cells, which are epithelial cells that are specialized in antigen uptake. Consequently DC pick up the antigen and present it to the lymphocytes. The lymphatics drain the PP and lamina propria, and DC from these sites traffic to the MLN. Regulatory T-cells or effector T-cells generated in the MLN gain gut homing markers and home back to the lamina propria (the effector compartment of the GALT) were they remain to exert their effector function. In addition, cells from the MLN traffic to the systemic compartment. In this way immune responses generated locally in the intestine can be transferred systemically contributing to optimal immune competence of the host.

### 3.1.1 Epithelial Lining

A monolayer of IEC separates the luminal contents from the mucosal immune system. The epithelial lining consists IEC of several subtypes such as goblet cells that produce mucins forming a protective mucus layer on top of the epithelial lining; Paneth cells that are located in the crypts and produce microbicidal products like beta-defensins, and absorptive enterocytes. IEC provide an intrinsic barrier since they are connected with "zipperlike" structures, the intercellular tight junctions. These prevent aspecific paracellular leakage of luminal antigens or bacteria into the mucosal immune system. These extrinsic (mucus layer) and intrinsic barrier properties are of great importance for intestinal homeostasis. Primary or secondary (e.g., as consequence of inflammation) defects in epithelial barrier properties have been shown to contribute to the pathogeneses of (chronic) inflammatory disorders such as IBD, allergic disease, and arthritis. Neonates have an immature intestinal immune system and leaky gut; hence in this phase of life critical developmental steps (such as gut maturation) have to be taken to ensure mucosal homeostases [40]. Pattern recognition receptors (PRR) such as toll-like receptors (TLR) and nucleotide oligomerization domain (NOD) receptors are present on IEC and immune cells. These receptors function in microbial recognition and selectively recognize several bacterial cell wall components and CpG DNA. Currently 14 TLR are known and 2 NOD receptors which have specific expression patterns on specific immune cells or IEC. In IEC the expression of membrane-bound receptors like TLR2 and TLR4 is constitutively low to limit immune activation. However during (allergic) inflammation receptor expression is enhanced enabling PRR signaling and activation of IEC [39]. In addition to PRR, the family of lectin receptors is specifically distributed amongst IEC and immune cells. These receptors consisting of the C-type lectin, siglec, and galectin family of receptors bind selective sugar moieties [41]. Within the IEC galectins are abundantly expressed. Currently ten different galectins have been recognized in human of which galectin $-1,-2,-3,-4$, and -9 have been detected within the epithelial layer of human and also in mice. Nowadays it is appreciated that IEC modulate the inductive as well as the effector immune response. This may have implications for both the local intestinal as well as the systemic immune response. IEC are capable of producing retinoic acid
(RA) from retinal, since they have the enzyme aldehyde dehydrogenase (ALDH). The level of expression and activity of ALDH is actively being regulated by environmental influences [42]. Together with transforming growth factor beta (TGF- $\beta$ ) epithelial derived RA is capable of instructing CD103+ dendritic cells that are capable of inducing de novo generated FoxP3+ regulatory T-cells that control tolerance induction and immune homeostasis [43-45].

### 3.1.2 Mucosal Antigen-Presenting Cells: Dendritic Cell and Macrophage Phenotype and Function

## GALT DC

In the intestine, DC are located in close proximity to IEC and the latter have been found to regulate DC function. DC are key directors for tolerance induction and maintenance of homeostasis. Several DC subsets have been defined in the different compartments of the GALT and short lived DC but also macrophages are replenished by bone marrow derived precursors. Lymphoid tissue DC, plasmacytoid $\mathrm{DC}(\mathrm{pDC})$, and monocytes are derived from the macrophage/DC precursor (MDP) [46, 47]. Monocytes only develop into DC in lymphoid organs under inflammatory conditions but a specific subtype of monocytes can also replenish DC under steady-state conditions [46, 47]. In the latter situation preDC derived from DC localized in the lamina propria are CD103+ and are able to migrate to the MLN. By contrast, monocyte-derived DC become fractalkine receptor (CX3CR1) positive and remain localized under the epithelial layer in the lamina propria [47-49]. CD103+CX3CR1- DC are located in the lamina propria and lie in close proximity to IEC ( $15 \mu \mathrm{~m}, 1$ cell per $200 \mu \mathrm{~m}$ villus) or in the PP [47, 49]. CD103 (integrin $\alpha E \beta 7$ ) is a ligand for E-cadherin expressed by IEC, and hence can be attracted to the epithelial layer. High constitutive chemokine receptor 7 (CCR7) levels ensure steady-state trafficking of this subpopulation to the MLN and are essential for oral tolerance induction [47, 49, 50]. This tolerogenic CD103+CD11b+ DC subset is able to instruct Forkhead box p3 positive (Foxp3+) regulatory T-cells (Treg) for example upon oral antigen exposure since they have the intrinsic capacity to produce retinoic acid [51]. By contrast, monocyte-derived DC/macrophages become fractalkine receptor (CX3CR1) positive and remain localized under the epithelial layer ( $10 \mu \mathrm{~m}, 4$ cells per $200 \mu \mathrm{~m}$ villus) in the lamina propria [47]. This CD103-CX3CR1+ DC/macrophage population in the small intestine and colon is known for its antigen sampling capacities via formation of protrusions through the epithelial layer [49, 52]. A process which depends on the expression of CX3CR1. The fractalkine positive DC/macrophages are capable of producing TNF- $\alpha$ under inflammatory conditions which may interfere with intestinal homeostasis and contribute or even drive intestinal inflammation in particular when CD103+ DC are absent [48]. By contrast, these fractalkine positive DC, which have also been phenotyped as being macrophages instead of DC , are capable of producing IL-10 as well which is involved in the establishment
of oral tolerance and Treg expansion hence protection of intestinal homeostasis [53]. Two types of tolerogenic DC, the CD103+ DC and plasmacytoid DC (pDC), are known to instruct the generation of Treg. IEC and DC are involved in the generation of tolerogenic T-cell responses since they contain RA-converting enzyme ALDH. Under influence of epithelial-derived RA and TGF- $\beta$, the CD103+ DC develop that express CCR7 and are attracted to the CCR7 ligands CCL19 and CCL21 in the MLN. Although studies in mice have generated most insight in the development and trafficking of tolerogenic DC in the GALT, also in human some of these principles have been confirmed.

Hence these CD103 + CCR7 + DC are able to pick up the antigen in the lamina propria and migrate to the MLN [49]. In the MLN in general they are thought to contribute to tolerance induction since they are able to induce Foxp3+ Treg via DC-derived RA and TGF- $\beta$ [43-45, 51, 54, 55]. These de novo-induced Foxp3+ Treg are instructed to express gut homing receptors CCR9 and integrin- $\alpha 4 \beta 7$, also under pressure of RA and via the blood stream they home back into the lamina propria. However, CD103+ DC not always generate tolerogenic responses. During mucosal inflammation the CD103+ DC drive an inflammatory T-cell (Th1, Th17, IL-6, IFN- $\gamma$ ) response instead of regulatory response as was established in a mouse model of T-cell-mediated colitis [42]. Besides RA recently DC-derived thymic stromal lymphopoietin (TSLP) was also shown to contribute to the instruction of Foxp3+ Tregs at the expense of Th17 cells [45]. Hence environmental factors determine whether a tolerogenic or inflammatory response is generated within the MLN by CD103+ cells [42]. In addition to the CD103+ DC, in mice tolerogenic capacities by pDC have been shown. pDC are continuously produced in the bone marrow, are TLR7 and TLR9 positive, and carry CCR9 which is responsible for homing to the small intestine since the IEC located here produce CCL25/TECK which serves as an chemoattractant [56].

## GALT Macrophage

In the lamina propria not only DC but also resident macrophages are involved in maintenance of homeostasis. The characterization of intestinal DC versus macrophages is challenging since they share several similar features. For example, classical DC integrin (CD11c) is highly expressed by DC while it remains low in macrophages except for CX3CR1+ myeloid DC/macrophages that also display macrophage marker F4/80+ [57]. These intestinal DC/macrophages have a strong capacity to destroy bacteria without inducing inflammation. Typically IEC express CX3CL1, the ligand for CX3CR1, which may contribute to the suppression of pro-inflammatory cytokine production by these DC/macrophages and support secretion of regulatory IL-10 by these cells [57]. IL-10 expressing CX3CR1+ gut-resident $\mathrm{DC} /$ macrophages have been shown to be essential for the local proliferation of Foxp3 + Treg which were generated within the MLN and had trafficked back into the lamina propria and contributed to the establishment of oral tolerance [53]. CX3CR1 was shown to contribute to IL-10 expression within these
macrophages [53]. Transmembrane receptor CX3CL1 is selectively expressed by epithelial cells of the small intestine and colon and can be released upon proteolytic cleavage providing a chemotaxis gradient [58].

### 3.1.3 T-Cell Response: Immunity Versus Tolerance

In the PP and MLN naïve T-cells are instructed towards an antigen-specific tolerogenic Treg response (tolerance via active immune suppression) or a Th1, Th2, or Th17 type of immune response (immunity) [36]. Several subsets of Treg are able to suppress excessive immune activation by means of cell-cell contact or TGF- $\beta$ and/or IL-10 secretion [38]. Natural arising CD4+CD25+FoxP3+ Treg develop from the thymus and have been shown essential for immune homeostasis. However CD4+CD25+FoxP3+ Treg can also be produced in the periphery such as the GALT. These induced or de novo generated Foxp3+ Tregs are generated within the naïve T-cell pool for example in the MLN by CD103+ DC [51, 54, 55]; alternatively pDC may be involved. The Treg home back in the lamina propria where they may further expand under influence of resident $\mathrm{DC} /$ macrophages [53]. Besides effector cells generated in the inductive sites that home to the lamina propria, intraepithelial lymphocytes contribute to the mucosal immune response and maintain homeostasis and oral tolerance [35, 59].

Preservation of intestinal homeostasis and oral tolerance induction is essential to prevent or cure food allergy. Oral tolerance is defined as the absence of a systemic reaction upon previous oral feeding of an antigenic protein as consequence of active immunological nonresponsiveness [37]. Oral tolerance to food proteins is established when a local intestinal immune response is absent or a suppressive antigen-specific immune response is generated within the intestine and systemic compartment [53]. Germfree mice do not develop tolerance but allergy upon oral delivery of a food protein and have impaired Treg function. Both can be restored by colonization with Bifidobacteria and Lactobacilli respectively (reviewed by van der Aa et al. [60]). In addition, it is hypothesized that commensal bacteria such as Bifidobacterium infantis in infants and Clostridium cluster IV and XIVa in adults are responsible for the instruction of Foxp3+ Treg in the intestinal mucosa [23].

Oral tolerance may involve deletion or anergy of reactive T-cells and the induction of antigen-specific Treg [61, 62]. Indeed allergen-specific Treg are expanded within a week upon oral allergen challenge in children who have outgrown cow's milk allergy while this did not occur in children that remained allergic [63]. Both CD25+ and CD25- T-cell populations may be involved in the instruction of a regulatory response; however recently it was shown that expression of Foxp3 is essential since loss of Foxp3+ Treg cells renders total loss of oral tolerance in mice resulting in a systemic IgE response and allergic symptoms [53]. The MLN that drain from the gut have been indicated as port that confers local tolerance for food protein to systemic nonresponsiveness. In particular FoxP3+ Treg that are induced in the MLN have been identified to confer oral to systemic tolerance. However, they are only able to do this when they are allowed to
home back to the lamina propria, a process requiring not only CCR7 expression by these Tregs but also MADCAM-1 [53]. Furthermore these FoxP3+ Tregs generated in the MLN need to be sustained in the lamina propria of the small intestine where its number remains increased 5 up until 12 days after oral antigen challenge [53]. Co-administration of the antigen with cholera toxin breaks oral tolerance induction which was associated with the lack of FoxP3 + Treg expansion within the lamina propria [53].

### 3.1.4 B-Cells and Humoral Response

One of the main functions of the mucosal immune system is to maintain mucosal homeostasis. Treg contribute to this via suppression of overrated immune activation via cell-cell contact or IL-10 or TGF- $\beta$ production. However TGF- $\beta$ derived from, amongst others Treg cells also contributes to the generation of $\operatorname{IgA} . \operatorname{IgA}$ is transported over the epithelial layer and secreted at the mucosal site as $\operatorname{sIgA}$. Here sIgA neutralizes and is involved in clearance of the antigen. Hence sIgA is very important in mucosal surveillance. Newborn depend on passive transfer of sIgA via breast milk and as the gut immune system matures the endogenous IgA production develops. Fecal sIgA not only serves as a marker for mucosal immune maturation it has also been associated with a reduced risk of IgE -associated allergic disease. In a cohort of 237 infants it was observed that high fecal sIgA levels at the age of 6 months associates with a $50 \%$ reduction in chance of developing IgE-associated allergic disease [64].

### 3.2 Effects of Dietary Nondigestible Oligosaccharides on the GALT

The main function of the GALT is to control homeostasis. Therefore the GALT is focused on instructing a self-regulating effector immune responses against offending pathogens and acquirement of tolerance against the commensal microbiota and harmless antigens such as food proteins. In the intestine tolerogenic DC instruct the generation of Treg which control excessive immune activation via cell-cell contact, IL-10, or TGF- $\beta$ secretion and have been shown to contribute to oral tolerance induction. In addition, local plasma cells produce IgA which has a major protective function. Although basic mechanisms on how nondigestible oligosaccharides affect the GALT are lacking, studies taking into account the abovementioned biomarkers of mucosal tolerance are mentioned below.

### 3.2.1 Nondigestible Oligosaccharides and the GALT in Healthy Rodents

In rats fed a $10 \%$ inulin/FOS (Synergy) diet for 4 weeks the IL-10 secretion by PP cells upon concanavalin A (conA) stimulation was found to increase in correlation with increase in IFN- $\gamma$. Furthermore sIgA concentrations in the ileum were enhanced [65]. Similar findings were done in mice receiving $10 \%$ FOS for 15 days. FOS enhanced the number of B-cells in the PP, and $2.5 \%$ FOS enhanced IgA secretion by PP cells and fecal IgA contents [66,67]. The dose of $7.5 \%$ FOS further enhanced IFN- $\gamma$, IL10, IL-5, and IL-6 secretion by PP cells upon stimulation with components from fragmented Bifidobacteria [67]. The increase in $\operatorname{Ig} A$ upon FOS feeding ( $5 \%$ ) was confirmed in another study with healthy mice. In tissue extracts of the jejunum, ileum, and colon, IgA levels were doubled as well as the percentage of IgA positive B-cells in the PP [68]. Supplementation of $20 \%$ isomalto-oligosaccharides for 4 weeks also enhanced IgA levels in feces in association with increased Lactobacillus counts [69]. In addition, ex vivo stimulation of intraepithelial lymphocytes resulted in increased IFN- $\gamma$ secretion by these cells. Also rats fed $5 \%$ pectin showed increased concentrations of IgA in MLN while IgE was reduced and upon stimulation MLN cells tended to produce more IFN- $\gamma$ and TNF- $\alpha$ [70].

### 3.2.2 Nondigestible Oligosaccharides and the GALT in Disease

In particular the combination of inulin/FOS has been used in mice and small clinical trials to prevent or treat IBD. IBD consists of Crohn's disease (CD) and ulcerative colitis (UC). During flare ups the Th1/Th17 prone inflammatory response in CD is transmural and may occur in the whole gastrointestinal tract, but mainly establishes in the terminal ileum. In active UC, the mucosal inflammation is superficially localized in the mucosa Natural killer cell/Th2 polarized and restricted to the colon. Prevention of dextran sodium sulfate (DSS) induced colitis in rats was observed using inulin/FOS (Synergy 1) resulting in strong reduction in disease activity and reduction in IL-1 $\beta$, neutrophil activation marker myeloperoxidase in colonic tissue while IL-10 and TGF- $\beta$ remained unaltered. Similar effects were shown in presence and absence of complimentary administration of commensal bacteria [71]. In a double-blind placebo-controlled trial with 18 patients with mild active UC the test group was supplemented with 6 g inulin/FOS per day in combination with Bifidobacterium longum for 1 month in combination with drug therapy. Colonic biopsies showed lower expression of epithelial derived beta defensins. Furthermore, inflammatory markers TNF- $\alpha$ and IL- $1 \beta$ reduced in the intervention group which was associated with an improved sigmoidoscopy score; however the clinical activity index did not improve [72]. In an uncontrolled trial, ten active Crohn's patients were provided with 15 g FOS per day for three consecutive weeks. FOS reduced the disease activity and enhanced fecal

Bifidobacteria counts. The percentage of IL-10+CD11c+ DC isolated from the lamina propria of rectal biopsies was increased upon FOS (inulin/oligofructose, Prebio $1=$ Synergy) supplementation ( $p=0.06$ ) [73, 74]. In addition to modulation of mucosal inflammatory markers in IBD, inulin/FOS (Synergy) was found to affect the GALT in rodent models for colon cancer. In azoxymethane (AOM, two times subcutaneous) installed colon cancer in rats, which were 2 weeks prior to up until 33 weeks after AOM treatment fed a $10 \%$ inulin/FOS diet, the tumor load was significantly lowered. The IL-10 secretion of conA stimulated cells of the PP and MLN was enhanced in the inulin/FOS rats sham or AOM treated, and IFN- $\gamma$ was increased in PP of sham rats [75]. A similar study by Femia et al. showed reduced colonic tumors in association with increased SCFA production in rats fed inulin/ FOS and a tendency towards reduced pro-inflammatory cyclo-oxygenase (COX) 2 and inducible nitric oxide synthase (iNOS) expression [76]. Also in Min mice that spontaneously develop intestinal tumors a $6 \% \mathrm{scFOS}$ diet reduced tumor formation in the colon but not in the small intestine. However in the small intestine the number of lymph node nodules increased which may relate to the decrease in colonic tumor formation [77]. After 12-week supplementation in a randomized, double-blind, placebo-controlled trial using inulin/FOS (Synergy) and Lactobacillus GG and Bifidobacterium B, fecal bifidobacteria and lactobacilli counts increased and colorectal proliferation in biopsies reduced [78]. However, 6-month inulin/FOS supplementation did not reduce mucosal proliferation in colon cancer patients in a Phase II clinical trial [79].

## 4 Dietary Intervention Using Nondigestible Oligosaccharides in Allergy Prevention and Treatment

In western developed countries the incidence of allergic diseases has been steadily rising during the past decades. It is hypothesized that immune maturation in early life is hampered by improved hygiene and reduced exposure to microbes rendering Th2 type immune polarization due to changes in intestinal colonization pattern (reviewed by van der Aa et al. [60]). Food allergy (in particular milk and hen's egg) and atopic dermatitis (AD) are the first diseases to establish in early infancy; affected children are more prone to develop allergic diseases like asthma and allergic rhinitis later in life (atopic march). Atopic dermatitis is one of the first manifestations of atopic constitution readily developing within the first year of life with a peak at sixth month; allergic airway diseases can be diagnosed at a later age. Currently the prevalence of atopic dermatitis is 5-20 \% in primary school children. Atopic dermatitis is often associated with food allergy and $40 \%$ of the children develop asthma later in life (reviewed by van der Aa et al. [60]). Nondigestible oligosaccharides in human milk are thought to contribute to gut and immune maturation of the new born either directly or via the establishment of a beneficial
intestinal microflora composition and may suppress the susceptibility to develop allergic disease in early childhood.

### 4.1 Allergy Rodent Models

Several studies have shown suppression of allergic disease by prebiotic oligosaccharides. Nagura et al. showed that a $5 \%$ raffinose diet, which was previously shown to reduce allergic airway eosinophilia in rats [80], increased IL-12 secretion by PP cells ex vivo in Balbc mice. DC from these mice stimulated IFN- $\gamma$ secretion by T-cells of ovalbumin receptor transgenic mice. Direct feeding in ovalbumin transgenic mice suppressed ovalbumin-induced IL-4 secretion by MLN cells and suppressed serum IgE levels [81]. scGOS/lcFOS +/- pAOS was demonstrated to prevent allergic symptoms in the mouse model for cow's milk allergy in association with reduced mast cell degranulation and scGOS/lcFOS/pAOS-enhanced Treg function [26, 82, 83]. Transfer of splenocytes from scGOS/lcFOS/pAOS-fed whey or casein-sensitized mice protected control diet-fed naive recipients from the development of allergic disease upon sensitization. This effect was lost upon in vivo and/or ex vivo CD25+ cell depletion, indicating functional Tregs to be involved in the protective effect generated by the scGOS/lcFOS/pAOS in the donor mice [82, 83]. Although mice were protected and functional Tregs were generated, antigen-specific whey IgE levels remained unaltered high. Recent studies revealed a novel mechanism of action of scGOS/lcFOS. scGOS/lcFOS have been shown to alter the bacterial microflora, but they may as well be able to affect the function of the epithelial layer. In an in vitro coculture model combining human intestinal epithelial cells (IEC) with activated leukocytes in a separate compartment [39, 84], scGOS/lcFOS were shown to further polarize the effector immune response towards a Treg and Th1 response, skewing away from the allergic phenotype, when combined with bacterial CpG DNA [93]. These effects were found to be generated via epithelial release of soluble-type lectin galectin- 9 which may contribute to the mechanism of action of oligosaccharides. Galectin-9 is known for its capacities to induce Treg and neutralize IgE [85, 86]. It was shown that dietary nondigestible oligosaccharides are able to enhance bacterial CpG DNA-induced galectin-9 expression in IEC [39, 93]. Indeed translational studies revealed this finding to be a major breakthrough in the understanding of the mechanism by which scGOS/lcFOS exert their protective effect in allergic disease. A scGOS/lcFOScontaining diet was found to enhance polarized galectin-9 expression in IEC of cow's milk allergic mice, and in addition, serum galectin- 9 levels were dramatically elevated, suggesting epithelial galectin- 9 release driven by the scGOS/lcFOS diet [87]. Indeed serum galectin- 9 levels were positively correlated with the reduction in allergic symptoms in the cow's milk allergic mice and contributed to reduced mast cell degranulation. Furthermore serum galectin-9 concentrations increased in infants affected with atopic dermatitis being supplemented with scGOS/lcFOS and Bifidobacterium breve in association with reduced symptom scores [87].

### 4.2 Human Studies Prevention

The risk of developing AD is associated with delayed maturation of a Th1 response and an atopic constitution (high total and specific $\operatorname{IgE}$ ). In a double-blind, placebocontrolled clinical trial aiming to prevent occurrence of atopic dermatitis $8 \mathrm{~g} / \mathrm{L}$ scGOS/lcFOS (9:1) was provided in extensively hydrolyzed milk formula for 6 months, starting within 2 weeks of life. The cumulative incidence of AD in the children at risk for atopy at the age of 6 months was over $50 \%$ reduced in the intervention group ( $n=10 / 102$ ) compared to the control group ( $n=23 / 104$ ) in association with higher Bifidobacteria counts in the feces. Children of the intervention group cried less and had improved stool consistency and frequency [88]. At 3 months of age all children received an Hexavac vaccination for diphtheria, tetanus, and polio (DTP). At 6 months of age DTP-specific immunoglobulins were established to similar levels in the scGOS/lcFOS supplemented children similar to the control group. By contrast total IgE and IgG1 and cow's milk protein-specific IgG1 were reduced in the supplemented group [89]. In addition to this reduction in atopic constitution of the intervention group, the oligosaccharides were found to reduce serum kappa as well as lambda immunoglobulin free light chains (IgfLC) in these children [90]. Like IgE, IgfLC can cause acute allergic symptoms upon antigen challenge and IgfLC levels were found to be enhanced in AD patients [90-92]. At the age of 2 years the cumulative incidence of AD was still $50 \%$ lower in the scGOS/lcFOS group ( $13.6 \%$ of $n=66$ ) compared to the placebo group ( $27.9 \%$ of $n=68$ ), while the supplementation was stopped at 6 months of age [94]. In addition, the supplemented group had a reduced incidence of over $50 \%$ in recurrent wheezing and allergic urticaria. Furthermore, the overall infection rate was lower which applied in particular to upper respiratory tract infections and the number of fever episodes and antibiotic prescriptions was lower. Similar results were obtained in a double-blind placebo-controlled trial with healthy term infants, using a formula milk enriched with $8 \mathrm{~g} / \mathrm{L}$ of a mixture of $\operatorname{scGOS} / \mathrm{lcFOS}(9: 1,85 \%)$ and pAOS $(15 \%)(n=414$ in prebiotic group and $n=416$ in the control group). Children were supplemented for 12 months, starting before 8 weeks of age. At the age of 12 months the prebiotic supplemented group showed over $40 \%$ reduced incidence of atopic dermatitis compared to the control formula group; the protective effect was comparable to that of the breast-fed control group. Also the severity of AD occurring in the prebiotic group tended to reduce compared to the control formula group, and the use of corticosteroids was reduced by over $50 \%$. Serum $\operatorname{IgE}$ concentrations did not differ between groups [95]. Atopic constitution may readily develop during gestation and dietary factors may affect immune imprinting of the fetus. In a placebo-controlled study, a mixture of four probiotic strains and scGOS was provided 2-4 weeks prior to delivery to pregnant women at high risk of delivering an atopic child $(n=461)$. This supplementation was continued in the infants for 6 months. Also in this study the incidence of atopic IgE-mediated eczema at the age of 2 years was reduced by $30 \%$ [96]. This effect was associated with enhanced colonization of lactobacilli and bifidobacteria species. The study
outcome was promising with regard to prevention of atopic dermatitis and also the cumulative incidence of IgE-associated allergic disease (food allergy, asthma, eczema, allergic rhinitis) tended to reduce. However, sensitization (positive skin prick test or $\operatorname{IgE}$ ) as such did not differ between groups.

### 4.3 Human Studies Treatment

Allergic disease is known to be orchestrated due to an over-reactive Th2 response on the one hand and compromised Th 1 and/or regulatory T cell activity on the other hand. This results in generation of allergen-specific IgE by B-cells, opsonizing mast cells which degranulate and cause allergic symptoms upon allergen challenge. Current drugs used, such as corticosteroids, mast cell stabilizers, or antihistaminica, are effective in suppression of symptoms. The allergy as such is not cured. Antigen-specific immunotherapy is used to treat allergies and for some allergens successful protocols are established to do so. Few studies are undertaken to use dietary nondigestible oligosaccharides in the treatment of allergic disease and in future these studies may be further expanded. In the Synbad study 90 infants with atopic dermatitis aged $<7$ months were randomized to receive either a placebo hydrolyzed milk formula or this formula supplemented with $8 \mathrm{~g} / \mathrm{L}$ scGOS/lcFOS and $B$. breve MV16. After 12 weeks of treatment the atopic dermatitis symptom scores were reduced by 13.5 points in the placebo group and 18.1 points in the subgroup of IgE-associated atopic dermatitis $(n=45)$ fed the synbiotic product ( $p=0.04$ ) while IgE was not affected [97]. Children treated with the synbiotics had increased fecal Bifidobacteria counts while pathogen counts were decreased. In the serum of synbiotic supplement infants the serum galectin- 9 levels were enhanced by the end of the intervention period [87]. Galectin-9 is known for its capacities to neutralize IgE [85] and may have contributed to the protective effect of the synbiotics. In the 1-year follow-up of the Synbad study population the prevalence of wheezing and noisy breathing apart from colds was assessed as early predictors for asthma, since $40 \%$ of children affected with atopic dermatitis develop asthma later in life. In the synbiotic group this was dramatically lower and use of asthma medication was reduced by $80 \%$ [98], even though these children did not receive synbiotics in the last 12 months. The same synbiotics, scGOS/lcFOS, and $B$. breve MV16 were supplemented to 29 adult asthmatics with house dust mite allergy in a randomized double-blind placebo-controlled trial. Although upon allergen provocation the synbiotics did not alleviate symptoms, the peak expiratory flow as measured by the patients in the morning and evening did improve after 3 and 4 weeks and serum IL- 5 levels reduced after 4 weeks of intervention [99].

## 5 Conclusion

HMOs are very complex and differ greatly between persons. Synthesized nondigestible galacto- and fractionated fructo-oligosaccharides mimic some functional aspects of HMOs. They induce growth of Bifidobacteria and Lactobacilli, reduce infections in infants, and increase fecal $\operatorname{IgA}$. In addition, evidence is building that nondigestible galacto- and fructo-oligosaccharides can modulate the GALT and systemic immune system in health and disease. In healthy rodents FOS was shown to enhance IL-10 secretion in the PP in association with increase in IFN- $\gamma$. FOS enhanced IgA in the PP, MLN, and intestinal tissues, while increasing numbers of IgA positive B-cells. In rodent models of IBD and colon cancer FOS/inulin improved clinical outcome, reduced inflammatory markers, and increased IL10 positive DC and IL-10 secretion by MLN cells. In clinical trials scGOS/lcFOS plus or minus pAOS have been shown to reduce the development of IgE-mediated AD by $50 \%$. scGOS/lcFOS was limited capable of reducing IgE, but may enhance serum galectin-9 known to neutralize IgE. Biomarkers of mucosal tolerance have been studied in rodent models. Besides the increase in IL-10 and IgA in healthy rodents, scGOS/lcFOS/pAOS was found to induce functional Treg in spleens of mice affected with cow's milk allergy, which may contribute to oral tolerance induction. Studies are warranted that elucidate the impact of dietary oligosaccharides and HMOs on GALT DC/macrophages and oral tolerance induction. Combining oral delivery of nondigestible oligosaccharides with allergenic epitopes could further improve effectiveness and may add to future oral immunotherapy strategies.

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# Chapter 9 <br> The Endocannabinoid System: A Dynamic Signalling System at the Crossroads Between Metabolism and Disease 

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## Abbreviations

| (LC-)PUFA | (Long chain-)polyunsaturated fatty acid |
| :--- | :--- |
| 2-AG | 2-Arachidonoylglycerol |
| AA | Arachidonic acid |
| AEA | $N$-arachidonoylethanolamine (anandamide) |
| CB (receptor) | Cannabinoid (receptor) |
| CBD | Cannabidiol |
| COX | Cyclooxygenase |
| DAGL | Diacylglycerol lipase |
| DHA | Docosahexaenoic acid (22:6n-3) |
| DHEA | $N$-docosahexaenoylethanolamine |
| ECS | Endocannabinoid system |
| FAAH | Fatty acid amide hydrolase |
| GPCR | G-protein coupled receptor |
| LOX | Lipooxygenase |
| MAGL | Monoacylglycerol lipase |
| NADA | $N$-arachidonoyldopamine |
| NAEs | $N$-acylethanolamines |
| OEA | $N$-oleoylethanolamine |
| PEA | $N$-palmitoylethanolamine |
| PPAR | Peroxisome proliferator-activated receptor |
| THCV | $\Delta 9$-Tetrahydrocannabivarin |
| TRVP1 | Transient receptor potential channel type V1 |
| $\Delta 9-T H C$ | $\Delta 9$-Tetrahydrocannabinol |

[^8]
## 1 Introduction: The Changing Views on the Endocannabinoid System

Endocannabinoids are signalling lipids playing important roles in a wide variety of biological processes. Together with their receptors and enzymes involved in their synthesis and breakdown they constitute the "endocannabinoid system" (ECS). By definition the term endocannabinoid is limited to those compounds displaying significant affinity to the cannabinoid receptors $\mathrm{CB}_{1}$ and $\mathrm{CB}_{2}[1,2]$. These receptors were discovered in the late 1980s [3, 4] and were shown to bind (-)-trans- $\Delta 9$ tetrahydrocannabinol ( $\Delta 9-\mathrm{THC}$ ) from the Cannabis plant. To date, nine "true" endocannabinoids are being distinguished (Fig. 9.1), which are all derived from long chain (C18 or longer) polyunsaturated fatty acids (LCPUFAs) [1]. The first two discovered and still the most studied are anandamide ( N -arachidonoylethanolamine (AEA)), its name originating from the Sanskrit word "ananda" meaning "the bliss", and 2-arachidonoylglycerol (2-AG).

However, more than two decades of research have shown that the ECS per se is less specific and distinct than originally assumed. It is now widely accepted that it is tightly intertwined with other signalling mechanisms and that endocannabinoids are part of a larger class of structurally related amides, esters and ethers of fatty acids which exist in a continuous dynamic equilibrium with each other. The vast majority of these molecules belongs to the fatty (acid-) amides like AEA, although analogues of 2-AG including 2-oleoylglycerol and 2-linoleoylglycerol have also been found (see Sect. 2.3). Fatty acid amides (Lipid Maps class FA08; http://www.lipidmaps.org) are conjugates of different long chain fatty acids and amines including ethanolamine, neurotransmitters (serotonin, dopamine) or simple amino acids. They are abundantly present in nature and involved in various regulatory processes. In animals, their molecular targets go far beyond the classical CB receptors and include a wide range of receptors including GPR55, GPR18, GPR119, TRPA1 (transient receptor potential ankyrin 1), TRPV1 (transient receptor potential channel type V1), PPARs (peroxisome proliferator activated receptors) as well as several non-receptor targets [2, 5-7]

It has also become clear that some (if not all) of the "true" endocannabinoids themselves display "promiscuous" behaviour by activating or blocking other receptors besides $\mathrm{CB}_{1}$ or $\mathrm{CB}_{2}$ with potencies that differ little from those with which they interact with "true" cannabinoid receptors [2, 6]. In addition, anandamide, 2-AG and other CB ligands interact directly or indirectly with non-receptor targets [5]. Biochemical pathways for synthesis and degradation of endocannabinoids and their congeners show several crossroads with those of other lipid mediators, in particular eicosanoids. This not only creates a number of regulatory nodes but also leads to the formation of "hybrid" structures including prostamides and other oxidation products, often with bioactivity [8-11]. Taken together, an "expanded" view of the ECS is increasingly considered a better concept to comprehend its full dimensions [12]. In line with this, it has been suggested to apply the term "endocannabinoidome" to describe this family of molecules (Fig. 9.2). Mediators that are part of this endocannabinoidome are fluctuating in a time and tissue-specific way, modulated by various endogenous (e.g. energy status, inflammation) and


Anandamide (AEA)


N -arachidonoyl dopamine (NADA)


Virodhamine


N -oleoyldopamine (OLDA)


2-Arachidonoyl-glycerol (2-AG)


Noladin ether

$N$-dihomo- $\gamma$-linolenoylethanolamine



Fig. 9.1 Structural formulas of the classical endogenous cannabinoid receptor agonists, anandamide ( $N$-arachidonoylethanolamine, AEA), 2-arachidonoyl glycerol (2-AG), $N$-arachidonoyldopamine (NADA), Noladin ether, $O$-arachidonoylethanolamine (Virodhamine), N -dihomo- $\gamma$-linolenoylethanolamine, $N$-oleoyldopamine (OLDA), $N$-docosatetraenoylethanolamine and oleamide
environmental factors, including diet. These network dynamics have major consequences for drug development. Soon after its discovery it became clear that the ECS is involved in a number of important processes and (chronic) diseases including pain, anxiety/depression, GI/liver diseases, cancer, metabolic disease and eating behaviour. Several promising new pharmacological targets were suggested which often indeed showed encouraging effects in animal studies. In particular in relation to weight management and metabolic diseases expectations were high to develop $\mathrm{CB}_{1}$ antagonists or inverse agonists into a completely new drug class. Therefore, the failure of the first in class compound rimonabant because of severe anxiety and depression-related side-effects in predisposed persons [13] shocked the pharmaceutical industry. By the end of 2008 at least nine companies terminated active development projects with $\mathrm{CB}_{1}$ blockers. These included some with compounds in a well-advanced stage of development such as Taranabant (Merck) and CP-945,598 (Otanabant, Pfizer). In retrospect these failures illustrate that initial


Fig. 9.2 Cartoon depicting the "expanding" view on the endocannabinoid system (ECS). The "classical" ECS (centre) is considered as part of an endocannabinoidome consisting of structurally related ligands, metabolites and enzymes involved. Endocannabinoids per se and their congeners interact with different non-cannabinoid receptors and other molecular targets
strategies to modulate a dynamic and pleiotropic like the ECS have been too narrow. The endocannabinoidome still holds many promises for both "food" and "pharmaceutical" applications. However, its complexity demands for more subtle multipletarget strategies instead of a classical one disease-one target-one drug approach. This chapter aims to illustrate some recent developments and activities in the field of the ECS, including some related receptors and mediators. Major therapeutic applications will be briefly illustrated. This will be elaborated in Sect. 4 by examples from two main domains, namely, "inflammation" and "weight management".

## 2 From Phytocannabinoids to Endocannabinoids: A classical Example of Reversed Pharmacology

### 2.1 Compounds with Pharmacological Activity from Cannabis spp.

Earliest written records on the physiological effects and medical use of Cannabis go back to about 2000 BC in the famous book Pe'n-ts'ao Ching attributed to the

Fig. 9.3 Examples of phytocannabinoids from Cannabis. The main psychoactive component is (-)-trans- $\Delta 9$ tetrahydrocannabinol ( $\Delta 9$ THC). Two other compounds discussed in this chapter are $(-)$-cannabidiol (CBD) and ( - )-trans- $\Delta 9$ tetrahydrocannabivarin ( $\Delta 9$-THCV)

$\Delta 9$-THC


Cannabidiol

$\Delta 9$-THCV

Chinese emperor Shen-nung [14]. This ancient pharmacopoeia describes a number of properties of Cannabis, including its capacity to "lighten one's body". Throughout history, medical use of Cannabis has been widely accepted and very common in different parts of the world until this began to decline around the beginning of the 1900s [15]. Since the last decades, there is a renewed interest in preparations and compounds prepared from the Cannabis plant. The term phytocannabinoids (phytoused here to distinguish them from endocannabinoids) refers to a group of terpenophenolic compounds with 22 carbons (or 21 carbons for neutral form) of which more than 70 have been found so far and which can be categorised into ten main structural types [16, 17]. In general, Cannabis refers to the species Cannabis sativa, although there is ongoing discussion whether the genus Cannabis comprises more than one species, i.e. Cannabis sativa and C. indica [16]. Preparations from different Cannabis breeds show a great variety in absolute and relative concentrations of phytocannabinoids [18], of which only a few are ligands for $\mathrm{CB}_{1}$ or $\mathrm{CB}_{2}$ receptors. However, as the adjective "cannabinoid" predates the discovery of cannabinoid receptors by many years this term is still commonly used to describe also other compounds with structures similar to the phytocannabinoid $\Delta 9-\mathrm{THC}$, irrespective of whether they are or are not cannabinoid receptor agonists or antagonists [2]. Recent breeding and selection of Cannabis for recreational purposes has primarily focussed on increasing the content of the psychotropic compound (-)-trans- $\Delta 9$-tetrahydrocannabinol ( $\Delta 9-\mathrm{THC}$, Fig. 9.3). At the same time, the renewed interest in Cannabis for medical use initiated the search for cultivars with completely different compositions and often much lower hallucinogenic activity. It is expected that the unravelling of the Cannabis sativa genome [19] will further stimulate these developments. In the plant, cannabinoids are produced as their carboxylic acid derivatives, known as cannabinoid acids. Their neutral counterparts can be formed through the action of heat (smoking), sunlight or during storage [20, 21]. Several cannabinoid acids themselves display biological activity, which are often distinct from those of their decarboxylated products [17, 20-22]. Chemical structures of some of the most studied phytocannabinoids are depicted in Fig. 9.3.

Although $\Delta 9$-THC (Dronabinol, Marinol ${ }^{\circledR}$ ) has been available as medicinal preparation for oral use since the 1980s, its therapeutic use initially remained rather limited. Efficacy was reported to be variable, at least partly due to significant firstpass metabolism [23]. Since the beginning of this century there has been a slow but steady growth in the development and application of medicinal products based on herbal Cannabis or natural cannabinoids [23-25]. Differences between regions and therapeutic viewpoints are among the factors which determine whether the focus is more on the herb or on specific phytocannabinoids. Some countries and regions (The Netherlands, Canada, several US states) have official medicinal Cannabis policies, often referred to as "medical marijuana". Herbal products are preferably taken by inhalation using special vaporizers, and there is an increasing trend towards "individualised" therapies using specially selected cultivars [18]. On the other hand, formulations with purified $\Delta 9-\mathrm{THC}, \mathrm{CBD}$ and/or THCV for oral or oromucosal delivery are also being developed and implemented [26, 27]. Cannabis or phytocannabinoid-based preparations are used for a number of indications, including pain, the amelioration of chemotherapy-induced nausea and vomiting, stimulation of appetite and management of spasticity in multiple sclerosis [25, 27, 28]. An in-depth discussion on the role of Cannabis or cannabinoid-based preparations in medical therapy is considered outside the scope of this chapter. It is obvious though that the debate on this issue continues until today.

From a scientific and potentially therapeutic perspective it is of interest to note that increasing data are becoming available on the activity of phytocannabinoids other than $\Delta 9-$ THC with only weak or no psychotropic effects. Compounds of interest include cannabidiol (CBD), cannabigerol (CBG), cannabichromene (CBC), $\Delta 9$-tetrahydrocannabivarin (THCV), cannabidivarin (CBDV) as well as (at least) some cannabinoid acids, $\Delta 9$-tetrahydrocannabinolic acid ( $\Delta 9-$ THCA) and cannabidiolic acid (CBDA). A detailed discussion of each of these molecules falls outside the scope of this chapter. For an excellent overview readers are referred to Izzo et al. [17]. However, two compounds are of specific interest and merit some extra attention here, namely, CBD and THCV.

Among the non-hallucinogenic phytocannabinoids, CBD (Fig. 9.3) is currently receiving the most attention. In dried Cannabis CBD contents range from very low ( $<1 \%$ ) to equal or even higher (up to around $8 \%$ ) compared to those of $\Delta 9-\mathrm{THC}$, depending on the cultivar and preparation. The oromucosal spray Sativex ${ }^{\circledR}$, prescribed for the treatment of spasticity due to multiple sclerosis contains CBD and $\Delta 9$-THC in a $1: 1$ ratio. Cannabidiol behaves like a typical multiple target compound. For reviews, see for example [17, 29]. It displays a highly diverse spectrum of activities including agonist activity for PPAR $\gamma$, TRVP1 and TRPA1 receptors, antagonist of GPR55, a complex antagonistic behaviour towards $\mathrm{CB}_{1}$ and $\mathrm{CB}_{2}$, etc. (see also Sect. 3.2). Specifically in relation to neurodegenerative diseases and (neuropathic) pain the effects of CBD on glia cells are of interest to note. Several preclinical and an increasing number of clinical studies have suggested at least promising activities in chronic inflammatory and autoimmune diseases including IBD [30], MS [31], cancer [5, 32, 33] and different CNS disorders [34-36]. Remarkably, there is increasing evidence that CBD and $\Delta 9-\mathrm{THC}$ interact within the CNS
thereby reducing the psychoactive effects of $\Delta 9-$ THC and possibly even its psychogenic risks [37-39]. Future clinical studies, of which several are presently ongoing (source: ClinicalTrials.gov) should further demonstrate the full therapeutic potential of CBD, alone or combined with other cannabinoids or other compounds.
$\Delta 9$-tetrahydrocannabivarin (THCV, Fig. 9.3) occurs in Cannabis as a minor component in varying amounts [18]. Interestingly, this compound has been found to possess $\mathrm{CB}_{1}$ antagonist properties [40, 41]. Therefore, it is receiving attention as a natural alternative to the $\mathrm{CB}_{1}$ blockers/inverse agonists like rimonabant. Recently, THCV has been found to ameliorate insulin sensitivity in two mouse models of obesity [42].

### 2.2 Phytocannabinoids from Plants Other than Cannabis

Remarkably, structures with affinity to $\mathrm{CB}_{1}$ and $\mathrm{CB}_{2}$ have also been found in plants other than Cannabis [43]. These molecules may be divided into two categories. First, plants like most other organisms contain lipid-derived structures which are chemically related to the endocannabinoids as those found in mammals (see also Sect. 2.3), albeit shorter acyl chains ( C 12 or C 14 ) appear to be more common in plant [43-46]. Next to this, an increasing number of other plant compounds with affinity for $\mathrm{CB}_{2}$ or $\mathrm{CB}_{1}$ have been characterised. Examples include (E)-ß-caryophyllene (present in many different spices and food plants including oregano, cinnamon and black pepper), falcarinol (found in carrots, parsley and celery), yangonin (present in Kava, Piper methysticum) and magnolol (from the medicinal plant Magnolia officinalis) (Fig. 9.4) [43, 44, 47-50].

Considering the wide abundance in nature and "promiscuity" of the ECS and related signalling systems, it does not seem unlikely that more natural compounds with similar properties will be found in common spices and herbs. It is tempting to speculate that such compounds may play a role in the culinary properties of some plants by inducing "hedonic" signals in the brain via $\mathrm{CB}_{1}$ receptor stimulation.

### 2.3 Endocannabinoids and Beyond

As mentioned in Sect. 1, the discovery of the prototypical endocannabinoids per se was followed by the finding that these molecules belong to much larger group of fatty acid-derived structures of which the biological effects go far beyond effects on $\mathrm{CB}_{1}$ and $\mathrm{CB}_{2}$.

The endocannabinoid anandamide (AEA) belongs to $N$-acylethanolamine (NAE) subclass of fatty acid amides. In addition to the NAEs, several other classes of fatty acid amides can be distinguished, including the primary fatty acid amides, the $N$-acylamino acids ( $=\mathrm{N}$-acylamines), N -acylarylalkylamines ( $N$-acyldopamines, $N$-acylserotonins) (Fig. 9.5) [51, 52].

(E)- $\beta$-Caryophyllene


N -alkylamide from Echinacea


Magnolol

Yangonin

Fig. 9.4 Some plant-derived compounds with $\mathrm{CB}_{1}$ or (and) $\mathrm{CB}_{2}$ affinity present in plants other than Cannabis

It has been shown that cells are able to "combine" different fatty acids and biogenic amines to make several possible permutations of different fatty acid amides [7,51]. Several studies have demonstrated that the local relative availability of fatty acid precursors, which in turn is modulated by dietary intake of lipids, plays an important role in determining the pattern of amide conjugates formed. For example, a number of studies in rodents and humans have shown that increasing the relative proportion of $n-3$ LC PUFAs in the diet can lead to a decrease in the formation of the "prototypic" endocannabinoids AEA and 2-AG, which are derived from the $\mathrm{n}-6$ fatty acid arachidonic acid [53-56]. These changes are a direct consequence from a shift in n-3-n-6 balance of membrane lipids, resulting in compensatory increases in n-3 LC-PUFA-derived acyl conjugates. The same holds true for the local availability of amines. For example, we showed that serotonin conjugates with fatty acids are formed by gut tissue, where most of the body's serotonin resides [57].


Fig. 9.5 Examples of fatty acid amide structures not belonging to the endocannabinoids per se (see also Fig. 9.1)

Compared to the many fatty acid amides, only little has been reported on 2-acylglycerol esters other than the endocannabinoid 2-AG. However, it is likely that several congeners will exist, for example formed out of triglycerides. In 1999, Ben-Shabat et al. reported the isolation of 2-linoleoyl-glycerol and 2-palmitoylglycerol (2-PG) from mouse spleen, brain and gut [58]. These two compounds did not directly bind to $\mathrm{CB}_{1}$ or $\mathrm{CB}_{2}$ receptors but apparently potentiated the binding of 2-AG and its capacity to inhibit adenylyl cyclase. Furthermore, both esters caused potentiation of some of the in vivo effects of 2-AG. Interestingly, 2-oleolyglycerol (2-OG) was found to stimulate GPR119 receptors (see Sect. 3.2.4) in vitro, and did this more potently than 2-AG, 2-PG and 2-linoleoyl-glycerol [59]. Subsequently 2-OG was given to human volunteers ( 2 g by jejunal delivery), which resulted in increased levels of plasma GLP-1 compared to administration of the precursor oleic acid. Triglycerides with oleic acid in the $s n-2$ position are very common in the diet and from these 2 -OG can be formed in the gut in amounts larger than the dose given in the study of Hansen et al. [59]. Very recently, the presence of 2-linoleoylglycerol and 2-oleoylglycerol (2-OG) has also been demonstrated in Drosophila [60].

## 3 Biochemistry and Pharmacology of the ECS

### 3.1 Endocannabinoid Formation and Breakdown

Synthesis and release of endocannabinoids and many related compounds are considered to take place "on demand" via different multi-step processes which are partly acting in parallel. For $N$-acyl-ethanolamines (NAEs) the most studied pathway is their formation from glycerophospholipids via $N$-acylphosphatidyl ethanolamines (NAPEs), present in phospholipid membranes. NAPEs function as stable precursors and source of their respective NAEs. Besides their role as precursor of NAEs, NAPEs have bioactive effects themselves [61]. NAPEs are formed by incorporation of fatty acids from the $s n-1$ position of a donor phospholipid like phosphatidylcholine and transfer to an ethanolamine phospholipid, e.g. phosphatidylethanolamine. This reaction is catalysed by a $\mathrm{Ca}^{2+}$-dependent $N$ acyltransferase [61, 62]. Next, free NAE can be generated by a NAPE-hydrolyzing phospholipase D (NAPE-PLD). In addition, other synthesis routes for NAEs have been found [61, 63]. The glycerol-ester 2-AG is synthesised from diacylglycerol (DAG), a very common second messenger, via the enzyme diacylglycerol lipase (DAGL), of which more than one form has been described [64]. Biosynthetic routes for other N -acylamides appear to be less well studied [52]. Huang et al. originally suggested that $N$-arachidonoyldopamine (NADA) may either be synthesised by condensation of dopamine with arachidonic acid (possibly via arachidonoyl-CoA) or via a pathway involving N -arachidonoyl-tyrosine [65]. Later, Hu et al. [66] reported that the latter may not be very likely. Instead they suggest a direct involvement of FAAH either as rate-limiting enzyme that liberates arachidonic acid from AEA, as a conjugation-enzyme, or both.

Conjugates of arachidonic acid (and possibly other fatty acids) with simple amino acids can be synthesised via the formation of the acyl-CoA thioesters, as was shown for N -arachidonoyl-glycine (NAGly) [67]. Interestingly, the arachidonic acid that conjugates with glycine appears to be a result of the hydrolysis of AEA [68]. An alternative pathway was proposed by Burstein et al. [69] who speculated that NAGly is produced by the oxidation of the ethanolamine in AEA, presumably through alcohol dehydrogenase. Evidence exists for both pathways [68].

Fatty amides and 2-acylglycerols are broken down by enzymatic hydrolysis. The primary NAE degrading enzyme is fatty acid amide hydrolase (FAAH, now also known as FAAH-1), localised on the endoplasmatic reticulum [70]. A second FAAH enzyme is found in humans located on cytoplasmic lipid droplets [70, 71]. Recently, a third NAE hydrolysing enzyme, $N$-acyl ethanolamine-hydrolysing acid amidase (NAAA) has been identified [72].

To reach their sites of catabolism within the cell, NEAs are bound to different proteins including fatty acid binding proteins 5 and 7 , heat shock protein 70, albumin and fatty acid amide hydrolase-like AEA transporter protein [73, 74]. Intracellular trafficking of NAEs is also important to reach those receptors that are located intracellularly [55, 75]. Next to hydrolysis, NAEs are substrates for oxidative
enzymes including cyclooxygenases (COXs), lipoxygenases (LOXs) and cytochrome P450 enzymes, yielding a range of prostaglandin-amides (prostamides), prostaglandin-glycerol esters and hydroperoxy-derivatives [5, 76, 77]. At least a number of these oxidation products show biological activity [76-78]. 2-AG is hydrolyzed via the enzyme monoacylglycerol lipase (MAGL), to a lesser extent by $\alpha / \beta$-hydrolase 12 (ABHD12) and $\alpha / \beta$-hydrolase 6 (ABHD6), and also by FAAH [79, 80]. Interestingly, AA in brain formed by hydrolysis of 2-AG via MAGL has been shown to serve as pool for pro-inflammatory eicosanoid synthesis, thus representing a connection between endocannabinoid and eicosanoid pathways [81]. 2-AG can also be oxygenated by COX-2 and LOX resulting in the formation of prostaglandin glycerol esters (PG-Gs) [76].

### 3.2 Cannabinoid and Related Receptors

According to the IUPHAR classification system the $\mathrm{CB}_{1}$ and $\mathrm{CB}_{2}$ receptors are the only bona fide cannabinoid receptors. They are phylogenetically restricted to the chordate branch of the animal kingdom [2]. Among other GPCRs, those structurally most closely related to $\mathrm{CB}_{1}$ and $\mathrm{CB}_{2}$ belong to the lysophospholipid receptors. These receptors for endocannabinoids or lysophospholipid-like molecules have evolved independently in different branches of the GPCR superfamily [1]. However, in terms of ligand binding characteristics the picture becomes more complicated. As mentioned before, endocannabinoids have a multitude of structural analogues. These compounds interact with different receptors and non-receptor targets. Several endocannabinoids per se, including anandamide, but also $\Delta 9$ THC and a number of synthetic $\mathrm{CB}_{1}$ or $\mathrm{CB}_{2}$ agonists and antagonists can activate or block different non-cannabinoid receptors with potencies that differ little from those with which they activate or block the "true" cannabinoid receptors [1]. According to nomenclature criteria of the NC-IUPHAR cannabinoid receptor subcommittee the TRPV1 channel might eventually come to be regarded as being either an "ionotropic cannabinoid $\mathrm{CB}_{3}$ receptor" or a dual TRPV1/CB $3_{3}$ receptor. In addition, some other receptors deserve further attention in this respect, namely, GPR18, GPR55, GPR119 and the peroxisome proliferator-activated receptors (PPARs) $\alpha$ and $\gamma$. Although these show little to no structural similarity to $\mathrm{CB}_{1}$ and $\mathrm{CB}_{2}$ they have been shown to respond to endocannabinoids, their endogenously present congeners and (or) plant-derived "phyto"-cannabinoids.

### 3.2.1 $\mathrm{CB}_{1}$ Receptors

$\mathrm{CB}_{1}$ receptors are presynaptically located at central or peripheral nerve terminals and act as modulators of synaptic transmission by a process which has been called retrograde signalling \{Wilson, 2002 \#4848; Cachope, 2012 \#3683; Vaughan, 2005 \#4853\}. Physiological stimulation of neurons induces the synthesis of


Fig. 9.6 Schematic representation of the mechanism of retrograde signalling by endocannabinoids at a synaptic cleft. Neuronal depolarization causes cleavage of membrane lipid precursors to induce de novo synthesis and release of endocannabinoids such as AEA, PEA, OEA and 2-AG into the synaptic cleft. These endocannabinoids activate cannabinoid $\mathrm{CB}_{1}$ receptors located on presynaptic terminals of neurons which reduces release of neurotransmitters (such as GABA or glutamate) onto the postsynaptic neuron. Endogenously released cannabinoids might also act via TRP ligand gated ion channels (e.g. TPRV1) and other GPCRs (e.g. GPR 119). Endocannabinoids are taken back up into neuronal and glial cells and then degraded by enzymes such as fatty acid amide hydrolase (FAAH) and MAG-lipase (MAGL)
endocannabinoids in the post-synaptic nerve terminal and this reduces synaptic inputs in a highly selective and restricted manner (Fig. 9.6).

The majority of $\mathrm{CB}_{1}$ receptors are coupled through $\mathrm{G}_{\mathrm{i} / \mathrm{o}}$ proteins. Their stimulation leads to inhibition of adenylate cyclase activity, effects on different $\mathrm{Ca}^{2+}$ and $\mathrm{K}^{+}$channels, and stimulation of mitogen-activated protein (MAP) kinase. In some cases $\mathrm{CB}_{1}$ receptors signal through $\mathrm{G}_{\mathrm{s}}$ proteins [1,2,80].

In contrast to what was originally assumed, the distribution of $\mathrm{CB}_{1}$ receptors is not limited to the CNS , and $\mathrm{CB}_{1}$ receptors are also found in the immune system, vascular endothelium, intestine, liver, skeletal muscles, peripheral nerve synapses and reproductive tissues. As a consequence of their localisation at the terminals of central and peripheral neurons where they mediate inhibition of neurotransmitter release, $\mathrm{CB}_{1}$ receptors are involved in a wide variety of biological processes [1] including learning and memory, anxiety, pain, eating behaviour, metabolism, reproduction and growth and development. As a result they have been associated
with different disorders and diseases (Sect. 4). For example, their involvement in food intake regulation (Sect. 4.2) takes place at different levels, starting from receptors within the GI tract to the regulation of hedonic rewarding in the brain [55, 82-84]. Its presence in peripheral tissues also provides an explanation for the sustained effects of the $\mathrm{CB}_{1}$ inverse agonist rimonabant on body weight and the improvement of insulin resistance and blood lipids, in addition to its short-term appetite-decreasing effect. On Vagal afferents $\mathrm{CB}_{1}$ expression was found to be regulated by CCK [85] and high/low fat diets [86]. Remarkably, peripheral stimulation of $\mathrm{CB}_{1}$ receptors on Vagal afferents by anandamide was shown to reduce appetite, whereas central stimulation of $\mathrm{CB}_{1}$ receptors increased food intake [87]. In the brain, the $\mathrm{CB}_{1}$ is now regarded the most abundant G-protein coupled receptor [2]. A pioneering study on its distribution in brain was published in 1990 by Miles Herkenbaum et al. [88]. More recent reviews include the following references [89, 90]. As mentioned before, the central regulation of energy intake and metabolism is one of the major functions of the "classical" ECS. Within the brain, $\mathrm{CB}_{1}$ receptors have been linked to several both homeostatic and non-homeostatic regulation mechanisms, with endocannabinoids acting as modulators of orexigenic and anorexigenic neurotransmitters and neuropeptides by presynaptic regulation of their release. The brain ECS shows numerous anatomical and functional connections with other signalling pathways including dopaminergic, opioid and GABA-ergic systems involved in pleasure and reward, pain, anxiety, fear, etc. [55, 91-95].

### 3.2.2 $\quad \mathrm{CB}_{2}$ Receptors

$\mathrm{CB}_{2}$ receptors are predominantly expressed on immune and haematopoietic cells, but functionally relevant expression has also been found in specific regions of the brain, other tissues and in various tumours. Like $\mathrm{CB}_{1}$ receptors they are coupled through $\mathrm{G}_{\mathrm{i} / \mathrm{o}}$ proteins, negatively to adenylate cyclase activity and positively to MAP kinase. Although several studies have suggested that $\mathrm{CB}_{2}$ activation is immunomodulatory and neuroprotective [96-98], some data remain inconclusive. This may be partly due to the fact that different components of the inflammatory cascade can be affected in a different direction [99]. Furthermore, discrepancies are caused by the use of different animal models, compounds and doses [100]. Diseaseinduced changes (usually increases) in $\mathrm{CB}_{2}$ receptor expression have been reported [101]. Furthermore, many synthetic $\mathrm{CB}_{2}$ receptor agonists have shown protective effects in a variety of preclinical disease models and pathological conditions (reviewed by ref. 101). Therefore, the application of selective $\mathrm{CB}_{2}$ agonists would be of interest for a number of disorders (Review: [28]). At the same time the wide abundance of $\mathrm{CB}_{2}$ receptors and the critical importance of retaining an adequate pro-inflammatory balance present challenges for their application as therapeutic targets [101]. Therefore, subtle and well-balanced approaches, including multiple targeted and/or localised therapies are likely to provide the best options [29].

### 3.2.3 Transient Receptor Potential (TRP) Cation Channels

Transient receptor potential (TRP) cation channels constitute a superfamily of receptors involved in the signal transduction of a wide range of stimuli, including effects elicited by endogenous lipids [2, 102-104]. Mammalian TRPs are subdivided into six protein families of which three are here considered of particular relevance because they bind endocannabinoids and related compounds and (or) phytocannabinoids. These are: the vanilloid receptor TRPs (TRPVs, in particular TRVP1), the melastatin or long TRPs (TRPMs, in particular TRPM8) and ankyrin transmembrane protein 1 (TRPA1).

The TRVP1 receptor is particularly known as the receptor for the vanilloid capsaicin present in red peppers. In addition it is perhaps the best established non-cannabinoid receptor for endocannabinoids, and for anandamide in particular [7, 95]. Several papers note the overlap between the ECS and what has been called "endovanilloid system" [95, 105-107]. Based on this it has been suggested to rename the TRVP1 receptor to "ionotropic cannabinoid $\mathrm{CB}_{3}$ receptor" or a dual TRPV1/CB $3_{3}$ receptor (see also Sect. 3.2). $N$-arachidonoyl-dopamine (NADA) was the first fatty acid amide shown to act as endogenous ligand of TRVP1 receptors [65]. Meanwhile several other $N$-acyl amides have also been demonstrated to activate TRPV1 [51]. TRVP1 is predominantly expressed in sensory neurons but also on non-neuronal cells including epithelial, endothelial and smooth muscle cells as well as in lymphocytes, hepatocytes and pancreatic cells [2, 5, 108]. Historically, TRPV1 has been considered a pro-inflammatory receptor in several conditions, including neuropathic pain, joint inflammation and inflammatory bowel disease. A number of TRVP1 antagonists have been developed as potential drugs against different forms of pain, but so far results in the clinic were not successful [108]. Recent evidence also demonstrates paradoxical, protective functions of TRVP1 in vivo [109]. The receptor also plays a role in energy metabolism and weight management as recently reviewed by Ahern [102]. For example, there is long-standing evidence that dietary consumption of chilli peppers can affect body weight. Treatment with capsaicin, or related "vanilloid" compounds, reduces weight gain and adiposity in animals consuming moderate to high-fat diets. An interesting finding was that the endogenous endocannabinoid congener N -arachidonoyl-serotonin (AA-5-HT) displays dual activity as both FAAH inhibitor and TRPV1 antagonist. The compound has shown marked effects against both acute and chronic peripheral pain in rodent models [110, 111]. Previous studies from our lab showed that this conjugate is particularly present in the gut, but so far its biological role has not been established [57]. In addition to TRPV1, other members of this family, including TRPV2-4 have been associated with, in particular effects of phytocannabinoids and (or) Cannabis extracts [2, 5, 112].

The TRPM8 receptor is involved in the detection of sensations such as cold. Activators include eucalyptol, menthol and icilin [113]. It is considered a therapeutic target for cold hypersensitivity and neuropathic pain [108]. Its expression was also found to be important for the survival of androgen receptor-dependent
prostate cancer cells [5]. Both anandamide and $N$-arachidonoyl dopamine, but not 2-AG, were shown to antagonise the stimulatory effect of menthol and icilin on TRPM8 [114]. In addition, several phytocannabinoids show activity on TRPM8 [112, 114].

The TRPA1 receptor is receiving increasing attention as a key regulator of neuropeptide release, neurogenic inflammation and pain. See [108, 115] for reviews. TRPA1 was found to be activated by CBD [114]. Another phytocannabinoid, cannabichromene can also act as TRPA1 agonist. The receptor was shown to be involved in the inhibition of nitric oxide production in macrophages and the amelioration of murine colitis by cannabichromene [116].

### 3.2.4 GPR119

GPR119 ([117] for a recent review) has been described as a class A (rhodopsintype) orphan GPCR but has no close primary sequence relative in the human genome. Two of its endogenous ligands discovered so far are the fatty acid amides oleoylethanolamide (OEA) and $N$-oleoyldopamine (OLDA). Furthermore the receptor can be activated, albeit with less potency, by PEA, EAE and linoleylethanolamine (LEA) [2, 118, 119]. As none of these compounds are ligands for $\mathrm{CB}_{1}$ or $\mathrm{CB}_{2}$ receptors, GPR119 is not considered a cannabinoid receptor per se [2]. Recently, GPR119 has also been found to respond to 2-oleoyl-glycerol, a compound formed out of common dietary triglycerides (described in Sect. 2.3). Following its de-orphanization in 2006 by Overton et al. [120] and the demonstration that small molecule agonists are able to reduce body weight gain in rodents, GPR119 has attracted considerable attention. The receptor is G $\alpha$ s-protein coupled and predominantly expressed in pancreatic islets and gastrointestinal tract in humans and rodents. GPR119 agonists were found to increase intracellular cAMP, which in turn leads to increased GLP-1 secretion from entero-endocrine cells. Following the synthesis of the first ligands, including PSN632408 and AR-231,453 several pharmaceutical companies became active in developing GPR119 agonists. Many of these compounds have shown interesting activities in animal models of type 2 diabetes and obesity, including a reduction of blood glucose without causing hypoglycaemia, a reduction of food intake and body weight, and reduced diabetes progression. Presently, a number of GPR119 agonists are in advanced stages of development [121].

### 3.2.5 GPR 55

The discovery of the orphan GPCR GPR55 was first described in 2007 [122]. The receptor was shown to bind some $\mathrm{CB}_{1}$ and $\mathrm{CB}_{2}$ ligands. Therefore, it has been considered a "novel" or "third" cannabinoid receptor for some time, but this viewpoint has been abandoned. Structurally the receptor has no significant sequence similarity with the CB receptors, in particular not in the areas responsible for ligand binding [2]. GPR55 is expressed in the gut and found in cells of the
immune system, including microglia in brain as well as in endothelial cells [123]. A recent study suggested that GPR55 regulates $\mathrm{CB}_{2}$ function in human neutrophils [124]. Following the report of Oka et al. [125] it is now assumed that its endogenous ligand is lysophosphatidylinositol (LPI). It was suggested by Ross [126] that LPI and GPR55 might play a role in driving cancer cell proliferation and migration. The phytocannabinoid CBD shows antagonist activity towards GPR55, which may of therapeutic relevance [127].

### 3.2.6 GPR18

The GPR18 gene was first cloned in 1997 [128] and at that time found to be highly expressed in human testis and spleen. In addition, its presence was shown in thymus, peripheral white blood cells and in the small intestine, whereas in many other tissues and organs it appeared to be absent. McHugh et al. [129] demonstrated that NaGly ( N -arachidonoylglycine, see Sect. 3.1) serves as an endogenous ligand. The same group also reported that two cannabinoid agonists, AEA and THC, are full agonists at GPR18, whereas CBD displays low efficacy as agonist [130]. Considering its location on microglia cells [131] and on peripheral macrophages, GPR18 and its endogenous ligand(s) are receiving increasing attention in relation to inflammation.

### 3.2.7 Peroxisome Proliferator-Activated Receptors (PPARs)

PPARs can be activated by some non-cannabinoid NAEs including OEA and PEA. The same has been shown for some 2-AG derivatives of the COX/LOX/CYPP450 pathways, and to a lesser extent also for AEA and 2-AG itself. PPARs are ligandactivated transcription factors that play critical roles in very different biological pathways such as lipid, protein, glycerol, urea, glucose, glycogen, and lipoprotein metabolism, adipogenesis, trophoblast differentiation and cell migration. For recent reviews see for example [132, 133]. Their best known agonists are various fatty acids and their derivatives. Therefore, PPARs are commonly regarded as generalnot very selective-lipid sensors monitoring local metabolic changes. The PPAR family consists of PPAR $\alpha, \operatorname{PPAR} \beta$ and PPAR $\gamma$. The three PPAR iso-types are similar in homology, but show their own distribution pattern. In humans PPAR $\alpha$ is localised in areas of high fatty acid catabolism (kidneys, liver, heart, brown adipose tissue and intestines). PPAR $\gamma$ is found as two isoforms: PPAR $\gamma 1$ (predominantly present in gut, brain, vascular cells and immune cells) and PPAR $\gamma 2$ (mainly in adipose tissue). PPAR $\beta / \delta$ has been found in many tissues and is particularly highly active in skeletal muscle, smooth muscle and skin [132].

The role of the PPAR $\alpha$ receptor as a pivotal switch in different inflammatory and pain signalling pathways in the CNS and periphery is widely acknowledged [132, 134, 135]. Two well-known $N$-acylamides that are linked to this PPAR are PEA and OEA. For PEA (see also Sect. 4.3.2) it is assumed that its anti-inflammatory activity
can largely be assigned to an agonist activity on PPAR $\alpha$ [135-137]. PPAR $\alpha$ is also playing a pivotal role in the satiety-inducing effects of OEA [138]. This NAE is formed form oleic acid in the epithelium of the proximal small intestine. PPAR $\gamma$ serves as the molecular target for the thiazolidinediones, an important class of antidiabetic drugs. Its major natural ligands and activators are PUFAs and fatty acidderived molecules. The beneficial action of PPAR $\gamma$ has typically been attributed to increased insulin sensitivity and reduced inflammation. Agonism of PPAR $\gamma$ is increasingly considered an important property of the phytocannabinoid CBD (Sect. 2.1). PPAR $\gamma$ and CBD are also receiving attention in relation to CNS diseases like Alzheimers' disease because of the role of PPAR $\gamma$ in stimulating microglial function [139, 140].

### 3.3 Interactions of Endocannabinoids with Non-receptor Targets

Several studies suggest that the biological activities of at least some of the endocannabinoids and their congeners are not exclusively mediated through GPCRs or nuclear receptors. An example comes from the anti-inflammatory effects of N -docosahexaenoylethanolamine (DHEA, Fig. 9.5), the ethanolamine conjugate of DHA (docosahexaenoic; 22:6n-3). Its concentration in animal tissues and human plasma increases when diets rich in fish or krill oil are consumed. Comparing a series of NAEs from n-3 and n-6 LC-PUFAs, we found DHEA to be the most potent anti-inflammatory compound in LPS-stimulated RAW264.7 macrophages [141]. Later studies suggested that anti-inflammatory effects of DHEA are at least partly independent from $\mathrm{CB}_{1}, \mathrm{CB}_{2}$ or PPARy receptors and probably take place via inhibition of eicosanoids produced through COX-2 [56]. Interestingly, DHEA was also reported to inhibit growth of prostate and breast cancer cell lines which was at least partly independent from $\mathrm{CB}_{1}$ or $\mathrm{CB}_{2}$ interaction [142, 143]. Similarly, DHEA was shown to stimulate neurite growth, synaptogenesis and glutamatergic synaptic activity in developing hippocampal neurons via (at least) cannabinoid receptorindependent mechanisms [144]. Another example is $N$-arachidonoyl dopamine (NADA). Like DHEA, NADA was found to be potent inhibitor of PGE2 synthesis in lipopolysaccharide (LPS) stimulated primary glia cells [145, 146].

## 4 Endocannabinoids and Targets in Disease

### 4.1 General Aspects, Targets and Examples

The broad involvement of the endocannabinoidome in various biological processes and its many connections with other systems in terms of ligands, receptors and
metabolic pathways explains why it is has been associated with so many disorders and diseases. However, it should be noted that associations with pathologies like those mentioned in Table 9.1 do not imply that suitable targets for prevention or intervention are at an easy reach. On the contrary, its wide abundance and high degree of pleiotropy present serious challenges to develop efficacious and specific drugs. It has also become clear that initial strategies to modulate the ECS have probably been too narrow and expectations too high. Two well-known examples in this respect are the experiences thus far with $\mathrm{CB}_{1}$ inverse agonists (Sect. 4.2) and FAAH blockers (Sect. 4.3). Furthermore, it is also clear that changes in the expression of certain receptors or ligands are often the result of other (patho-) physiological processes instead of being part of a modifiable cause of a disease. As can be seen from the list of disease areas of interest (Table 9.1) many of these are of a chronic and multifactorial character. It is increasingly acknowledged that such disorders are often better managed by multiple target strategies, instead of a "one disease-one target" approach. This involves the use of promiscuous drugs or targeted drug (of drug-food) combinations [147]. These developments stimulated by the evolution of "omics" technologies, system biology and bioinformatics and the endocannabinoidome lends itself well for such an approach [148]. Table 9.1 lists a non-exhaustive overview of disease areas of interest. In the next sections, two of these are further elaborated viz weight management (Sect. 4.2) and inflammation (Sect. 4.3). For other field readers are referred to the literature.

### 4.2 The Endocannabinoidome in Weight Management

The modulation of food intake and energy metabolism is generally considered one of the most pivotal roles of the ECS. It has also been the most intensively studied topic in this field, in particular until 2008 when the withdrawal of rimonabant caused a dramatic change. The ECS modulates food intake and energy metabolism at different levels, starting from $\mathrm{CB}_{1}$ receptors within the GI tract to the regulation of hedonic rewarding of foods in the brain [82-84, 190, 191]. From an evolutionary perspective it is thought that one of its main functions is as a pleiotropic regulator of energy uptake and storage and of non-homeostatic eating behaviour [192, 193]. In the past these mechanisms were biologically advantageous in order to survive periods of food shortage [194]. The discovery of the high abundance of $\mathrm{CB}_{1}$ in brain, and the observation that $\mathrm{CB}_{1}$ antagonists and reverse agonists induce a reduction of appetite and food-intake in animals fuelled an enormous activity of research in academia and industry, resulting in the market introduction of rimonabant 2006. Expectations, therapeutic and financial, were very high. The failure of rimonabant because of depression-related side-effects [13] shocked the research community and the pharmaceutical industry. By the end of 2008 at least nine companies terminated active development projects of $\mathrm{CB}_{1}$ blockers. Next to rimonabant, which has been on the market in Europe but not in the USA, several related compounds were in advanced stages of development, including taranabant

Table 9.1 Non-exhausting overview of main disease areas in which the endocannabinoidome is of potential interest

| Obesity and metabolic syndrome | See Sect. 4.2 |
| :--- | :--- |
| Cardiovascular disorders | $[149-154]$ |
| CNS disorders ${ }^{\text {a }}$ | $[155-161]$ |
| Neurodegenerative diseases (general) | $[162,163]$ |
| Alzheimer's disease | $[164,165]$ |
| Trauma/brain injuries | $[166,167]$ |
| Cancer | $[5,32,168-171]$ |
| Intestinal diseases $_{\text {Inflammation }^{\text {b }}} \quad[30,172-178]$ |  |
| Pain | Section 4.3 |
| Skin diseases | $[29,136,148,179-182]$ |
| Liver diseases | $[183-185]$ |

${ }^{\text {a }}$ Here used as a collective term for various disorders (psychosis, stress, anxiety, fear, addiction); only a few references are mentioned
${ }^{\mathrm{b}}$ Inflammation in a general sense. Often there are links with (chronic) pain
${ }^{\mathrm{c}}$ Here used as a collective term for different forms of pain (nociceptive, hyperalgesia, neuropathic pain)
(Merck), surinabant (Sanofi) and CP-945,598 (otanabant, Pfizer). In the meantime it has become clear that $\mathrm{CB}_{1}$ receptors are also abundantly present outside the CNS [12, 191]. In fact, it is now assumed that the central effects of rimonabant are responsible for the short-term reduction of food-intake, whereas the more sustained effects on body weight and the improvement of insulin resistance and blood lipids are largely due to its peripheral actions. In the gut, CB receptors show a specific distribution, being largely distributed in the enteric nervous system (ENS) [178]. Both $\mathrm{CB}_{1}$ and $\mathrm{CB}_{2}$ receptors are found on enteric neurons, nerve fibres and nerve terminals in the ENS. The $\mathrm{CB}_{1}$ receptor is found on nerve fibres throughout the wall of the gut, but with the highest density in the two ganglionated plexuses, the myenteric and submucosal plexus, of the ENS. $\mathrm{CB}_{1}$ expression on Vagal afferents was found to be regulated by CCK [85] and high/low fat diets [86]. Stimulation of central $\mathrm{CB}_{1}$ receptors, for example by anandamide has been shown to increase food-intake. Remarkably, stimulation of $\mathrm{CB}_{1}$ receptors on Vagal afferents seems to do the opposite [87].

Notwithstanding the failure of rimonabant and other $\mathrm{CB}_{1}$ blockers/inverse agonists, $\mathrm{CB}_{1}$ receptors remain of interest as a pharmacological target. The presence of $\mathrm{CB}_{1}$ receptors outside the CNS offers possibilities for treatment of type 2 diabetes and other complications of the metabolic syndrome. To improve tissue specific activity and reduce CNS side-effects so-called peripherally restricted $\mathrm{CB}_{1}$ antagonists are under investigation [12, 28, 121, 191, 195]. Furthermore, the use of $\mathrm{CB}_{1}$ neutral antagonists or partial agonists as opposed to inverse agonists such as rimonabant has been proposed as a strategy [191].

As described in Sect. 2.1 there exist also (at least) one natural weak $\mathrm{CB}_{1}$ antagonist, THCV from Cannabis which might offer possibilities in this respect [17, 41, 42].

In addition to CB receptors, related receptors may offer interesting targets in weight management, including TRVP1 (Sect. 3.2.3) and GPR119 (Sect. 3.2.4).

Although not discussed in further detail are the possibilities to target the ECS in order to increase appetite or food-intake in general. The use of Cannabis preparations in AIDS and cancer patients for this purpose has already been introduced in Sect. 2.1. The ECS is also receiving interest in relation to eating disorders like anorexia and bulimia nervosa [161, 196, 197].

### 4.3 Inflammatory Processes

Several receptors which are modulated by endocannabinoids or their structural analogues are involved in the regulation of inflammation, pain and immunefunctions in a broad sense [100]. Of particular interest are $\mathrm{CB}_{2}$ (Sect. 3.2.2), TRVP1, TRPA1 and other TRP cation channels (Sect. 3.2.3), GPR18 (Sect. 3.2.6) and PPARs (Sect. 3.2.7), and this list is likely to increase. Furthermore, a number of endocannabinoids per se (Anandamide, 2-AG) and related compounds (PEA, SEA, OEA, DHEA, etc.) have shown anti-inflammatory and (or) immune modulating properties. Finally, the endocannabinoidome is deeply intertwined with other important lipid-based signalling systems including those regulated by COX and LOX. On the one hand, this broad involvement offers several potential targets for intervention. On the other hand, this complexity provides challenges in terms of specificity and side-effects. Some examples will be highlighted in this Section.

### 4.3.1 Modulators of Endocannabinoid Turnover

Inhibition of enzymes involved in the synthesis or breakdown of endocannabinoids, in particular DAGL, MAGL, FAAH or NAAA ( $N$-acylethanolamine acid amidase) has been considered a manner to modulate inflammation and (or) pain. Diacylglycerol lipases (DAGL $\alpha$ and DAGL $\beta$ ) are involved in the synthesis of 2-AG. Inhibition of DAGL $\beta$ has been found to lower 2-AG, as well as AA and eicosanoids, in mouse peritoneal macrophages in a manner that was distinct and complementary to disruption of cytosolic phospholipase-A2 [198]. Mono-acyl glycerol lipase (MAGL) catalyses the hydrolysis of 2-AG to arachidonic acids (AA). Inhibition of peripheral MAGL in rats using the selective MAGL inhibitor JZL184 was found suppressed LPS-induced circulating cytokines which in turn was suggested to modulate central cytokine expression [199]. In brain, AA formed by hydrolysis of 2-AG has been shown to serve as pool for pro-inflammatory eicosanoid synthesis, thus representing another crossroads between endocannabinoid and eicosanoid pathways [81]. MAGL-disrupted mice displayed neuroprotection in a model for Parkinson's disease but showed no haemorrhaging in the gut as seen with COX inhibitors [200]. Inhibition of Fatty Acid Amide Hydrolase (FAAH) aiming to increase fatty amide levels has also been considered as intervention strategy in
inflammation and (or) pain. A number of animal studies, for example with the inhibitor URB597, indeed showed reduction of inflammatory pain or modulation pro-inflammatory gene induction, although results were not always unambiguous [201, 202]. It has been suggested that inactivation of FAAH can modulate 2-AG tissue levels as well, either up or down, depending on the location [203]. Studies in human volunteers with FAAH inhibitors confirmed increased NAE levels, including that of AEA, OEA and PEA [204]. However, in a recent phase II clinical trial in patients with osteoarthritic knee pain the FAAH inhibitor PF-04457845 failed to show any effect [205, 206]. As FAAH activity was strongly inhibited and plasma NAE concentrations consistently elevated, it was suggested that alternative targets and pathways for breakdown might have counteracted the potentially beneficial effects of elevated anandamide levels on pain and inflammation [207]. Inhibition of NAAA provides an alternative approach to increase levels of for example PEA and OEA. Recently, the selective NAAA inhibitor ARN077 has been found to inhibit hyperalgesia and allodynia caused by inflammation or nerve damage [208]. Interestingly, the antinociceptive effects of ARN077 were prevented by the selective PPAR- $\alpha$ antagonist GW6471 and did not occur in PPAR- $\alpha$ knockout mice.

### 4.3.2 Endocannabinoid Congeners as Potential Anti-inflammatory Compounds

Several individual endocannabinoids, fatty amides and phytocannabinoids have been demonstrated to possess anti-inflammatory properties [100]. The n-3 LC-PUFA derived N -docosahexaenoylethanolamine (DHEA, Fig. 9.5) has already been described in Sect. 3.3. The same holds true for the Cannabis-derived compound CBD (Sect. 2.1). An interesting compound which is receiving increasingly attention is $N$-Palmitoylethanolamide (PEA, Fig. 9.5), an endogenous NAE originating from palmitic acid (C16:0), the most common saturated fatty acid found in animals [209]. Earliest reports on its anti-inflammatory properties date back to 1957. PEA shows a broad diversity of receptor affinities, including interactions with PPAR $\alpha$, GPR55 and TRVP1, as well as indirect activity via an "entourage" effect [137, 210]. The latter refers to a mechanism in which PEA reduces the enzymatic breakdown of AEA through competition for FAAH, resulting in higher AEA concentrations [211, 212]. The compound is presently receiving attention as potential drug or nutraceutical against chronic pain, (neuro-)inflammation and degenerative diseases of the central nervous system [137, 167, 209, 213, 214]. Increasing evidence indicates that non-neuronal cells within the CNS are crucially involved in mediating the effects of PEA [137, 215, 216]. These non-neuronal cells regulate inflammatory processes in the CNS and are key players in the communication between the immune system and the CNS during neurodegenerative disorders and in neuropathic pain. The C18 homologue of PEA, $N$-stearoyl ethanolamine (SEA) has also been associated with anti-inflammatory effects but this compound has been far less investigated [217].

## 5 Conclusions and Perspectives

More than two decades of research have changed our early view of the ECS. Initial expectations on the possibilities to develop new drug classes based on its key molecular targets have proven to be too high. It is now obvious that the "prototypical" ECS is deeply intertwined with other important signalling systems. Endocannabinoids have numerous bioactive congeners and metabolites, which often show "promiscuous" behaviour towards their receptors and other targets. This so-called endocannabinoidome is modulated by various endogenous (e.g. energy status, inflammation) and environmental factors in a time- and tissuespecific manner. The complexity and dynamics of the endocannabinoidome presents technical challenges and its understanding and modulation demands for a systems biology approach. At the same time the endocannabinoidome still holds many promises for both "food" and "pharmaceutical" applications as it is crucially involved in many disorders. Chronic diseases often involve tissue degeneration and remodelling, inflammation and pain, and are orchestrated by different interacting metabolic processes in which the "expanded" ECS is centrally involved.

Significant progress in their prevention and modulation is likely to come from a paradigm shift as it is currently taking place in the discovery and development process of drugs and nutritional products. These involve more subtle multiple-target strategies instead of a classical one disease-one target-one drug approach.

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# Chapter 10 <br> Effects of Natural Products on Pharmacokinetics and Pharmacodynamics of Drugs 

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## 1 Introduction

The popularity of dietary supplements and natural products is rapidly increasing in terms of the promotion of health and the prevention and treatment of diseases. Herbal ingredients have received a great deal of attention in complementary and alternative medicine and are used as dietary supplements or natural products in many countries. Herbal ingredients are perceived as safe because they are natural and have been used for centuries in Asian cultures. Elderly people frequently take dietary supplements and natural products with prescription drugs, and this will increase in the near future. A major concern is the adverse events caused by a large excess intake or the interactions of dietary supplements and natural products (including beverages such as fruit juices and green tea) with drugs. The potential for their interaction with drugs is considerable because a large number of constituents are contained in dietary supplements and natural products [43, 117]. Possible changes may occur in the pharmacokinetics and pharmacodynamics of drugs: absorption in the small intestine, metabolism in the intestine and liver, distribution to target organs, transport across the cell membrane, and binding to specific

[^9]

Fig. 10.1 Schematic representation of pharmacokinetic and pharmacodynamic interactions between the ingredients of functional foods or herbs and drugs
receptors (Fig. 10.1). The induction and inhibition of hepatic drug-metabolizing enzymes by herbal ingredients or dietary compounds have been investigated. For example, St. John's wort, a herbal medicine used to treat mild depression, has been shown to decrease the blood concentrations of drugs by inducing hepatic cytochrome P450 (CYP) 3A4 activity and thereby attenuates the efficacy of drugs such as cyclosporin, indinavir, and digoxin [5, 27, 89] (Fig. 10.2). Furthermore, ginkgo biloba extract (GBE) and saw palmetto extract (SPE) are commonly prescribed in some European countries for the treatment of cerebral insufficiency and peripheral vascular diseases [51,67] and reduce the symptoms of benign prostatic hyperplasia (BPH) [28], respectively. Coleus forskohlii extracts (CFE) contain the diterpene forskolin, an activator of adenylate cyclase, and are expected to have various therapeutic [7, 6] and weight loss effects [41, 35]. These herbs are used as dietary supplements and natural products in the USA and Japan. Common beverages such as fruit juice, green tea, and cranberry juice have been reported to affect the pharmacokinetics and pharmacodynamics of drugs [71, 81, 104, 115].

This chapter focuses on the possibility of the pharmacokinetic and pharmacodynamic interactions of GBE, SPE, CFE, grapefruit juice, and green tea with drugs.

## 2 Ginkgo Biloba Extract

### 2.1 Induction of CYP by GBE

GBE is one of the most popular herbal ingredients and is used to improve cognitive function and peripheral arterial disease [96]. Recent randomized control trials failed


Fig. 10.2 Effects of the intake of St. John's wort (SJW) on plasma concentrations of cyclosporine and its immunodepressant effect in patients with renal transplantation (cited from Barone et al. Ann. Pharmacother. 34: 1013, 2000)
to confirm the effectiveness of GBE in reducing the incidence of dementia in elderly individuals with normal cognition or mild cognitive impairment [19, 97]. Nevertheless, GBE has remained popular among the elderly. As elderly people frequently take prescription drugs with dietary supplements [74], GBE-drug interactions may represent a major concern.

GBE is a natural plant product containing many chemicals. Most commercially available GBE products are standardized according to the amount of ginkgo flavonol glycosides (glycosidic derivatives of quercetin, kaempferol, and isorhamnetin) and terpenoids (ginkgolides A, B, C, and bilobalide), which comprise $22-27 \%$ and approximately $5-7 \%$ of GBE, respectively, and less than 5 ppm of ginkgolic acid [10]. GBE products also contain $0.5-1 \%$ of organic acids, such as vanillic acid and $p$-hydroxybenzoic acid. The exact constituents of GBE may vary among products due to the time and place of harvest and the extraction methods used.

In pharmacokinetic studies with rats and mice, GBE induced the expression of hepatic drug-metabolizing enzymes, particularly CYP, in a dose- and timedependent manner (Fig. 10.3) without causing hepatic damage [110, 111]. Significant increases in the concentrations and activities of CYP enzymes were detected on day 1 of feeding of a $0.5 \%$ GBE diet and after the administration of 10 mg GBE/kg body weight for 5 days by intragastric gavage. The human equivalent dose, determined by the body surface normalization method [85], is $1.62 \mathrm{mg} / \mathrm{kg}$ body weight, which is approximately 100 mg GBE/60 kg body weight and within the recommended dose range (up to 240 mg ) taken from dietary supplements. Among


Fig. 10.3 Dose-dependent changes in hepatic CYP activities in rats administered various doses of GBE. Rats were orally administered GBE at doses of $0,1,10,100$, and $1,000 \mathrm{mg} / \mathrm{kg}$ body weight for 5 days. Subtypes of CYP enzymes were determined by HPLC. Each column represents the mean $\pm$ S.E. *Significantly different from untreated controls, $\mathrm{p}<0.05$ (cited from Umegaki et al. Jpn J Pharmacol. 90: 345-351, 2002)
the CYP enzymes, the activity of pentoxyresorufin O-dealkylase, a CYP2B enzyme, was markedly increased, as confirmed by Western blot analysis and expression of mRNA. GBE also increased CYP2B1/2, CYP3A1, and CYP3A2 mRNA levels and related CYP activities in the rat liver [95, 110]. A similar induction of hepatic CYPs by GBE in rats was observed with EGb761, a standardized GBE extract [14, 127].

It is important to identify which substances in GBE are involved in the induction of CYPs. In vitro and in vivo studies revealed bilobalide to be a major substance inducing hepatic CYPs [13, 20, 91, 107, 111] (Fig. 10.4). Although the contribution of bilobalide is unclear, GBE activated mouse and human PXR, a nuclear receptor involved in the transcriptional regulation of drug-metabolizing enzymes and transporters [121]. The reported half-life of bilobalide in the blood is approximately 2 h in rats and humans [9, 66], indicating that it is eliminated easily from the blood. A single dose by gavage of bilobalide ( $30 \mathrm{mg} / \mathrm{kg}$ ) in rats was found to produce a timedependent induction of hepatic CYP activity and protein expression, and mRNA expression of CYP2B, which was maximal at 6 h and showed a similar response to


Fig. 10.4 Content of hepatic CYPs in mice given bilobalide or GBE containing an equivalent amount of bilobalide. Mice were administered either bilobalide ( $10.5,21,42 \mathrm{mg} / \mathrm{kg}$ ) or GBE ( $1,000 \mathrm{mg} / \mathrm{k} ; 42 \mathrm{mg} / \mathrm{kg}$ as bilobalide) for 5 days. Each value is expressed as the mean $\pm$ S.D. for five mice. *Significantly different from untreated controls, $p<0.05$. NS: Not significantly different from GBE ( $1,000 \mathrm{mg} / \mathrm{kg}$ ) (cited from Umegaki et al. J Pharm Pharmacol. 59: 871-877, 2007)
that exhibited by plasma and liver bilobalide concentrations [106]. These findings suggested that bilobalide markedly induced CYPs; however, the induction was quickly turned off due to bilobalide's rapid elimination from the liver. The rapid recovery of CYPs was confirmed in rats given excess GBE [100]; continuous and excess feeding of GBE (approximate dose: 500 mg GBE/kg and 21 mg bilobalide/ kg ) for 1 week to rats markedly induced hepatic CYPs, while discontinuation of the treatment led to normal levels of CYPs within 1 week. These findings suggest that interactions with drugs could be avoided by discounting the GBE treatment.

### 2.2 CYP-Mediated Interactions

The induction of CYP by GBE suggested an interaction with various drugs. In rats, GBE at $0.5 \%$ in the diet for 2 weeks increased hepatic CYPs and reduced the hypotensive effect of nicardipine, which is metabolized by CYP3A, with a decrease in the maximal nicardipine plasma concentration $\left(C_{\max }\right)$ and 23-h area under the curve $\left(\mathrm{AUC}_{0-23}\right)$ [53]. Similarly, 0.5 and $1.0 \%$ GBE diets given to rats for 2 weeks shortened the sleeping time of phenobarbital, which is known to be metabolized by CYP2B, with a reduction in the maximal phenobarbital plasma concentration ( $C_{\max }$ ) and 24-h area under the curve $\left(\mathrm{AUC}_{0-24}\right)$ [54]. The interaction of GBE with tolbutamide, an oral antidiabetic agent, was also detected in young and aged rats, where a 5-day pretreatment with a $0.1 \%$ GBE diet attenuated the hypoglycemic action of tolbutamide and corresponded well to the enhanced activity of


Fig. 10.5 Effects of a simultaneous treatment and 5-day pretreatment with GBE on the hypoglycemic effect of tolbutamide in young (a) and old (aged) (b) rats. Young rats ( 7 weeks old) or old rats ( 19 months old) were administered tolbutamide ( $40 \mathrm{mg} / \mathrm{kg}$, p.o.) with or without GBE treatment. The GBE-pretreated group was given feed containing $0.1 \%$ GBE for 5 days, and the simultaneous GBE-treated group was given a single dose of GBE ( $100 \mathrm{mg} / \mathrm{kg}$, p.o.) with tolbutamide. After the administration of tolbutamide, blood was collected for the analysis of blood glucose concentrations. Each point represents the mean $\pm$ S.D. for six rats. Filled circles: control group; filled triangles: GBE-pretreated group; and open squares: GBE-simultaneous-treated group. Each point is expressed as the mean $\pm$ S.D. for five rats. *Significantly different from controls, $p<0.05$ (cited from Sugiyama et al. Life Sci. 75: 1113-1122, 2004)
(S)-warfarin 7-hydroxylase, which is a CYP2C subtype and a major isoform metabolizing tolbutamide [99] (Fig. 10.5). It is noteworthy that the interaction of GBE with tolbutamide was clearly observed in aged rats, which have a lower basal activity level of CYP subtypes in the liver, while induction by the GBE treatment was greater than that in young rats. The effects of GBE on the pharmacokinetics and pharmacodynamics of tolbutamide were significantly enhanced in rats maintained on a low-protein diet [105]. In mice, GBE interacted with (s)-warfarin through the induction of hepatic CYP2C by bilobalide, which resulted in increased warfarin metabolism, thereby decreasing the concentration of warfarin and its anticoagulant action [107] (Fig. 10.6).

Species differences exist for drug-metabolizing enzymes; thus, it is important to investigate whether GBE has the potential to interact with drugs in humans at the current recommended doses. In contrast to studies with rats and mice, reports of GBE-drug interactions in clinical studies are inconsistent; some show interactions [87, 108], while others do not [26, 40, 45, 59, 125]. The intake of GBE ( $240 \mathrm{mg} /$ day for 28 days) slightly decreased midazolam's $\mathrm{AUC}_{0 \text {-infinity }}$ and $C_{\text {max }}$ in 14 healthy subjects, indicating the interaction of GBE with CYP3A4 drugs [87]. The intake of GBE at $360 \mathrm{mg} /$ day for 28 days slightly lowered the area under the concentration versus time curve ( $\mathrm{AUC}_{0-\text { infinity }}$ ) of tolbutamide and blood glucose-lowering effect of tolbutamide in healthy male volunteers [108]. On the other hand, GBE $400 \mathrm{mg} /$ day for 13 days did not influence the elimination half-life of antipyrine in a human study [26]. The administration of GBE of $240 \mathrm{mg} /$ day for 28 days to healthy subjects caused no alteration in the activities of CYP3A4, CYP1A2, CYP2E1, or


Fig. 10.6 Effects of the GBE pretreatment on changes in anticoagulation parameters induced by (S)-warfarin or (R)-warfarin. Mice were orally administered GBE ( $100 \mathrm{mg} / \mathrm{kg}$ ) for 5 days and (S)warfarin or (R)-warfarin at a dose of $0.75 \mathrm{mg} / \mathrm{kg}$ for the last 3 days of the 5 -day regimen. The coagulation parameters shown are prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombotest (Owren) (TTO). Each column represents the mean $\pm$ S.E. for 5-6 mice (cited from Taki et al. Phytomed. 19: 177-182, 2012)

CYP2D6 assessed using a cocktail of specific substrates for individual CYPs [40]. A 7-day pretreatment with the recommended doses of GBE did not influence the pharmacokinetics or the pharmacodynamics of warfarin in 12 healthy male subjects [45]. The intake of GBE of $240 \mathrm{mg} /$ day for 12 days did not affect the pharmacokinetics of voriconazole, a substrate of CYP2C19, in Chinese volunteers genotyped as either CYP2C19 extensive or poor metabolizers [59]. No relevant effect of GBE for the major CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A) was observed following an 8-day pretreatment with GBE at $240 \mathrm{mg} /$ day in 18 healthy men and women [125].

According to the above reports, the interaction of GBE with drugs appears to be slight. In a study of GBE-atorvastatin interactions, treatment with 360 mg of GBE daily for 14 days slightly decreased plasma atorvastatin concentrations, but had little significant effect on its cholesterol-lowering efficacy [38]. The different influences of GBE-drug interactions between humans and rats may be due to species differences in CYPs, the dose of and intake periods of GBE, and the amount of the active substance that induces CYPs.

One of the most concerning adverse events associated with GBE is bleeding, which has been reported in those simultaneously taking GBE and anticoagulant drugs such as aspirin and warfarin [109]. Although an in vitro study showed the PAF antagonistic action of ginkgolide B [18, 57], human studies failed to demonstrate enhanced bleeding by the intake of GBE [52, 55, 107]. In animal studies, GBE attenuated rather than promoted the anticoagulant action of warfarin through the induction of hepatic CYPs by bilobalide [107]. Nevertheless, careful observations for bleeding and hemorrhage and interaction with drugs related to GBE-containing products are needed in clinical practice because of individual differences in sensitivity.

Fig. 10.7 Effects of the repeated treatment ( 320 mg / day, 14 days) with saw palmetto on the plasma concentration of alprazolam (CYP3A4 activity) in normal volunteers (cited from Markowitz et al. Clin Pharmacol Ther 74, 536-542, 2003)


## 3 Saw Palmetto Extract

The ripe berries of the American dwarf palm (Serenoa repens) have been traditionally used to treat genitourinary problems; to enhance sperm production, breast size, or libido; and as a mild diuretic [28]. SPE is almost exclusively used to treat BPH. Fujino et al. [32] showed that the repeated oral administration of SPE in rats had little significant influence on the content and activities of hepatic drugmetabolizing enzymes. Markowitz et al. [65] reported that SPE ( $320 \mathrm{mg} /$ day for 14 days) for the treatment of lower urinary tract symptoms (LUTS) suggestive of BPH did not alter the plasma concentrations of probe drugs for CYP2D6 and CYP3A4 activities in normal volunteers (Fig.10.7). No effect of the repeated treatment with SPE ( $160 \mathrm{mg} /$ day for 28 days) was shown using each probe drug for CYP1A2, CYP2D6, CYP2E1, and CYP3A4 [39]. Therefore, it is unlikely that SPE at generally recommended doses alters the disposition of co-administered drugs.

SPE has been shown to significantly improve urinary dysfunction possibly through the direct action of drug targets such as pharmacological $\alpha_{1}$-adrenoceptors and muscarinic cholinoceptors in the prostate and bladder [79, 101, 102]; and thus, the combination of SPE and medicines ( $\alpha_{1}$-blockers or antimuscarinics) may be advantageous in terms of a reduction in the dosage, cost, and adverse effects of drugs with pharmacodynamic interactions.

## 4 Coleus forskohlii Extract

Coleus forskohlii is a member of the mint family and is native to India [8], where it has been used for centuries in Ayurvedic medicine to treat various diseases of the cardiovascular, respiratory, gastrointestinal, and central nervous systems
[1]. Extracts of C. forskohlii (CFE) roots contain the diterpene forskolin, which increases cAMP concentrations via the activation of adenylate cyclase, resulting in various therapeutic effects against asthma and idiopathic congestive cardiomyopathy [6, 7]. Theoretically, an increase in cAMP induced by forskolin should enhance lipolysis, leading to elevated fat degradation and physiological fat utilization, thereby promoting fat and weight loss. CFE standardized with forskolin was shown to induce favorable effects on body fat in overweight women and obese men [35, 41]. Thus, CFE standardized with $10 \%$ forskolin is a popular herbal ingredient for commercial weight-loss dietary supplements.

Feeding mice a diet containing CFE (standardized with $10 \%$ forskolin) was clearly shown to dose and time dependently induce hepatic CYP enzymes such as CYP2B, CYP2C, and CYP3A [112]. Significant induction was observed at a CFE dose of $60 \mathrm{mg} / \mathrm{kg}$ body weight in mice, which corresponded to approximately $5 \mathrm{mg} /$ kg body weight of a human equivalent dose when calculated using the body surface normalization method [85]. Furthermore, CFE also induced hepatic steatosis in mice, although the effective dose was ten times higher than the dose that induced CYPs [113]. CFE is composed of various substances; however, forskolin was not involved in CYP activation or hepatic steatosis [112,113], indicating the contribution of unidentified substances. A study of the solvent fractionation of CFE revealed that the unidentified substances involved in CYP induction were mainly distributed in the diethyl ether fraction [122]. The route of CFE administration, by a diet or an intragastric gavage, did not influence the induction of CYPs as long as the CFE dose and feeding diet were the same. In addition, the level of hepatic CYP in CFE-treated groups was positively correlated with the level of starch in a semi-purified diet, which indicated that dietary starch enhanced CYP induction by CFE [124].

Activation of the nuclear receptors pregnane $X$ receptor (PXR) and constitutive androstane receptor (CAR) was shown to regulate drug-metabolizing enzymes as well as glucose and lipid metabolism [34]. Ding and Staudinger clearly demonstrated that the constituents of CFE, namely, forskolin and 1,9-dideoxyforskoiln, induced CYP3A gene expression through the PXR in cultured hepatocytes [21]. Therefore, the activation of PXR and/or CAR may be involved in the mechanism of action of CFE-induced drug-metabolizing enzymes and steatosis.

The induction of hepatic CYPs by CFE suggests the interaction of CFE with prescribed drugs. Warfarin has a powerful anticoagulant action and is metabolized by the CYP2C subfamily of enzymes, which were induced by CFE [112]. As expected, CFE pretreatment attenuated the anticoagulant action of warfarin via the induction of hepatic CYP2C in mice in vivo [123] (Fig. 10.8). CFE also directly inhibited CYP2C activity in human and mouse microsomes to a similar extent in vitro. These findings suggest the interaction of CFE and warfarin and that the intake of warfarin together with dietary supplements containing CFE increases the risk of thrombus formation. As CFE also induced CYP3A, which catalyzes $50 \%$ of prescribed drugs [86], the interaction of CFE and other prescribed drugs may also occur. Healthcare professionals should observe and communicate with patients who are receiving warfarin or other drugs metabolized by CYP2C and CYP3A while consuming dietary weight-loss supplements containing CFE.


Fig. 10.8 Effects of the Coleus forskohlii extract (CFE) pretreatment and/or warfarin administration in mice on (S)-warfarin 7-hydroxylase activities in the liver and prothrombin time in blood. Mice were fed various doses of CFE standardized with $10 \%$ forskolin for 1 week and were administered warfarin by gavage on the last 2 days of the treatment regimen (cited from Yokotani et al. J Pharm Pharmacol. 64: 1793-1801, 2012)

## 5 Grapefruit Juice

Grapefruit juice was shown to increase the bioavailability of drugs such as calcium channel blockers, benzodiazepines, and immunosuppressants [4, 3, 25, 31, 33, 56]. The main mechanism for this interaction is considered to be the irreversible inhibition of CYP3A, a major drug-metabolizing enzyme in the small intestine, by the furanocoumarins present in grapefruit juice [82]. Grapefruit juice inhibits not only metabolic enzymes but also drug transporters such as P-glycoprotein [23, 64, 104], which play important roles in the function of the intestinal barrier in a coordinated manner with CYP3A [103]. The inhibitory effect of grapefruit juice on the intestinal barrier may enhance the oral bioavailability of drugs, which has been associated with a higher incidence of side effects.

Morphine is the most commonly used opioid analgesic for the treatment of pain associated with cancer. The antinociceptive effect of morphine, a substrate of P-glycoprotein [92], was enhanced by the knockout of the P-glycoprotein gene in mice and the administration of a P-glycoprotein inhibitor in rats [60, 118, 129]. Furthermore, P-glycoprotein may be partly associated with morphine tolerance [2, 80], which limits the clinical use of morphine. We examined the effects of grapefruit juice on oral morphine antinociception and pharmacokinetics in morphine-tolerant rats [81]. Morphine tolerance was developed by the repeated oral administration of morphine for 5 days, and grapefruit juice significantly potentiated the antinociceptive effect. Morphine concentrations in blood and intrathecal cerebrospinal fluid (CSF) were gradually reduced by the repeated treatment with morphine. Grapefruit juice significantly increased the blood concentration of morphine in morphine-tolerant rats. These results suggest that grapefruit juice enhances antinociception by increasing the intestinal absorption of morphine. The inhibition
of intestinal P-glycoprotein by grapefruit juice may partly overcome morphine tolerance. However, there is absence of clinical evidence demonstrating an enhancement in the effects of morphine by grapefruit juice. In humans, the absorption of morphine is regulated by the intestinal P-glycoprotein [48]. Thus, further clinical studies are needed to examine the clinical effects of grapefruit juice on intestinal P-glycoprotein activity.

## 6 Green Tea (Catechins)

Over recent decades, green tea has been recognized as a healthy beverage for the prevention of cancer, cardiovascular disorders, and infectious diseases [47, 119] and is consumed by a large proportion of the world's population. Accordingly, it is anticipated that opportunities for the concomitant use of various drugs with green tea are increasing; thus, the evaluation of scientific evidence on possible drug interactions with green tea catechins is of importance to reduce the risks of unwanted side effects. Green tea catechin-drug interactions mediated by the inhibition or the induction of enzymes such as drug-metabolizing enzymes and drug transporters need to be considered.

Green tea (Camellia sinensis) is the most abundant source of catechins and consists of (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), $(-)$-epicatechin (EC), and (-)-epicatechin-3-gallate (ECG) [37], which accounts for $30-42 \%$ of the dry weight of the solids in brewed green tea [47]. In general, a cup of infused green tea ( 150 mL ) supplies $30-40 \mathrm{mg}$ of EGCG [42]. After the ingestion of a green tea extract containing 375 mg of EGCG, the plasma concentration of EGCG reached approximately $4 \mu \mathrm{M}$ [75].

### 6.1 Cytochrome P450 Enzymes

Many drug interactions are attributed to the inhibition or the induction of CYP enzymes [116]. The effects of green tea on the activity of CYP enzymes were first reported in rodents: drinking green tea significantly increased rat CYP1A1, 1A2, and 2B1 activities, but not CYP2E1 or 3A activities [98]. Park et al. recently demonstrated that repeated treatment with a green tea extract up-regulated CYP2B1 and downregulated CYP3A mRNA expression in the rat liver [83]. Pharmacokinetic interactions between green tea catechins and CYP substrate drugs including clozapine [44], diltiazem [61], midazolam [77], nicardipine [15], tamoxifen [94], and verapamil [17] have been studied in rats. The main findings are summarized in Table 10.1. These animal studies suggest that green tea catechins markedly inhibit CYP3A activity in the liver or the intestine and increase the plasma concentrations of its substrates. Regarding human CYP enzymes, Muto et al. reported that ECG and EGCG inhibited CYP1A1, 1A2, 2A6, 2C9, 2E1, and
Table 10.1 Effects of green tea catechins on cytochrome P-450 (CYP) enzymes in rats and humans in vivo

| CYP | Drug | Subject | Treatment | Effects | Comment | Reference |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| CYP1A2 | Clozapine, p.o. | Rats | GTE ( $175 \mathrm{mg} / \mathrm{kg}$ ), 4 days | ```Cyp1a2 protein level 2.0- fold}\uparrow\mathrm{ , C fold }``` |  | [44] |
| CYP1A2 | Caffeine, p.o. | Humans | $\text { EGCG ( } 800 \mathrm{mg} / \text { day }),$ $28 \text { days }$ | No effect |  | [16] |
| CYP2B |  | Rats | GTE (100 mg/kg), 7 days | Cyp2b1 mRNA levels 2.2fold $\uparrow$ |  | [83] |
| CYP2C9 | Losartan, p.o. | Humans | $\begin{aligned} & \text { EGCG ( } 800 \mathrm{mg} / \text { day }), \\ & 28 \text { days } \end{aligned}$ | No effect |  | [16] |
| CYP2D6 | Dextromethorphan, p.o. | Humans | Decaffeinated GTE, 14 days | No effect |  | [22] |
| CYP2D6 | Dextromethorphan, p.o. | Humans | EGCG ( $800 \mathrm{mg} /$ day ), 28 days | No effect |  | [16] |
| CYP3A |  | Rats | GTE ( $100 \mathrm{mg} / \mathrm{kg}$ ), 7 days | Cyp3a mRNA levels 0.5fold $\downarrow$ |  | [83] |
| CYP3A | Diltiazem, p.o. | Rats | EGCG (12 mg/kg) | AUC 1.8-fold $\uparrow$, BA 1.8fold $\uparrow$ | P-gp inhibition may be involved | [61] |
| CYP3A | Midazolam, p.o. | Rats | GTE (400 mg/kg), 7 days | $C_{\max } 2.1-\mathrm{fold} \uparrow, \text { AUC 3.0- }$ fold $\uparrow$ | Intestinal Cyp3a expression was decreased | [77] |
| CYP3A | Nicardipine, i.v. | Rats | EGCG (10 mg/kg) | No effect |  | [15] |
| CYP3A | Nicardipine, p.o. | Rats | EGCG (10 mg/kg) | $C_{\text {max }} 1.5-$ fold $\uparrow$, AUC 1.8fold $\uparrow$ |  | [15] |
| CYP3A | Tamoxifen, p.o. | Rats | EGCG (10 mg/kg) | $\begin{aligned} & C_{\max } 1.9-\text { fold } \uparrow, \text { AUC 1.8- } \\ & \quad \text { fold } \uparrow \end{aligned}$ | P-gp inhibition may be involved | [94] |
| CYP3A | Verapamil, p.o. | Rats | EGCG (10 mg/kg) | $\begin{aligned} & C_{\max } 2.3 \text {-fold } \uparrow \text {, AUC 2.1- } \\ & \quad \text { fold } \uparrow \end{aligned}$ | P-gp inhibition may be involved | [17] |
| CYP3A | Alprazolam, p.o. | Humans | Decaffeinated GTE, 14 days | No effect |  | [22] |
| CYP3A | Buspirone, p.o. | Humans | $\begin{aligned} & \text { EGCG ( } 800 \mathrm{mg} / \text { day }), \\ & 28 \text { days } \\ & \hline \end{aligned}$ | AUC 1.2-fold $\uparrow$ |  | [16] |

[^10]

Fig. 10.9 Effects of the ingestion of green tea on the plasma concentrations of simvastatin lactone and simvastatin acid in hypercholesterolemic patients (cited from Werba et al. Ann Internal Med. 149: 286-287, 2008)

3A4 activities in a concentration-dependent manner in a human CYP-expressing membrane fraction [72]. Contrary to its inhibitory effects, treatment with green tea extract for 6 h induced CYP1A1 and 1A2 expression and increased the mRNA levels of CYP2E1, 2D6, and 2C isoforms in human tongue cells [120]. In a clinical study, the chronic consumption of decaffeinated green tea extract did not alter either the pharmacokinetics of alprazolam, a CYP3A4 probe drug, or the metabolic ratio of dextromethorphan, an index of CYP2D6 activity, in healthy volunteers [22]. Chow and colleagues conducted a clinical trial to determine the effects of repeated green tea catechin administration on in vivo CYP activities using a drug cocktail containing caffeine (CYP1A2), dextromethorphan (CYP2D6), losartan (CYP2C19), and buspirone (CYP3A) [16]. Among the phenotypic indices investigated, only the area under the plasma concentration-time curve (AUC) of buspirone was significantly higher (by 1.2 -fold) than the baseline value by green tea catechins, suggesting that green tea caused a small reduction in CYP3A activity but had no effects on CYP1A2, 2D6, or 2C19. More recently, Werba et al. showed that green tea intake doubled the AUC of simvastatin, a cholesterol-lowering agent, and led to intense leg muscle cramps and pain in a hypercholesterolemic patient [115] (Fig. 10.9). Because simvastatin is mainly metabolized by CYP3A [76], this interaction may be due to a reduction in CYP3A activity by green tea. Further studies are required to clarify whether these interactions stem from the modulation of CYP3A activity. Collectively, green tea catechins may have an inhibitory effect on CYP3A in humans. However, considering the low bioavailability of catechins [58], the pharmacokinetic interactions of green tea catechins with CYP3A substrate drugs may mainly occur in the gastrointestinal tract, similar to grapefruit juice [68]. It also cannot be excluded that green tea catechins have an inductive effect on some CYP subtypes, especially during the chronic consumption of green tea. Further investigations of the interactions between green tea catechins and CYP
substrates including the underlying mechanisms will help toward optimal pharmacotherapy in patients who drink green tea.

### 6.2 Other Enzymes

Interactions mediated by other drug-metabolizing enzymes, such as phase II conjugating enzymes, have received less attention than CYP enzymes [49]. However, because many drugs and their metabolites undergo conjugation reactions, it is important to enhance our understanding of phase II enzyme-mediated drug interactions. Since green tea catechins undergo conjugate metabolism by UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT), and catechol- $O$ methyltransferase (COMT) in rodents and humans [29], the coexistence of green tea catechins and their substrates may cause drug interactions through the inhibition of these enzymes. Zhu et al. reported that a green tea catechin mixture and EGCG inhibited the glucuronidation of estrone in a concentration-dependent manner with $\mathrm{IC}_{50}$ values of 12.5 and $10 \mu \mathrm{~g} / \mathrm{ml}$, respectively, in rat liver microsomes [128]. Moreover, recent studies revealed that UGT1A1 and UGT1A4 activities were markedly inhibited by EGCG with $\mathrm{IC}_{50}$ values of 7.8 and $34.4 \mu \mathrm{~g} / \mathrm{mL}$ in human liver microsomes [69, 70]. EGCG showed weak inhibitory activities toward UGT1A6 and UGT1A9 [69]. On the other hand, EGCG was shown to have no effect on the mRNA expression of UGT1A1 [11]. To the best of our knowledge, there is currently no clinical evidence regarding UGT-mediated drug interactions with green tea catechins. As for the other phase II enzymes, EGCG has been reported to inhibit COMT activity with $\mathrm{IC}_{50}$ values ranging from 0.07 to $0.2 \mu \mathrm{M}$ in human liver cytosol $[63,73]$. In in vitro experiments using human recombinant SULT1A1 and SULT1A3, green tea catechins, particularly ECC and EGCG, inhibited SULT1A1 and SULT1A3 activities at around $10 \mu \mathrm{M}$ [78]. In addition to the inhibition of phase II enzymes, Golden and colleagues reported that EGCG directly reacted with bortezomib, an anticancer drug, and blocked its antiproliferative function in preclinical in vitro and in vivo models [36]. This interaction may have arisen as a result of a physicochemical interaction leading to the formation of a covalent cyclic boronate between EGCG and bortezomib. In summary, the findings described above highlight the possibility of green tea catechin-drug interactions through the modulation of not only phase II enzymes but also the chemical structures of co-administered drugs.

### 6.3 Transporter-Mediated Interactions

Many findings from in vitro and in vivo studies suggest that drug transportermediated drug interactions are of clinical importance [24]. The recognition of drug interactions that lead to negative clinical outcomes, i.e., decreased
effectiveness or tolerability, should support better medication and improve patient care. Several dietary flavonoids were found to modulate the efflux transporter, P-glycoprotein [12, 93]. Concerning green tea catechins, EGCG in green tea was shown to inhibit the efflux of drugs mediated by P-glycoprotein in vitro [46]. Qian et al. also demonstrated that EGCG modulated the function of P-glycoprotein and reversed P-glycoprotein-mediated multidrug resistance in human cancer cells [84]. To date, there is a lack of in vivo evidence to support the inhibitory effects of green tea catechins on P-glycoprotein. A recent study showed that the intravenous administration of EGCG ( $20 \mathrm{mg} / \mathrm{kg}$ ) to rats inhibited the transport of irinotecan and its active metabolite, $\mathrm{SN}-38$, into the biliary tract, and prolonged their half-lives in plasma, possibly by modulating P-glycoprotein activity [62]. BCRP is another efflux transporter involved in cross-resistance to chemotherapeutic agents [88]. Some flavonoids have been identified as potent inhibitors of BCRP; however, EGC and EGCG did not exhibit such inhibitory activity in vitro [126].

Uptake carriers such as OATP represent another class of drug transporters [50]. Although no data are available regarding the in vivo impact of green tea catechins on OATP activity, a few in vitro studies may facilitate further investigations of OATP-mediated drug interactions with green tea catechins. Using OATP1B1-expressing HeLa cells, Wang et al. found that EGCG, but not EGC, was a potential inhibitor of OATP1B1 with an $\mathrm{IC}_{50}$ of $14.1 \mu \mathrm{M}$ [114]. As for OATP2B1, which is expressed on human intestinal epithelia, green tea itself and green tea catechins including EC, ECG, and EGCG, significantly inhibited the OATP2B1-mediated transport of estrone-3-sulfate in human embryonic kidney 293 cells at concentrations considered likely to be attainable in the human intestine [30]. Recently, Roth et al. showed that ECG and EGCG inhibited the uptake activities of OATP1A2, 1B1, and 2B1 in a concentration-dependent manner, while EC and EGC had minimal effects on OATPs [90]. Interestingly, EGCG was found to be a potent stimulator of OATP1B3-mediated uptake for one substrate examined, whereas EGCG behaved as an inhibitor of OATP1B3 for another substrate [90]. This study suggested that potential inhibitors should be examined using multiple and clinically relevant substrates when screening for OATPmediated drug interactions. In summary, more in vivo evidence is needed for a better understanding of drug transporter-mediated drug interactions between green tea catechins and drugs. We also note that such interactions may have beneficial properties. For example, given that green tea catechins have potent inhibitory effects on efflux transporters such as P-glycoprotein in vivo, drinking green tea may be valuable for patients receiving cancer chemotherapy because P-glycoprotein inhibition by catechins could suppress multidrug resistance in cancer cells.

## 7 Conclusions

The oral intake of some natural products has been shown to significantly influence the pharmacokinetics and pharmacodynamics of co-administered drugs. Such interactions may be partly mediated through the significant inhibition or induction of drug-metabolizing enzymes and transporters in the small intestine, liver, kidney, and brain. Whether the interactions of natural products with medicines have clinically harmful or beneficial effects for drug therapies is needed to be clarified. Furthermore, some ingredients of natural products may directly affect the pharmacological targets of medicines, thereby causing a significant augmentation or deterioration in therapeutic effects. Further scientific and clinical evaluations of the pharmacokinetic and pharmacodynamic interactions of natural products with prescription drugs are prospectively encouraged to establish their proper uses in clinical settings. Finally, it should be kept in mind that combinations of natural products (including beverages) and medicines should be viewed cautiously in terms of potential adverse interactions in patients treated with drugs, such as warfarin, that have relatively narrow therapeutic windows.

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# Chapter 11 <br> Nutrition and Gastrointestinal Health as Modulators of Parkinson's Disease 

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## 1 Definition, Etiology, and Symptoms

Parkinson's disease (PD) is the second most common neurodegenerative disease of aging, affecting about $1 \%$ of the population over 60 years old in North America [94], and is projected to affect nearly 10 million citizens of the world's most populous countries by 2030 [38, 77]. Parkinson's disease is a relentlessly progressive disease, and the societal and personal burden of disability from PD is considerable [59]. Parkinson's disease diagnosis also results in reduced life expectancy, ranging from 4 to 10 years depending on age of diagnosis, with a greater reduction in life expectancy with earlier diagnosis [146]. Clinical symptoms vary depending on disease state and include both motor and non-motor symptoms. Non-motor symptoms such as constipation, loss of sense of smell, and rapid eye movement (REM) behavior disorder may manifest years before PD diagnosis. Diagnostic clinical symptoms of advanced PD include motor impairments involving resting tremor, bradykinesia, postural instability, gait difficulty, and rigidity. Unfortunately, there is no curative treatment for PD, and this is at least partly because the majority of patients with PD will be diagnosed and receive treatment after the onset of neurological symptoms when substantial neuronal dysfunction and neuronal loss

[^11]has already occurred. The technology to identify PD before it reaches symptomatic Braak Stage 3 (substantia nigra compacta [SNc] involvement) already exists [125]; thus, a more successful approach could be to diagnose and start treatment before neuronal degeneration results in the emergence of clinical signs of PD.

It is believed that PD pathology is a consequence of interaction between genetic susceptibility and toxic environmental factors [134]. Specific genes related to dopamine metabolism such as Parkin and leucine-rich repeat kinase 2 (LRRK2) have been associated with PD risk, and those with a first-degree relative with PD have a 4-9 \% increased risk of developing the disease. Therefore, risk is largely influenced by environment; environmental factors that influence risk include age (older age), gender (male), and exposure to environmental toxins such as pesticides and certain solvents. While it is not yet known how dietary intake impacts PD risk, it is thought that dietary intake may also play a role. The combination of these genetic and environmental risk factors is thought to increase neuronal oxidative stress; despite this, the exact etiology of PD is not known. However, the pathobiology of neuronal loss in PD is well characterized. It is now well established that the pathological hallmark of PD is neuronal inclusions termed Lewy bodies (LB) or Lewy neurites (LN) whose main component is aggregated and phosphorylated $\alpha$-synuclein [18, 123]. It is believed that these $\alpha$-synuclein aggregates are the first steps resulting in neuronal loss that is responsible for neurological symptoms and signs of PD [18]. Recent studies have shown that inoculation of $\alpha$-synuclein aggregates can transfer the disease to wild-type mice [79]. These and other recent studies support that PD is a prion-like illness, and that $\alpha$-synuclein is a prion-like protein. This hypothesis suggests novel targets for the development of putative neuroprotective therapies [96, 105].

In addition, many recent studies support a model in which PD pathogenesis begins in the peripheral autonomic nervous system and/or the enteric nervous system (ENS) and that the substantia nigra is spared in early stages of the disease [37]. This theory explains why autonomic symptoms, especially gastrointestinal symptoms such as constipation, occur early in the disease, as much as 20 years before the onset of motor deficits [1, 37].

## 2 The Gastrointestinal Tract in the Pathogenesis of Parkinson's Disease

Although intestinal symptoms are described by Parkinson in his original writing [102], Braak was the first to propose a possible direct role of the gastrointestinal tract in PD pathogenesis. Braak originally suggested that the GI tract might be a portal of entry for a putative PD pathogen, triggering pathological changes in the submucosal/myenteric neurons, which then spread through the vagus nerve to the dorsal motor nucleus and the medulla oblongata [19, 58]. From there, pathological changes may move rostrally, ultimately resulting in the clinically defining motor
symptoms of PD when there is extensive involvement at the level of the midbrain substantia nigra [18]. Thus, the involvement of the GI tract in PD is of great interest as a contributing factor to the development and progression of PD [73].

Consistent with the model of a possible GI tract origin for PD, GI symptoms of PD are an important element of disease manifestation and represent a major complication of PD [25, 73]. Gastrointestinal symptoms in PD include reduced salivation, dysphagia, impaired gastric emptying, impaired GI motility, constipation, and defecatory dysfunction. These could be due to both central and peripheral processes. It is generally believed that these symptoms are a consequence of PD and are the result of intestinal motility disorders and associated intestinal bacterial overgrowth. However, over the last decade there has been mounting evidence that supports a role for the GI tract and the ENS in the pathogenesis of PD [58, 73]. Dysphagia occurs in about $80 \%$ of patients with PD [37], and one of the earliest descriptions of ENS Lewy bodies was esophageal staining in patients with achalasia [106]. Only three reports of Lewy bodies in the stomach are known [17, 103, 142]. However, erratic gastric emptying is a well-described problem in PD and can result in poor levodopa distribution [68]. The presence of Lewy bodies in the colonic myenteric and submucosal plexuses in patients with PD was first described in 1987 [67] and later substantiated and shown especially to occur in vasoactive intestinal polypeptide-reactive neurons in the ENS [141] and substance P-containing neurons [118]. Additionally, dopaminergic defects in the ENS of patients with chronic constipation have been seen [127]. Several neuropathological studies show accumulation of abnormal $\alpha$-synuclein-containing inclusions (Lewy neurites) in the ENS and dorsal motor nucleus of the vagus nerve, both in PD and in incidental Lewy body disease (ILBD) [24]. Colonic biopsies in PD patients with chronic constipation all showed significant $\alpha$-synuclein staining [72], indicating constipation as one key possible biomarker of PD, with significant constipation occurring more than 20 years before the onset of motor symptoms [1, 116]. Indeed, constipation is known to be overrepresented among individuals who later develop PD [1], and colonic biopsies obtained 2-5 years prior to the onset of PD features demonstrate $\alpha$-synuclein aggregates in colonic submucosal neurons [118]. It should be noted that $\alpha$-synuclein aggregates in colonic submucosal neurons can occur in PD patients even in the absence of constipation. Indeed, we recently showed the presence of $\alpha$-synuclein aggregates in Substance P containing neurons in the sigmoid colonic submucosal neurons in newly diagnosed patients with PD who did not complain of constipation [48]. These human studies provided compelling evidence that the GI tract might be the initial site for neuronal damage in PD; however, the data are still indirect.

To further determine whether the GI tract is involved in the initiation and/or progression of PD, a series of animal studies were performed by several investigators. For example, Pan-Montojo et al. administered the mitochondrial toxin rotenone locally into the intestine and reported $\alpha$-synuclein aggregation in the intestinal wall that, over time, propagated to and caused neurodegeneration in the dorsal motor nucleus of the vagus nerve and eventually in the substantia nigra [99]. In a follow-up study, the same team reported that cutting the vagus nerve or partially
removing sympathetic ganglia could block the spreading of Lewy-like pathology from the gut to the central nervous system [100]. However, it does not appear that the vagus nerve is the only path for spread of $\alpha$-synuclein aggregation because $\alpha$-synuclein aggregation is found in the sigmoid colon, which is not innervated by the vagus nerve [119]. Lee and coworkers have also addressed the idea that the gut could be a starting point for $\alpha$-synuclein misfolding. They showed that injection of brain extracts prepared from patients with dementia with Lewy bodies, but not from normal brains, induced the deposition of $\alpha$-synuclein aggregates in myenteric neurons of transgenic mice that overexpressed human A53T $\alpha$-synuclein [76]. Thus, routine colonic biopsies have been proposed as a tool to monitor PD patients [74]. This group has already shown that the degree of ENS Lewy body staining in colonic biopsies correlates with patient constipation [75] and that rectal biopsies are less sensitive at detecting $\alpha$-synuclein pathology in the same PD patient [103]. Thus substantial evidence supports a role for colonic inflammation and $\alpha$-synuclein pathology in PD that could be a valuable biomarker for not only diagnosis of PD but also as a target of therapy [35].

While intestinal phosphorylated $\alpha$-synuclein aggregates may be formed as a consequence of oxidative injury [61, 124], the source of neuronal oxidative stress in PD is not known. It is highly plausible that the GI tract is a major site and source of oxidative stress in neuronal tissue based on several factors. First, the GI system and the brain are directly linked anatomically through the dorsal motor nucleus of the vagus nerve, a brain region proposed to express Lewy pathology very early in the disease process [18]. In addition to the vagus nerve, the GI tract is connected to the CNS by the sympathetic and parasympathetic neuronal network in the spine. Second, the GI tract is the largest interface between neural tissue and the environment. These enteric neuronal cells are large in number in the submucosal plexus and myentric plexus, large enough that the GI neuronal network is called the "second brain" [73]. More importantly, this neuronal network is in close proximity to the potentially injurious factors such as bacterial products capable of inducing oxidative stress [117]. Indeed, the GI lumen harbors the largest and most diversified human-associated microbiota community with the capability of inducing inflammatory and oxidative pathways [55].

### 2.1 Intestinal Barrier Function and Parkinson's Disease

Central to the regulation of exposure to pathogens and oxidative processes is the GI tract's semipermeable barrier, which allows regulation of nutrient, ion, and water absorption and regulates host contact with a large number of dietary antigens and bacterial products [86]. Intestinal permeability can be defined as the facility with which the intestinal epithelium allows molecules to pass through by non-mediated passive diffusion. Several chronic autoimmune intestinal diseases including inflammatory bowel disease and celiac disease are associated with increased intestinal permeability, also known as "leaky gut" [46, 62]. One particularly detrimental
consequence of increased intestinal permeability is the translocation of bacteria (e.g., E. coli) and bacterial products (e.g., lipopolysaccharide [LPS], also known as endotoxin) that are key components of the outer membrane of gram-negative bacteria. Lipopolysaccharide binds to Toll-like receptor 4 (TLR4) and stimulates inflammation on a variety of intestinal cells including intestinal epithelial cells, enteric microglial cells, and cells of the immune system. Other bacterial products may also promote inflammation and oxidative stress through additional mechanisms (e.g., other TLRs, NOD-like receptors) [50]. The result of this stimulation is a proinflammatory environment, increasing the oxidative stress burden in the ENS.

Parkinson's disease and other neurodegenerative diseases such as Alzheimer's to a large degree have now been shown to be associated with low-grade systemic and neuronal inflammation, oxidative stress, and proinflammatory cytokines (TNF- $\alpha$, IL-6, IL-1 $\beta$ ). Indeed, endotoxin-induced systemic inflammation has been shown to involve microglia [57]. Thus, gut leakiness in patients with a genetic susceptibility to PD or previous exposure to environmental insult independent of the gut may be a pivotal early step promoting a proinflammatory/oxidative environment, contributing to the initiation and/or progression of the PD process. Studies from our laboratory have recently shown that newly diagnosed and untreated PD patients' tissue stains positive for colonic biopsy $\alpha$-synuclein aggregates [119] and that these patients have significantly increased intestinal permeability [48]. In addition, increased intestinal permeability in these PD patients correlated with increased colonic biopsy markers for bacterial translocation and oxidative stress as well as $\alpha$-synuclein staining. These data thus support a model in which increased intestinal permeability (leaky gut) could result in exposure to luminal bacterial products resulting in inflammation-triggered oxidative stress and $\alpha$-synuclein misfolding in susceptible individuals. We have proposed that a leaky gut may play a role in PD by the resulting effects on systemic inflammation and oxidative stress that may promote $\alpha$-synuclein misfolding [48, 120]. Likewise, recent research on major depression has shown an associated increase in intestinal permeability, blood endotoxin, and inflammatory markers, as well as oxidative stress [80, 81, 108]. Similar data for increased intestinal permeability and proinflammatory bacterial endotoxemia have been shown in amyotrophic lateral sclerosis (ALS) and Alzheimer's disease [151], as well as autism [144]. In addition, the relationship between neurological disease and inflammation may be bidirectional, creating a vicious cycle [87].

As there is evidence for GI tract involvement in PD, specifically alterations in intestinal permeability, we propose that such individuals might therefore benefit from therapeutic interventions that positively impact the intestinal milieu by either changing microbiota to produce less proinflammatory/injurious products or preventing gut leakiness. These interventions include dietary or pharmacologic therapies including diet, prebiotics, probiotics, and synbiotics directed at reducing intestinal inflammation and hyperpermeability which might break this pathologic vicious cycle in the gut. In the following sections, we discuss the scientific evidence that supports possible nutritional therapy (i.e., whole foods, dietary patterns, and supplemental nutrition such as probiotics and prebiotics) for PD and other
neurodegenerative diseases, primarily through modulation of the gut milieu. We will primarily focus on the dietary components in terms of PD risk, highlighting the importance of mitigating exposure to neuro-oxidative substances for PD prevention. We recognize that our knowledge of these mechanisms and thus these proposed therapies are still at very early stages of development. However, we propose that considerable solid scientific evidence has now been gathered to support two important themes. First, there is a clear relationship in animal models and human studies between the GI tract, intestinal inflammation and hyperpermeability (especially in conjunction with the microbiota), and neurological diseases. Second, evidence supports that certain nutritional therapies can have a beneficial effect to alleviate microbiome dysbiosis (shift from healthy state), ameliorate intestinal hyperpermeability, and/or inhibit intestinal inflammation or oxidative stress. Specific nutritional therapies of focus for direct modulation of the gut microbiota are products either containing "good" bacteria (lactobacilli, bifidobacteria) such as cultured milk or yogurt products and probiotic products, or prebiotics which are complex carbohydrates (fiber) that form the fuel for "good" fermentative bacteria. We also include such traditional foodstuffs as found for example in the so-called Mediterranean diet such as whole grains, unsaturated fats such as olive oil, and fish. These may have anti-inflammatory/antioxidative stress properties related to their modulation of the microbiota or may have direct beneficial anti-inflammatory or antioxidative stress effects. In any case, the nutritional therapeutic themes we propose are normalization of the microbiota diversity and promotion of lactobacilli and bifidobacteria, restoration of normal intestinal barrier function, and prevention of intestinal inflammation and oxidative stress. In addition to this novel, potential key role of nutrition, the impact of these nutritional measures on symptom management will be discussed in brief.

## 3 Nutrition and Parkinson's Disease

### 3.1 Evidence for the Role of Nutrition in Parkinson's Disease Risk

Specific dietary components that impact the risk of PD development are not clear, but research indicates the potential for certain dietary components, as well as overall dietary patterns, may modulate PD risk. Currently, many different dietary components such as dairy products, fat, alcohol, coffee and tea, antioxidants, and minerals have all been investigated for their role in PD risk. A select presentation of these dietary components is in the following sections.

The most thoroughly studied dietary macronutrient in regard to PD risk is dietary fat. In a population-based case-control study, energy-adjusted fat intake was significantly associated with PD (OR, $5.3 ; 95 \% \mathrm{CI}, 1.8-5.5$ ) when comparing the lowest quartile to the highest quartile of fat intake. Specifically, intake of animal fat
was associated with PD risk when comparing the lowest quartile to the third (OR, 3.61; $95 \% \mathrm{CI}, 1.32-9.83$ ) and fourth quartiles (OR, $5.28 ; 95 \% \mathrm{CI}, 1.80-15.49$ ) [78]. In a retrospective assessment of past food intake in a case-control study, intake of foods high in animal fat such as ice cream, hard and soft cheeses, milk, liver, pork, and beef was associated with an increase in PD risk (OR, 3.30; 95 \% CI, $1.43-7.61$ ), specifically when comparing $3.2-4.8$ servings and greater than 4.8 servings versus 2.1 servings or less per day [5]. However, this association between PD and animal or saturated fat intake was no longer seen when a later analysis was conducted using a larger sample of the same group [104]. A meta-analysis of prospective studies investigating the contribution of dairy to PD showed a relative risk of 1.6 ( $95 \%$ CI 1.3-2.0) for the highest versus lowest quintile for milk or dairy products overall; the relative risk was 1.8 in men and 1.3 in women [26]. Kyrozis et al. examined dietary variables associated with PD development using the EPICGreece cohort. Dairy products, specifically milk, were associated with PD risk (HR, $1.34 ; 95 \%$ CI, 1.14-1.58; $p<0.001$ ), while polyunsaturated fat intake was inversely associated with PD [69]. While a recent case-control study reported a null association of total and saturated fat with PD risk, when saturated fat intake was examined in those with pesticide exposure, saturated fat intake (low versus high tertile) was positively associated with risk of PD; it was suggested that pesticide exposure modified the relationship between saturated fat and PD by increasing oxidative stress [63]. Indeed, fat-induced increases in oxidative stress are known, specifically saturated fat, providing a mechanism for an increased risk of PD and other neurodegenerative disorders with saturated fat intake. Mechanistically in PD, the potential for specific fats to impact oxidation status is suggestive in animal studies as an intake of $60 \%$ of kcalories from fat exacerbated the effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity [16]. Interestingly, the authors suggested that it was due to high-fat diet-induced obesity, indicating a potential role for obesity in PD risk.

While it is suggestive that saturated fats may increase risk of PD, other categories of fatty acids may impact PD risk differently; polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids (MUFAs) may reduce oxidative stress and have neuroprotective effects. In the Nurse's Health Study and the Health Professionals Follow-Up Study, while overall fat intake was not associated with PD risk, replacement of PUFA with saturated fat (5 \% of the diet) in statistical models increased risk of PD in men (RR, 1.83; CI, 1.10-3.03) but not women [27]. In addition, omega- 3 PUFA and $\alpha$-linolenic acid were both inversely associated with PD risk when dietary intake was reported in tertiles (low versus high tertiles) [63]. Specific PUFAs such as omega-3 fatty acids may beneficially impact the brain through protection against a decrease in tyrosine hydroxylase and dopamine [15], or through reduction in neuroinflammation [97]. In addition, one PUFA, the omega- 6 arachidonic acid, may actually increase the risk of PD; $>0.17 \mathrm{~g} /$ day of arachidonic acid (highest quartile) was significantly associated with an increased risk of PD in humans ( $\mathrm{OR}, 2.09 ; 95 \% \mathrm{CI}, 1.21-3.64$ ) as compared to the lowest quartile [88]; however, this association was reversed in the Nurse's Health Study [27]. While a diet high in PUFAs and MUFAs may be protective against PD [33],
the amount of MUFAs and PUFAs to impart this benefit is unclear. In addition, no other lipid intake, whether categorized as total fat, categories based on saturation status, or individual fatty acids, has been examined for impact on PD risk.

In addition to dietary fat intake, other dietary components and patterns may influence PD risk. Data from the Nurse's Health Study and the Health Professionals Follow-Up Study was used to examine the associations between dietary patterns and PD risk. Two dietary patterns were identified: a prudent diet with high intakes of fruits, vegetables, legumes, whole grains, and low intakes of saturated fat; and a Western diet with high intakes of saturated fat from red and processed meats, refined grains, French fries, desserts, and sweets. The prudent diet was inversely associated with PD risk (RR, $0.78 ; 95 \% \mathrm{CI}, 0.56-1.07$; $p$ for trend $=0.04$ ), while the Western dietary pattern was not associated with PD risk. In addition, diet was categorized through two dietary quality scores, Alternate Healthy Eating Index (AHEI) and the alternate Mediterranean Diet score (aMed). The study indicated that plant-based dietary patterns that included some fish and poultry may protect against PD development as indicated by the relative risk for AHEI ( $0.70,95 \%$ CI $0.51-$ $0.94 ; P$ for trend $=0.01$ ) and aMed $(0.75,95 \%$ CI $0.57-1.00 ; P$ for trend $=0.07)$ when comparing the bottom quintile (least accordant) to the top quintile (most accordant) [51]. In addition, data from this study used in a meta-analysis indicated that the Mediterranean diet may reduce incidence of PD and Alzheimer's disease by $13 \%$ [128]. This is further supported in a case-control study that assessed diet using the Willett semiquantitative questionnaire that quantified dietary intake over the past year; this information was used to generate a Mediterranean diet score. For each additional point on the Mediterranean diet score (indicating higher adherence), the odds of having PD were lower by 14 \% (OR 0.86, $95 \%$ CI 0.77-0.97; $p=0.010$ ). In addition, a lower diet score was associated with earlier age of PD onset. The specific dietary components that were responsible for this association were unable to be detected, possibly due to the lack of an adequate sample size [3].

While there is an indication that the Mediterranean diet may be protective against PD risk, the mechanism of action is currently unknown. Recently, it was suggested that the nicotine component in peppers could reduce PD risk [93]; this food along with other foods containing nicotine such as tomatoes and potatoes are components of the Mediterranean diet. In addition, smoking is strongly negatively associated with PD risk, and this has been partially attributed to the nicotine component. It is reasonable to consider that the Mediterranean diet could be reducing oxidation and increasing neuroprotection from specific antioxidant containing foods. Indeed, the Mediterranean diet pattern has been shown to reduce both risk factors associated with cardiovascular disease (CVD) intermediate outcomes, as well as limited evidence for reducing CVD and related death [43]. Foods common in this eating pattern are high in polyphenols and include, but are not limited to, tea, coffee, blueberries, dark chocolate, green olives, red wine, and almonds. These foods function to benefit CVD through their antioxidant activities and regulation of cellular activities of inflammation-related cells and their molecular targets [132]. It is possible that these foods may be impacting risk or progression of PD through similar mechanisms.

For example, the Honolulu Asia-Aging study compared alcohol use (ever) versus never; those that consumed alcohol had a relative risk PD of 0.76 (95 \% CI, 0.45-1.28) compared to those that never drank alcohol, but this was not significant [56]. However, overall analyses of alcohol intake on PD risk do not strongly suggest that it is protective. Both tea and coffee consumption has been examined for its impact on PD, with some evidence for coffee consumption and reduced PD risk. Ten or more cups of coffee a day was negatively associated with PD risk as compared to no coffee intake (OR, 0.26 ; $95 \% \mathrm{CI}, 0.07-0.99$ ) [114]. Specifically, it is thought that the caffeine component contributes to reduced risk compared to those that do not consume caffeine; this may occur due to caffeine's effect on adenosine A2a receptors [89]. The association between tea intake and PD is less defined, and it is also unknown if it is the caffeine component or the polyphenols that may be beneficial in these beverages. Despite this potential beneficial impact, the majority of studies associating single antioxidants such as vitamins $\mathrm{E}, \mathrm{A}$, and C to PD have largely found that intake is not associated with PD risk ([90], [153]). This lack of association may indicate that whole foods, rather than individual components, are most influential for reduction of PD risk. It is tempting to think, as discussed later, that the foods emphasized in a Mediterranean eating pattern, and other healthy dietary patterns, beneficially alters microbiota, intestinal permeability, or endotoxemia. Unfortunately, no current literature exists that investigates the impact of the Mediterranean diet itself on these factors.

In summary, the available literature provides evidence, albeit limited, for the role of specific dietary components in PD development. There is potential that certain dietary components can have a negative impact (dietary fat and saturated fat), or may provide benefit (omega- 3 fats, coffee), as seen in a recent study investigating the Mediterranean diet. While these components may systemically reduce inflammation and oxidative stress as evidenced by reductions in CVD risk, it is unknown if they have a similar role in PD.

In addition to dietary quality, quantity of dietary components may be important in PD in relation to body weight status. Indeed, obesity is not only a risk factor for other diseases (e.g., CVD, diabetes), but is deemed a disease itself by the American Medical Association. Some evidence exists for a positive association between BMI and PD risk [60], but this association was not seen in several other studies [28, 98]; therefore, it is unknown if obesity increases risk for PD, and further research should be done to determine if the increased inflammation often seen in obese individuals can have a detrimental effect on PD risk. We suggest that an important source of inflammation is gut-derived, and that this modulation through diet, dependent or independent of obesity, may influence PD risk.

### 3.2 Dietary Intake for Parkinson's Disease Progression and Symptom Relief

While there is potential importance for diet in PD development, specific dietary components have yet to show that they can slow disease progression or improve motor function. The American Academy of Neurology does not recognize any dietary therapies to slow progression of disease or improve motor function, with more research needed on specific dietary components [130]. In contrast, modulation of non-motor symptoms (GI-centered symptoms such as constipation) may be benefitted by dietary components such as fiber. Briefly discussed is the current literature on impact of anti-inflammatory foods and supplements for disease progression, and then fibers for GI-related symptom management.

### 3.2.1 Role of Nutrition in Disease Progression and Motor Function

Currently, there is no cure for PD, though lifestyle components, medications, or medical procedures may slow the onset or severity of some PD symptoms. Amongst the dietary components investigated include a decrease of pro-oxidant foods such as saturated fat, and an increase in dietary compounds with purported antioxidant activity such as creatine, CoQ10, vitamin E, and omega-3 fatty acids. The ability to detect differences in PD progression by alterations in dietary intake is difficult, and as mentioned, currently no dietary alterations have definitively shown to beneficially alter disease course; however, current strategies under investigation are discussed here in brief. While it is known that the timing of food, and specifically protein intake, is important in respect to Levodopa absorption [95] and consequently impacts the ability of the drug to benefit motor function, this aspect of nutrition and PD will not be discussed.

Due to the potential connection between animal fat and PD risk, it has been suggested that limiting dietary animal fat may be effective in reducing PD symptoms. In a case report study, a diet of minimally processed foods with less than 25 g of animal fat per day was not seen to prevent decline due to PD, with worsening motor symptoms and tremors despite alterations in the dietary pattern. However, when the same diet was supplemented with the antioxidants fisetin and hexacosanol through ingestion of strawberries and wheat germ, respectively, there was a clinically significant improvement in motor symptoms such as cogwheel rigidity micrographia, bradykinesis, dystonia, hypomimia, and retropulsion [110]. However, this was a case report, and when weighing the significance of the data, this should be considered. One category of fatty acids, omega-3's, may have therapeutic potential for those with PD due to their influence on dopaminergic activity and neuroprotection; this evidence largely stems from mechanistic studies using animal models and is not yet routinely recommended in those with neurodegenerative diseases such as PD [14].

As mentioned, foods with antioxidant capacity have been targeted in those with PD as oxidation and inflammation are thought to play roles in the etiology and progression of the disease. Several foods such as creatine have been investigated for their ability to modify the trajectory of PD. Creatine is an organic acid that is both endogenously synthesized and exogenously obtained primarily from dietary intake of meat. While creatine's most well-known role is to phosphorylate ADP to create ATP, it also functions as an antioxidant, impacts mitochondrial energy production, and attenuates the loss of dopamine caused by MPTP [85, 133]. Despite the potential for creatine to positively impact the mitochondria, study results supporting its use are mixed. The Deprenyl And Tocopherol Antioxidate Therapy Of Parkinsonism (DATATOP) trial failed to identify supplemental creatine ( $5 \mathrm{~g} /$ day) as effective, with effectiveness set at a $30 \%$ improvement in the Unified Parkinson's Disease Rating Scale (UPDRS) score. However, a futility trial by the Neurological Disorders and Stroke Exploratory Trials in Parkinson's Disease determined that further study of creatine (but not minocycline, coenzyme Q10 [CoQ10], and GPI-1485) was warranted to determine its efficacy in slowing PD progression. As a result, a Phase III clinical trial is underway to determine the impact of $10 \mathrm{~g} /$ day creatine versus placebo in slowing clinical decline in PD between baseline and the 5-year follow-up visit against the background of dopaminergic therapy and best PD care [41].

CoQ10, also known as ubiquinone, is a cofactor in the electron transport chain and is found largely in the liver and the brain. CoQ10 accepts electrons in the mitochondria from complex I and II and acts as an antioxidant by reducing the oxidized form of $\alpha$-tocopherol in the mitochondria and the lipid membrane. Thus, due to the role of mitochondrial dysfunction and oxidative stress in PD, CoQ10 may have a role in treatment or symptom relief. Indeed, CoQ10 levels in the mitochondria were significantly lower in patients with Parkinson's disease compared to ageand sex-matched controls [121].

A neurotoxin that selectively damages the nigrostriatal dopaminergic system, MPTP, causes clinical, biochemical, and neuropathologic changes similar to those seen in PD. To determine the effect of CoQ10 on MPTP administration, mice were either fed a control diet or a diet supplemented with CoQ10 ( $200 \mathrm{mg} / \mathrm{kg} / \mathrm{day}$ ) for 5 weeks. After MPTP treatment at week 4, striatal dopamine concentrations and dopaminergic axons were significantly higher in the group treated with CoQ10 versus the control [11]. In addition, supplementation of CoQ10 in mice resulted in significant protection against loss of dopamine, the loss of tyrosine hydroxylase neurons, and the induction of $\alpha$-synuclein inclusions [30].

In a double-blind, randomized, placebo-controlled study, 80 subjects with early PD that did not require medication were randomly assigned to either a placebo group or CoQ10 at 300,600 , or $1,200 \mathrm{mg} /$ day. The subjects were evaluated using the change in total UPDRS score at baseline, $1,4,8,12$, and 16 months after treatment administration. While there was less increase in disability in the CoQ10 groups versus the control, only the $1,200 \mathrm{mg}$ CoQ10 group showed significantly less increase in the UPDRS score as compared to placebo ( +11.99 vs +6.69 , placebo and control, respectively; $p=0.04$ ) [122]. Another study $(n=28)$ aimed
to determine the symptomatic response of daily consumption of $360 \mathrm{mg} /$ day CoQ10 supplementation for 4 weeks on PD-associated symptoms and visual function. After 4 weeks, the CoQ10 supplementation group had mild symptomatic benefit ( $p=0.01$ ) and significantly better Farnsworth-Munsell 100 Hue test (measurement of color discrimination) performance compared to the placebo group [91]. Despite these encouraging data, CoQ10 is not currently recommended for PD as a standard of practice, and larger trials with CoQ10 should be conducted to tease out its role in PD progression and motor function.

In addition to creatine and CoQ10, vitamin E also has been investigated to determine if consumption can reduce PD progression. While observational studies indicate that vitamin E may reduce PD risk [44], little benefit of vitamin E intake exists once PD is diagnosed. The DATATOP trial indicated that vitamin E at $2,000 \mathrm{IU}$ did not slow the progression of PD over a period of 14 months as compared to the control group. This is despite the more recent in vitro and in vivo work supporting its ability to reduce oxidation and associated neuronal death [23].

Although no conclusive data exist to support the routine use of dietary components in PD progression or motor function, further investigation into the specific food components or dietary patterns that impact progression is reasonable; the antiinflammatory nature of many foods gives hope that evidence will support consumption of these foods in the future.

### 3.2.2 Role of Nutrition in the Relief of Gastrointestinal Symptoms

As mentioned, symptoms in PD are varied, ranging from motor symptoms such as dyskinesia, tremors, and rigidity, to non-motor symptoms such as depression, cognitive impairment, and gastrointestinal complaints. These GI complaints include constipation, dysphagia, and lack of appetite, among others. It is generally believed that these symptoms are a consequence of PD and are the result of intestinal motility disorders caused by the impact of PD on the enteric autonomic nervous system and the associated intestinal bacterial overgrowth and delayed gastric emptying. This slows transit time and can cause defecatory dysfunction such as constipation. While there is limited evidence for diet in the nutritional management of non-motor symptoms, changes in dietary intake (i.e., fiber inclusion) can be impactful for those PD patients with constipation-predominant GI symptoms.

While constipation is one of the most common complaints for PD patients, it is to be noted that these symptoms vary substantially by disease state; therefore nutritional intervention for GI-related symptoms varies as well. While complications such as dry mouth and swallowing difficulties in advanced stage PD make adequate nutrient intake difficult, even those primarily with Hoehn \& Yahr Stage II symptoms (and MDS-UPDRS part II mean [SD] subscore of 27.3 [12.1]) experience sialorrhea ( $89 \%$ ), constipation ( $67 \%$ ), and incomplete bowel emptying ( $51 \%$ ) [65]. Indeed, $59 \%$ of patients with PD meet the Rome III criteria for functional
constipation versus $21 \%$ of older adults without known neurological conditions [64]. In addition, constipation frequently manifests before PD diagnosis. A total of 96 men from the Honolulu Heart Study developed PD; those with $<1$ bowel movement (BM)/day had a 2.7 -fold excess risk of PD versus men with $1 \mathrm{BM} /$ day ( $95 \% \mathrm{CI}, 1.3-5.5 ; p=0.007$ ), and this risk was increased when $<1 \mathrm{BM} /$ day was compared to greater BMs per day (4.1- and 4.5 -fold risk when compared to $2 \mathrm{BM} /$ day and $>2 \mathrm{BM} /$ day, respectively). In an incident cohort of PD, constipation was reported by $42 \%$ of early PD subjects ( $p<0.001$ compared to healthy controls), and $60 \%$ of patients with advanced disease suffer from constipation that adversely affects quality of life and is resistant to therapy [115]. These GI symptoms negatively impact health-related quality of life (HRQoL); for example, incomplete bowel emptying had a negative impact upon HRQoL in early PD patients [39]. As these non-motor symptoms are important determinants of PD patients' quality of life, appropriate treatment of constipation in PD will increase quality of life and may enhance the absorption and therapeutic effect of PD medications [82]. Although these intestinal symptoms are described by Parkinson in his original writing [102], there is as yet no effective treatment for these symptoms.

To alleviate this condition and help keep impact of drug intake consistent, various means for reducing constipation are recommended. The American Academy of Neurology supports the use of polyethylene glycol (Miralax ${ }^{\circledR}$ ); increases in water and dietary fiber have also shown clinical benefit for constipation relief [150]. Exacerbation of constipation may also occur due to the inability of those with PD to consume adequate fiber and water due to PD-induced eating difficulties (dysphasia, lack of appetite).

While nearly $\$ 300$ million was spent on fiber supplement products in the United States in the past year, limited literature exists that describes the effectiveness of fiber supplementation specifically in PD patients. A diet supplemented with 28 g insoluble fiber (wheat bran, pectin, and dimethyl-polyoxylhexane-900) was provided to PD patients with severe constipation. Constipation improved significantly, with frequency of bowel movements increasing to four or more times per week. A significantly increased UPDRS motor score was seen after both two weeks and two months of fiber supplementation. Furthermore, there was a relationship between the reduced constipation and higher bioavailability of Levodopa, which suggests that Levodopa activity was increased by a decreased gastric emptying time and increased intestinal motility [9]. Provision of 10.2 g psyllium per day increased stool frequency (increase of $3 \times /$ week) and stool weight in constipated PD patients [8]. As limited evidence for relief of constipation through fiber intake has been documented in the literature, it is unknown what specific type of fiber is most beneficial to relieve PD symptoms. In concept, individual or combinations of fibers could be designed and utilized in a particular illness to alter the composition of the gut microbiota, its function, and/or fermentation end products such as SCFAs towards a desired condition-specific effect. Fibers such as prebiotics could meet this need, by both reducing constipation and improving gut milieu to limit the uptake of oxidative and inflammatory components through the gut barrier.

### 3.3 Novel Proposed Role of Nutrition on Gut Health and Systemic Inflammation for Parkinson's Disease

As stated, PD risk is a combination of genetic and environmental factors. Known environmental factors that truly impact disease risk are limited, with evidence strongest for the inverse relationship of smoking and PD risk. While evidence for dietary factors and PD risk in inconclusive, the data are also encouraging that nutrition can play a role in modulation of PD risk. However, once one has the disease, progression is difficult to slow, and symptoms are hard to manage. Therefore, searching for novel mechanisms for PD prevention is essential to reduce disease burden. Our recent work indicates that newly diagnosed and untreated PD patients stain positive for colonic biopsy $\alpha$-synuclein aggregates [119] and have significantly increased intestinal permeability [119], which correlated with increased colonic biopsy markers for bacterial translocation and oxidative stress. Therefore, an intestinal-focused therapeutic intervention that could correct abnormal milieu (including gut microbiota), normalize the intestinal barrier, and mitigate ENS oxidative injury could be a paradigm shift approach for treatment of not only GI symptoms of PD patients and possibly CNS symptoms of PD, but most importantly reduce risk of PD. While it is too early to identify the magnitude of this dietary impact and whether this may play a larger role in disease risk or modification of disease course, we propose that the aforementioned factors may be mitigated through dietary means. Discussed will be both whole foods and supplements that modify gut health and therefore have the potential to reduce PD-related neuronal oxidation and inflammation. Specifically manipulation of the gut bacteria and associated gut milieu and barrier function through probiotic, prebiotic, and symbiotic administration will be discussed.

### 3.3.1 Dietary Patterns and Whole Foods to Modulate Gut Health and Systemic Inflammation

While the impact of specific dietary components and dietary patterns on the gut microbiota, gut milieu, and endotoxin is not completely delineated, it is known that what we consume can have profound effects on these parameters. Overall dietary patterns can influence microbiota composition, with those eating more animal protein and saturated fat resulting in a Bacteroides enterotype, and those eating a dietary pattern high in plant-based foods, carbohydrates, and low in meat and dairy exhibiting a Prevotella-dominated enterotype [149]. Despite the lack of literature examining the impact of the Mediterranean diet on gut microbiota and associated health, dietary components of the Mediterranean diet such as whole grains, unsaturated fat, polyphenol (e.g., olive oil, fruits, vegetables, coffee, tea) intake, and low animal and saturated fat intake have been linked to a beneficial microbiota profile.

The commensal gut microbiota thrive on the substrates that escape absorption in the small intestine and are available for colonic bacterial fermentation [147]. These
substrates, often found in whole grain carbohydrates rich in fiber, can increase the commensal gut microbiota and positively impact host health. A recent randomized, crossover trial provided subjects with whole-grain barley ( 18.7 g fiber), brown rice ( 4.4 g fiber), or a combination of both ( 11.5 g fiber) for 4 weeks each. All treatments increased microbiota diversity; whole grain barley increased beneficial bacteria such as Roseburia and Bifidobacterium. Both whole grain barley and the combination of both whole grain barley and brown rice reduced IL-6 concentrations compared to baseline [84]. While fiber can modulate gut bacteria, other components in the whole grains may also be beneficial. Indeed, polyphenols from various dietary sources may reduce intestinal mucosal inflammation and permeability [84]. Even the consumption of cocoa flavanols can selectively alter the gut bacteria (increase bifidobacteria and lactobacilli) and promote systemic health [137].

In regard to evaluating dietary intake's impact on gut milieu, a majority of the focus has been on dietary fat and its role in compromising microbiota composition, the intestinal barrier, and resultant endotoxemia. Several studies in murine models indicate that a high-fat diet induces dysbiosis [21, 136]. Compared to a highcarbohydrate diet, a high-fat diet (38-62 \% fat) or a Western diet (high in saturated fat and refined carbohydrates) may result in dysbiotic microbiota (e.g., lower bifidobacteria, higher firmicutes and proteobacteria) [20, 32, 47]. Acute effects of high calorie, high-fat intake have been investigated in healthy subjects. A single meal consisting of toast and 50 g butter increased plasma LPS by $50 \%(8.2 \mathrm{pg} / \mathrm{mL}$ vs $12.3 \mathrm{pg} / \mathrm{mL}$ ) [42]. In addition, a breakfast of egg and sausage muffin and hash browns increased LPS as compared to a meal similar in energy but high in fiber and fruit [52], indicating the type of foods eaten differentially impact gut health and subsequent endoxotin levels. Ingestion of liquid glucose, orange juice, or cream resulted in increased inflammatory makers (e.g., TNF- $\alpha$, IL-1 $\beta$ ), LPS concentrations, and TLR-4 expression with cream, while glucose only increased inflammatory markers; no increase in these measures were seen with orange juice intake [34]. This increase in inflammatory markers by glucose also highlights the potential for refined carbohydrates (e.g., sucrose, fructose, glucose) to negatively alter gut bacteria. While most studies utilized high quantities (as a percent of total) of fat, even a moderate fat meal ( $33 \%$ of energy as fat) may increase LPS [70]. As the average fat intake in the United States is $33 \%$ and saturated fat intake is $11 \%$ [138], both above recommended intake levels ( $30 \%$ and $10 \%$ respectively for healthy individuals), a better understanding of the impact of dietary fat intake on health (PD included) is important. While high-fat diets may cause oxidative damage independent of LPS, this gut-mediated mechanism could also contribute to dietary fat-induced PD [26, 78].

While a high-fat diet, particularly in animal models, has been linked to dysbiosis and endotoxemia, fewer studies have differentiated between types of dietary fat and these outcomes. A recent study using a porcine model compared consumption of coconut oil, fish oil, and olive oil on LPS concentrations [83]. Pigs fed a high saturated fat meal (coconut oil) had a 2 -fold increase in circulating endotoxin compared to those fed fish and vegetable oil. In addition, fish oil-treated ex vivo
porcine ileum transported less endotoxin than control or the other fatty acid-treated groups [83].

Not only the type of food can impact microbiota composition, intestinal barrier, and thus the level of endotoxemia, but the time of eating can also impact endotoxemia through its impact on intestinal and liver circadian rhythms. It is well known that the time of eating impacts intestinal circadian rhythm [7, 49]. We recently showed that disruption of circadian rhythms in mice causes intestinal hyperpermeability and endotoxemia [131]. We have also found that disruption of circadian rhythms by light/dark phase inversion in mice on high-fat diet causes dysbiosis (unpublished data). It is intriguing that core circadian genes like Per and disruption of circadian organization have been proposed to be involved with PD pathology [145]. Thus, it is reasonable to consider that time of eating could be an important factor for PD course. Further studies are needed to assess the impact of circadian rhythms and impact of time of eating on PD symptoms and PD course. In the meantime, it is prudent to suggest that patients with PD consider consuming majority of their daily calorie during sun light (breakfast and lunch) in order to optimize circadian alignment.

While this evidence is limited, it is tempting to think that these aforementioned dietary factors can influence PD through this gut-mediated mechanism. We now understand the importance of the gut microbiota and how its disruption, specifically through dietary intake, can have negative health impacts. As our recent work indicates that microbiota differs between those with PD and healthy controls (unpublished data), it is essential to continue to research specific foods and dietary patterns that can improve gut health for PD risk reduction.

### 3.3.2 Probiotics, Prebiotics, and Synbiotics to Modulate Gut Health and Systemic Inflammation

While modulation of the health of the GI tract by whole foods is of primary importance, it is possible that dietary supplementation may be beneficial in some individuals. Supplementation of probiotics, prebiotics, and synbiotics has recently been investigated for their ability to beneficially modulate gut milieu and systemic health.

The World Health Organization (WHO) defines probiotics as "live microorganisms which when administered in adequate amounts confer health benefits to the host" [148]. These live microorganisms generally are comprised of various bacterial strains such as the lactic acid-producing bacteria within Lactobacillus, Bifidobacterium, and Streptococcus strains [40], but technically include other microorganisms such as yeast. Strains within these groups of bacteria are selected due to their ability to reach the large intestine, promote bacterial growth, and produce related beneficial effects. Probiotics are available in both food and supplement form and are increasingly in demand due to the marketing opportunity made available by recent research supporting their health benefits. Most commonly, probiotics are found in specific yogurts with added bacterial strains beyond that found as a result of normal fermentation. Also, multiple supplements are available
to consumers; however, until specific strains and quantity of those strains are identified as being most appropriate for gut health and specific health conditions, supplementation may not be as beneficial.

Gibson et al. first introduced the concept of prebiotics in 1995 as a way to increase the survival rate of probiotics in the GI tract [54]. Since then, the definition has evolved, and in 2010, Gibson et al. defined prebiotics as "a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host heath" [53]. In order for a dietary ingredient be labeled a prebiotic, it must meet certain criteria. Nondigestibility of the prebiotic needs to occur in the small intestine for the prebiotic to reach the lower GI tract, fermentability has to be demonstrated, and the prebiotic has to function as a selective substrate and stimulate the growth of the microbiota. Presently, there are a limited number of prebiotics which meet these criteria of reaching the lower GI tract; fructooligosaccharides (FOS), inulin, galactooligosaccharides (GOS), and lactulose meet these criteria as these prebiotics are selective towards bifidobacteria and lactobacilli [111]; however, more recent identification of butyrate-producing strains exist, which are promoted through prebiotic supplementation. With this and other new findings, those substances categorized as a prebiotic are likely to change. As with probiotics, prebiotics can be consumed as supplements or added to foods. Frequently inulin or fructooligosaccharides will originate from chicory root, and will be listed as such on food labels. While bananas, onions, garlic, and artichokes are examples of foods that naturally contain prebiotic fiber, most appreciable amounts of prebiotics are found through adding prebiotics to cereals, cereal bars, breads, and yogurt drinks during food production.

A synbiotic is the combination of a probiotic and a prebiotic. Not only do the synbiotics promote growth of the resident microbiota in the host, but they also promote the survival of the new microbiota from the added probiotic strain; the prebiotic can promote the growth of both the resident microbiota and the supplemented probiotic [66]. For example, a particular strain of bifidobacteria may be coupled with GOS as GOS can promote the growth of bifidobacteria both originating from the supplement or in the GI tract. Currently, the majority of synbiotic foods available on the market are yogurts or dairy drinks, but synbiotics are also available in a supplement form.

## Probiotics

The benefits that probiotics impart on the host are dependent on the strain and the quantity of probiotic consumed. If the right strain is taken in adequate quantities, probiotics can beneficially modify microbiota, stimulate the production of SCFA, specifically butyrate, as well as improve intestinal barrier function.

In vitro experiments have identified bacterial isolates (Lactobacillus plantarum 299, L.rhamnosus HN001, and Bifidobacterium lactis) that increase transepithelial electrical resistance (TEER) in Caco-2 cells challenged with $1 \%$ penicillin-
streptomycin, indicating tight junction integrity [6]. Incubation of T84 cells with Lactobacillus casei DN-114 001 protected against the increase in paracellular permeability induced by Escherichia coli as assessed by TEER, as well as by monitoring zonula occludens-1 distribution [101]. Additional research has supported the use of Lactobacillus for promotion of normal intestinal permeability [2], as well as both lactobacillus and bifidobacterium for inflammation reduction in RAW 264.7 macrophages [112].

In vivo experiments, primarily murine models, have been used to determine the impact of probiotics on gut microbiota, barrier function, and endotoxemia. Mice fed a high-fat diet for 5 weeks with Lactobacillus casei strain Shirota (Lcs) had lower levels of lipopolysaccharide-binding protein (LBP) compared to the control fed a high-fat diet without Lcs, indicating reduced high-fat diet-induced endotoxemia through probiotic consumption [92]. In addition, supplementation of Bifidobacteria adolescentis in rats improved gut barrier function and reduced bacterial translocation and endotoxemia after thermal injury; plasma endotoxin was significantly negatively correlated with Bifidobacteria counts ( $r=-0.4912 p<0.001$ ) [143]. This was supported in a study by Chen et al; rats fed a high-fat diet (72 \% fat from corn oil and lard) supplemented with Bifidobacterium spp. had lower levels of endotoxemia, metabolic endotoxemia and intestinal inflammation compared to the high-fat group ( $p=0.006$ and $p<0.001$, respectively) [29].

While specific probiotic strains reduce intestinal permeability in vitro and in animal models, limited evidence exists in humans. Supplementation of Lactobacillus casei Shirota did not improve intestinal permeability in those with metabolic syndrome [71]. In 41 preterm infants, adding $2 \times 10^{7} \mathrm{cfu} / \mathrm{g}$ Bifidobacterium lactis decreased intestinal permeability. Compared to matched preterm infants without provision of Bifidobacterium lactis, the lactulose/mannitol ratio was significantly lower in the probiotic group compared to the control group after 30 days of supplementation [129]. In relation to the interconnectivity of the brain and the gut, recent research has focused on probiotic supplementation and mood [12], anxiety [107], and brain activity [135]. Chronic ingestion of a fermented milk product containing five probiotic strains for 4 weeks modulated brain activity, providing evidence of the potential for probiotics to influence brain-gut interactions, especially in the context of PD. However, to date, there is no study that provided any direct evidence for use of probiotics in PD.

## Prebiotics

The commensal gut microbiota thrive on the substrates that escape absorption in the small intestine and are available for colonic bacterial fermentation [147]. These substrates, or prebiotics, can improve and stabilize the composition of gut microbiota, which can lead to fortification of the intestinal barrier. In addition to decreasing intestinal permeability, prebiotic effects also include reduction of metabolic endotoxemia, reduction of obesity risk, and reduction of metabolic syndrome risk [111].

In ob/ob mice, oligofructose fed for 5 weeks significantly decreased Firmicutes and increased Bacteroidetes phyla, as well as changed 102 distinct taxa. Also, the prebiotic diet improved glucose tolerance, increased $L$ cells, and reduced fat-mass development, oxidative stress, and low-grade inflammation ( $p<0.05$ ) [45]. High-fat-fed mice ( $60 \% \mathrm{fat}$ ) fed oligofructose for 8 weeks had improved leptin sensitivity, improved glucose tolerance, reduced fat mass, and increased muscle mass compared to the control group ( $p<0.05$ ) [45].

In addition, oligofructose restored the levels of Bifidobacteria in high-fat-fed mice ( $72 \%$ fat from total energy) which negatively correlated with endotoxemia ( $r=-0.41, p=0.025$ ) [21]. Oligofructose can also increase intestinal epithelial ZO-1 and occludin as assessed by immunofluorescence analysis [22]. This beneficial effect of prebiotics may be mediated in part through a mechanism involving increases in GLP-2 production, thus improving gut barrier during obesity and diabetes [22]. In rats fed an enteral formula ( 18.5 g protein, 53.5 g carbohydrate, 17.5 g lipid) with GOS, Bifidobacteria and sIgA levels were significantly higher in the GOS-fed group compared to the enteral-fed group without GOS. In addition, small intestinal epithelium apoptosis was lower ( $p<0.01$ ) and occludin was higher ( $p<0.01$ ) in the GOS-fed group after 7 days compared to control, indicating that GOS can significantly improve intestinal barrier function in rats [152].

While animal models provide important mechanistic insight, dietary interventions, including probiotics and prebiotics, can have divergent impacts in humans. Intake of $2.5,5.0,7.5$, or $10 \mathrm{~g} / \mathrm{d}$ of short-chain fructooligosaccharides (scFOS) for 7 days increased Bifidobacteria compared to the placebo group ( $\mathrm{p}<0.03$ ) in 40 human volunteers. While subjects experienced significantly more bloating during scFOS consumption at doses of 2.5 and $5 \mathrm{~g} /$ day ( $p=0.03$ ), no significant increases in bloting were experienced at doses of 7.5 and $10 \mathrm{~g} / \mathrm{day}$ [13]. A mix of 16 g inulin and oligofructose ( $50 / 50 \mathrm{mix}$ ) for 3 months in obese women increased Bifidobacterium and Faecalibacterium prausnitzii; these bacteria also negatively correlated with serum LPS levels ( $p<0.05$ ), indicating that consumption of a prebiotic lad to modest changes in host metabolism as evidenced by the correlation of certain bacterial species with metabolic endotoxemia [36].

Vulevic et al. conducted a crossover study to examine the effects of a trans galactooligosaccharide mixture on gut microbiota, immune function, and markers of metabolic syndrome in overweight adults ( $\mathrm{BMI}>25 \mathrm{~kg} / \mathrm{m}^{2}$ ) that had $>3$ risk factors for metabolic syndrome. After 6 weeks and at the end of the study (12 weeks), B-GOS increased the number of bifidobacteria in the feces and decreased Bacteroides spp. and C. histolyticum ( $p<0.0001$ ). C-reactive protein was lower at the end of 12 weeks in the B-GOS group ( $p<0.0012$ ), and sIgA was significantly greater in the B-GOS group at treatment end compared to the placebo $\operatorname{group}(p<0.0001)$ [140]. Intake of GOS at $0.0,2.5,5$, or 10 g of GOS by 18 healthy subjects for 3 weeks each increased bifidobacteria in the $5 \mathrm{~g}(p<0.05)$ and the 10 g ( $p<0.001$ ) GOS groups compared to the control group. The bifidogenic effect was inversely correlated with the bifidobacteria levels at baseline, indicating that the subjects that started with lower numbers of bifidobacteria had a higher potential for the prebiotic to induce a 100-1,000-fold increase [31]. In a crossover study, a total
of 27 volunteers consuming bread with arabinoxylan oligosaccharide for 3 weeks had increased SCFA, specifically $70 \%$ higher butyrate, than at the end of the treatment period of bread without the added arabinoxylan oligosaccharides ( $p=0.05$ ). In healthy volunteers consuming inulin-enriched pasta for 5 weeks, serum zonulin was lower and serum GLP-2 was higher when compared to baseline and the control pasta group without inulin [113].

While the importance of the brain-gut axis is known, and that the microbiota impact this relationship, investigation of prebiotics to impact the brain-gut axis is limited. Intake of GOS in patients with irritable bowel syndrome resulted in lower anxiety scores than at baseline [126]. The apparent beneficial impact of prebiotics on gut microbiota, barrier function, and endotoxemia shows promise that prebiotics may also play a role in modulating the ENS and reduce neuronal oxidation and inflammation. However, to date, there is no study that provides any direct evidence for use of prebiotics in PD.

## Synbiotics

Because a synbiotic contains both a probiotic and a prebiotic that is intended to increase that probiotic, synbiotics should be more beneficial compared to its prebiotic and probiotic counterparts. Van Zanten et al. examined the effects of eight different synbiotic combinations on the composition and activity of human fecal microbiota. The well-studied probiotic Lactobacillus acidophilus NCFM was combined with either isomaltose, cellobiose, raffinose, or an oat $\beta$-glucan hydrosylate. Another commonly used probiotic Bifidobacterium animalis subsp. lactis B1-04 was combined with melibiose, xylobiose, raffinose, or maltotriose. All combinations of the synbiotics were tested in a model of the human colon; all combinations significantly increased both Lactobacillus acidophilus NCFM and Bifidobacterium animalis subsp, lactis B1-04 ( $p<0.05$ ). Also, all of the synbiotic combinations significantly decreased the ratio of Bacterioidetes/Firmicutes ( $p<0.05$ ). Short-chained fatty acid levels increased, specifically acetic and butyric acid by three- to eightfold compared to the control ( $p<0.05$ ). The decrease in the ratio of Bacterioidetes/Firmicutes correlated with the increase of acetic and butyric acid production ( $p=0.04$ and $p=0.03$ respectively) [139].

Baffoni et al. conducted two trials on chickens to test the impact of a prebiotic or a synbiotic on modulating the gut microbiota with an increase in beneficial bacteria such as bifidobacteria and lactobacilli with a decrease in the pathogenic bacteria Campylobacter jejuni. Campylobacter spp. was significantly decreased in the synbiotic group after 14 days of supplementation, and the reduction was maintained even after the washout period ( $p<0.05$ ) [10].

Reddy et al. investigated if the combination of synbiotics, antibiotics, and mechanical bowel preparation (MBP) would preserve intestinal barrier function during colorectal surgery in a randomized control trial. Approximately half of the 88 surgical patients enrolled received synbiotics, consisting of 15 g oligofructose and $4 \times 10^{9}$ of Lactobacillus acidophilus, Lactobacillus bulgaricus,

Bifidobacterium lactis BB-12, and Streptoccocus thermophiles. The researchers took two bowel samples to determine bacterial translocation and intestinal permeability was determined using a sugar test. The synbiotic group had the lowest incidence of bacterial translocation ( $p<0.001$ ) after surgery as measured by urine sugar test, but there was no difference in intestinal permeability between groups [109]. A total of 20 patients with at least a 4 -day ICU stay with intragastric tube feedings were provided synbiotics ( $10^{10}$ Pediococcus pentosaucus 5-33:3, $10^{10}$ Lactococus raffinolactis 32-77:1, $10^{10}$ Lactobacillus paracasei subsp paracasei $19,10^{10}$ Lactobacillus plantarum, and 2.5 g of $\beta$ glucan, inulin, pectin, and resistant starch), as well as a mixture of micro and macronutrients. After 7 days, intestinal permeability decreased in the synbiotic group ( $p<0.05$ ) [4]. However, to date, there are no studies that provide any direct evidence for use of synbiotics in PD.

## 4 Conclusions

Parkinson's disease is the second most common neurodegenerative disease of aging, and is characterized by neuronal inclusions comprised of $\alpha$-synuclein aggregates contributing to debilitating motor and non-motor symptoms. Parkinson's disease is thought to be caused by neuronal oxidative stress, and more recently, it has been suggested that this oxidative stress may originate in the GI tract and be the initial site for neuronal damage; this is based in part on the exposure of the GI tract to potentially injurious factors such as bacterial products capable of inducing oxidative stress. Those with PD have been shown to have increased intestinal permeability, intestinal $\alpha$-synuclein aggregates, and increased bacterial translocation and oxidative stress. While impact of diet on PD risk is limited, it is suggestive that diets high in saturated fat from animal sources have a negative impact, and unsaturated fats, and foods containing antioxidants may be protective as evidenced by analysis of dietary patterns. Altering PD course through dietary means is difficult, and further experimentation should be done to determine if this modulation is impactful; however, potential exists for fibers such as prebiotics to beneficially modify the gut milieu, reducing constipation in individuals with PD. Due to the potential role of the gastrointestinal barrier in exposure to injurious factors, therapeutic intervention through whole foods, dietary patterns, and supplemental nutrition (probiotics, prebiotics, and synbiotics) may positively impact intestinal milieu and result in reduced inflammation and oxidation and reduced risk for PD.

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# Chapter 12 <br> Eat to Heal: Natural Inducers of the Heme Oxygenase-1 System 

Matheus Correa-Costa and Leo E. Otterbein

## 1 Introduction

Tissue injuries can be assessed by a number of factors including hypoxia, drug toxicity, diabetes, shock, and hormonal axes such as the renin-angiotensin system, among others. Most of these lesions are characterized by increased oxidative stress, exaggerated inflammatory sequelae, and pro-fibrotic stimuli that can lead to organ damage and failure if not resolved efficiently. These factors influence local homeostasis and can increase cell death and/or transdifferentiation. In response to changes in the environment, both intra- and extracellular, nature designed intricate and effective solutions, hardwired into all cells that offer elaborate defense mechanisms against overwhelming conditions where the cell is placed in peril. The greater the resilience of the tissue compartment the better the cell is able to manage the stressful insult, and with it, better recovery from disease. Induction of HO-1 is one such modality that offers profound protective effects and may explain one mechanism as to how immune tolerance is achieved [1].

Heme (iron protoporphyrin IX) is part of the prosthetic group contained within various proteins and enzymes including hemoglobin, nitric oxide synthase, mitochondrial oxidases, cytochrome P-450, cyclooxygenase, and catalase. Heme is involved in critical functions, such as oxygen delivery, mitochondrial respiration, and signal transduction [2, 3]. As such the need for heme processing is essential. HO, described by Tenhunen in 1968, is the rate-limiting enzyme responsible for heme degradation [4]. Heme oxygenase cleaves the heme ring, and, as a result, biliverdin is generated, releasing as products iron and carbon monoxide (CO) in equimolar quantities. Biliverdin is subsequently converted to bilirubin by biliverdin reductase, which is located as a surface receptor and intracellular kinase in most cells [5, 6].

[^12]The HO enzyme exists as one of the two distinct isoforms, HO-1 (inducible) and HO-2 (constitutive), which are products of different genes. HO-1 is primarily localized in microsomes and is ubiquitously present in mammalian tissue, and under normal physiologic conditions, its expression is relatively low. The only exception is the spleen, where HO-1 is important for the recycling of iron from senescent erythrocytes. Recent studies show that HO-1 deficiency affects stress erythropoiesis and leads to reduced function and viability of erythrophagocytosing macrophages, resulting in tissue damage and iron redistribution [7, 8]. Importantly, these HO-1-deficient animals are exquisitely sensitive to stress of any kind, but particularly so to heme.

HO-2 also functions as a regulator of cell function. It is present in mitochondria and generally expressed in the brain, testis, endothelium, nephron distal segments, liver, and gastrointestinal tract where it regulates vasomotor tone, neuronal signaling, and circadian rhythms [9]. HO-2 shares $40 \%$ of amino acid homology with HO-1 [10].

## 2 Protective Effects of Heme Oxygenase-1

Of the two isoforms, HO-1 is the most studied and, when increased, provides greater cytoprotection. Therefore in the context of this chapter, we focus on this isoform. HO-1 functions primarily as an antioxidant indirectly first by removing excessive heme from the milieu, the iron of which can act as a pro-oxidant by generating hydroxyl radicals through Fenton chemistry [11]. The free iron released from heme stimulates the expression of ferritin, an intracellular iron reservoir, that sequesters the iron [12]. Furthermore, biliverdin and bilirubin formation display important antioxidant effects, as both molecules are potent peroxyl radical scavengers [13].

The effects of HO-1 on proliferation are intriguing as there are clear differences depending on the cell type. Antiproliferative effects are observed in primary cells including vascular smooth muscle cells, $T$ cells, and epithelial cells but also cancer cells [14-18]. A recent study showed that rapamycin could induce HO-1 expression, and this upregulation led to protection in a model of pulmonary disease. The same work showed that smooth muscle cells derived from animals deficient for HO-1 were not responsive to the antiproliferative or the cell cycle inhibition actions of rapamycin [19]. Moreover, studies have shown that a possible mechanism related to inhibition of cell growth by HO-1 could be the upregulation of inhibitory protein $\mathrm{p} 21^{\text {cip }}$; HO-1-deficient mice show profound hyperproliferative effects in response to vascular trauma [15]. Interestingly, this pathway also contributes to antiapoptotic effects of HO-1 [20, 21]. HO-1 can also exhibit pro-proliferative effects when induced in endothelial cells and hepatocytes [22, 23]. Such difference must be due to the fact that, in some diseases, proliferation is beneficial for organ recovery. A recent study showed that treatment with CO increased the protein levels of cyclins D1 and E, with reduction of p21, leading to a better outcome after hepatectomy [22].

HO-1 can also act as an immunomodulatory enzyme, especially in T lymphocyte-mediated diseases [24]. Burt et al. proposed that HO-1 contributes to T cell homeostasis, maintaining these lymphocytes in a nonactivated state while pharmacological inhibition of HO-1 leads to T cell activation and proliferation [18]. The importance of HO-1 in Treg cells was described by a couple of works showing that $\mathrm{CD} 4^{+} \mathrm{CD} 25^{+}$Treg cells constitutively express HO-1 and that this enzyme could be induced by FoxP3 expression in $\mathrm{CD} 4^{+} \mathrm{CD} 25^{-}$cells, thereby conferring the regulatory phenotype [25, 26]. Others showed in a murine model of colitis that treatment with hemin, to induce HO-1, resulted in expansion of Treg cells while decreasing the levels of Th17-related molecules. Inhibition of HO-1 led to opposing effects and aggravated the disease [27]. The immunomodulatory effect of HO-1 also influences the priming of T cells. Cheng et al. showed that deletion of HO-1 or use of small interfering RNA in dendritic cells promoted upregulation of major histocompatibility complex class II, enhancing the alloantigen presentation to CD4 ${ }^{+}$T lymphocytes [28].

Finally, the anti-inflammatory properties of HO-1 are perhaps the most well described. Many have shown that the upregulation of HO-1 can directly inhibit the inflammatory process [29-31] triggered by seminal early studies by Nath and Tyrell who showed that administration of heme or hemoglobin in vitro or in vivo prior to insult resulted in modulation of cellular activation and inflammatory or stress responses. These works led to a tidal wave of reports showing that induction of HO-1 decreased the intensity of the inflammatory response and in many cases completely prevented it from occurring with decreased gene expression and protein expression of the prototypical inflammatory cytokines, e.g., TNF- $\alpha$, IL- 6 , and IL-1 $\beta$. Importantly, what was later discovered was that in these cells and animal models, the decrease in pro-inflammatory molecules corresponded to a concomitant increase in protein levels of immunomodulatory or anti-inflammatory cytokines and mediators such as IL-10 [32]. How HO-1 was able to regulate for example defined response remained clouded for years and was concluded to be due to the enzymatic degradation of pro-inflammatory heme molecule and generation of bilirubin. It was not until the early 2000s that each of the products of HO-1 activity possessed powerful modulating effects on cells and tissues. The reader is directed to excellent reviews in the literature on carbon monoxide and the bile pigments, the discussion of which is outside the scope of this chapter [33-35].

As described above, free heme is a highly toxic compound driven by the release of ferrous $(+2)$ iron, which increases the oxidative burden and stress to the cell. Heme when present increases the influx of leukocytes into organs during experimental inflammation [36]. In addition, heme is part of many pro-inflammatory enzymes, like cytochrome p450 mono-oxygenases, inducible nitric oxide synthase, and cyclooxygenase [37]. Therefore HO-1, by removing excessive free heme, influences the optimal activity of those enzymes and their ability to contribute actively to inflammation [38]. Degradation of heme by HO-1 liberates equimolar concentrations of carbon monoxide, biliverdin, and iron, each of which has profound effects on the cell to regulate function. HO-1 expression, induced by heme, is in part regulated by p38 mitogen-activated protein kinase (MAPK). Lee
et al. demonstrated that inhibition of this kinase results in impaired HO-1 induction, and consequently, the protection of human proximal tubular epithelial cells is abrogated [39]. One of the main chemoattractant proteins in the body is monocyte chemotactic protein-1 (MCP-1), which can recruit leukocytes to the site of injury (mainly macrophages, memory T cells, and natural killer cells) [6]. A recent study in mice showed that renal epithelial cells directed to constitutively overexpress HO-1 presented with decreased production of monocyte chemotactic protein-1 (MCP-1) after stimulation with albumin [40]. Moreover, in mice deficient in HO-1 the basal levels of MCP-1 were significantly increased compared to wildtype animals and the levels become even higher in response to nephrotoxic and ischemic insults [41]. Importantly, given that HO-1 is accepted as a stressresponsive enzyme, recent work showed that urinary HO-1 could be a useful and sensitive biomarker for tubule interstitial inflammatory damage in renal diseases [42].

HO-1 has largely been associated with organ protection, and one of the most studied models where beneficial effects have been most clearly observed is in ischemia reperfusion injury (IRI). In this model there is a break of ionic homeostasis due to ATP depletion within the cells, which then initiate programmed cell death. All this is accompanied by an initial intense vasoconstriction, increase in adhesion molecules, reactive oxygen species production, and expression of pro-inflammatory cytokines and chemokines [43]. HO-1 induction with cobalt protoporphyrin prophylactically has been shown to protect animals from IRI [44, 45] [46]. Administration of cobalt chloride protected rats from IRI by increasing hypoxia inducible factor (HIF)-1 $\alpha$, erythropoietin (EPO), glucose transporter (Glut)-1, and vascular endothelial growth factor (VEGF) resulting in diminished macrophage infiltration into the kidney and renal protection [47].

Provided above are a few examples of what has been very well dissected in the literature regarding the salutary effects of having elevated HO-1 expression in models of acute and chronic inflammation. There is no doubt that HO-1 has direct links and regulates the innate inflammatory response. The role of HO-1 as a unique immunomodulatory enzyme system continues to be described, and the reader is referred to excellent reviews that summarize the voluminous number of reports and reviews dedicated to this remarkable enzyme [38, 48-52].

## 3 Natural Heme Oxygenase-1 Inducers

Like many other investigators that study inducible gene expression, the field of heme oxygenases has struggled with how best to take advantage of this powerfully beneficial system. As described above, administration of heme to induce HO-1 carries potential detrimental effects and is costly. Gene therapy remains fraught with many roadblocks. Many pharmaceutical companies are investing in smallmolecule inducers. One possibility with potential promise is to bypass the enzyme itself and administer one or more of its products. While potentially useful this


Fig. 12.1 Natural inducers of heme oxygenase-1 promote protection and improve health
brings its own challenges to substitute for the pleiotropic effects of endogenously generated products by HO-1 itself. What has exploded however is the discovery that a variety of natural compounds present in foods and plants are proving to be very effective inducers of HO-1 in a non-stressful and non-cytotoxic way when taken through the diet (Fig. 12.1). Some of these substances have been used for centuries as alternative medicines and are constituents of a variety of spices and herbs used

Table 12.1 Natural inducers of HO-1 and subsequent cytoprotective effects

| Compound | Mechanism of action | Reference(s) |
| :---: | :---: | :---: |
| Curcumin | $\downarrow$ ROS, TNF- $\alpha$, and lipid peroxidation | [52-54] |
| Flavonoids | $\downarrow$ ROS production | [59-62] |
| Isothiocyanates | $\downarrow$ Inflammatory cytokines and nuclear translocation of p65 | [63, 64] |
| Resveratrol | $\uparrow$ Glucose signaling and $\downarrow$ apoptosis, iNOS, lipid peroxidation, VSMC proliferation, and inflammation | [66-71] |
| Ginkgo biloba | $\downarrow$ Liver damage | [73] |
| Garlic-derived organosulfur compounds | $\downarrow$ Apoptosis | [75] |
| Polyunsaturated fatty acids | $\uparrow$ Activation of antioxidant response elements and $\downarrow$ MMP9 expression | [76, 77] |
| Kahweol (coffee) | $\downarrow$ ROS production and apoptosis | [79] |

worldwide [53, Table 12.1]. The remainder of this chapter is dedicated to discussing what is known regarding natural inducers of HO-1.

## 4 Curcumin

Curcumin, a member of the ginger family, is a popular Indian spice turmeric. This compound comes from a plant named Curcuma longa, and if it is not used fresh it is usually boiled for several hours and dried in hot ovens. Afterwards, the plant is ground into a deep orange-yellow powder commonly used as a spice in curries. Typically found as a yellow powder, it is used as food flavoring primarily by tropical Asian cultures. It is perhaps the most infamous well-studied natural inducer of HO-1. In vitro administration of curcumin upregulates HO-1 in a dose- and timedependent manner [54]. Although it is known that excessive curcumin is cytotoxic, its beneficial effects at low doses are well studied.

Zhong et al. showed in macrophages that curcumin decreases the generation of MCP-1 in a dose-dependent manner and attenuated the generation of reactive oxygen species (ROS) induced by LPS. After treating cells with an HO-1 inhibitor, the protective effects of curcumin were abrogated [55]. Further, curcumin treatment was able to upregulate HO-1 and significantly decreased ROS production, TNF- $\alpha$ expression, and paw thickness in a carrageenan-induced model of paw inflammation. Moreover, the combined treatment of curcumin with quercetin (another flavonoid) enhanced the protective effects of HO-1 overexpression [56].

Curcumin-induced HO-1 expression has been shown to be protective in different organs, indicating that the compound is not tissue specific. To corroborate this fact, in a model of high-fat diet, curcumin, via Nrf2 (a master regulator of HO-1 transcription), was able to attenuate glucose intolerance and increase insulin sensitivity. The authors suggested that this phenomenon was due to the HO-1
upregulation and subsequent decrease in mitochondrial oxidative stress [57]. Cerny and colleagues showed that administration of curcumin decreased transaminase levels in a liver failure model. In this same work, they observed higher HO-1 levels in liver tissue after curcumin treatment and, as a consequence, decreased levels of lipid peroxidation and increased hepatocyte viability [58].

Curcumin showed protective effects in a simulated cold preservation and warm reperfusion injury model of the liver. To further elucidate the role of $\mathrm{HO}-1$ in this model, the authors performed an in vitro $\mathrm{H}_{2} \mathrm{O}_{2}$-mediated oxidative injury. In such set of experiments, the authors pretreated hepatocytes with curcumin and then they added $\mathrm{H}_{2} \mathrm{O}_{2}$ and observed that such compounds were able to upregulate $\mathrm{HO}-1$ and provided a striking protection. The addition of ZnPPIX (a known selective HO-1 inhibitor) decreased cell protection otherwise afforded by curcumin treatment. On the other hand, addition of CO or bilirubin in the same conditions listed above substituted and reversed the deleterious effects of HO-1 inhibition [59]. Finally, one of the possible molecular mechanisms that could be mediating this protection and attenuating cell death is linked to autophagy. Such a process, which allows cells to degrade and recycle damaged organelles, proteins, and other cellular components, has been shown to be upregulated by HO-1 [60]. Curcumin induced a beneficial form of autophagy in human endothelial cells in an oxidative stress model of $\mathrm{H}_{2} \mathrm{O}_{2}$ exposure, enhancing cell survival and potentially becoming a therapeutic target for the treatment of oxidative stress-related diseases [61].

## 5 Flavonoids

Flavonoids are natural antioxidants that belong to the family of polyphenols. These compounds are largely present in plants and include citrus fruits, berries, onions, parsley, legumes, green tea, and cocoa and are used as food or medicine. It has been well described that flavonoids possess important therapeutic properties [53]. One of the most studied flavonoids is quercetin, which has been shown to ameliorate ethanol-induced hepatic disease through HO-1 induction via p38 and ERK/Nrf2 pathways [62]. In a model of hepatic injury, Huang and collaborators observed that the flavones (a subtype of flavonoids), chrysin, apigenin, and luteolin, were protective and decrease oxidative burst with a concommitant dose-dependent upregulation of HO-1 mediated through ERK and Nrf2 signaling. Again, the cytoprotective effects were reversed when HO-1 is inhibited [63]. Soy isoflavone treatment was linked to improvement of antioxidant capacity in the mitochondria of rat brain damaged by injection with beta-amyloid peptides. This compound was even able to reverse beta-amyloid-induced downregulation of Nrf2 and HO-1 protein expression in brain tissue [64]. Corroborating this observation, the same protective pattern was observed with anthocyanin-enriched bilberry extracts, which halted oxidative stress and improved HO-1 levels in cultured human retinal pigment epithelial cells [65].

## 6 Isothiocyanates

Isothiocyanates are important inducers of cytoprotective enzymes, beyond known anti-carcinogenesis properties. Brassica vegetables including cabbage, cauliflower, broccoli, and Brussel sprouts contain high concentrations of glucosinolates, which are the precursors of isothiocyanates. To test that such protection is, at least in part, mediated by HO-1, an elegant series of experiments in macrophages stimulated with endotoxin showed that addition of allyl-isothiocyanates reduced inflammatory cytokines as well as decreased the nuclear translocation of p65, a subunit of NF-кB, a well-described transcription factor that regulates the expression of many inflammatory proteins. The addition of the compounds was accompanied by increased protein expression of Nrf2 and subsequent upregulation of HO-1 [66]. Of note, in another study the authors showed that although this compound is an effective inducer of Nrf2, it has little effect on delaying Nrf2 protein degradation as a potential mechanism of action [67]. Ernst and collaborators evaluated the Nrf2inducing activity of the isothiocyanates iberverin, iberin, and cheirolin. The authors were able to assess that all compounds effectively induced Nrf2 nuclear translocation with consequent expression of HO-1 and $\gamma$-glutamylcysteine synthetase ( $\gamma \mathrm{GCS}$ ), an enzyme important in regulation of the antioxidant glutathione. Moreover, the same study suggested that Nrf2 induction occurred via ERK-dependent signal transduction [68].

## 7 Resveratrol

Resveratrol (3,5,49-trihydroxystilbene) is a natural polyphenol and a member of the phytoalexin family. It can be isolated from the roots of Japanese knotweed, but it is also present in several foods, like peanuts, blueberries, bilberries, and red grapes. And last, but not least, resveratrol constitutes one of the valuable ingredients in red wine. Its cytoprotective effects have been shown to modulate several diseases with the HO-1 system shown to be an important mediator of such protection. Resveratrol has been linked to slowing of the aging process by inducing members of the sirtuin gene family [69, 70]. HO-1 expression has been linked to both sirtuins and resveratrol, so one might posit by inference that HO-1 induction is in some manner linked to the aging process by modulating oxidative stress.

In an in vitro model of pancreatic injury, Cheng and collaborators showed that treatment with resveratrol increased glucose uptake and activated insulin signaling, through an Nrf2-HO-1-dependent pathway [71]. Further, in a model of doxorubicin-induced cardiomyocyte toxicity, the use of resveratrol increased HO-1 expression and decreased cardiac injury and cardiomyocyte apoptosis. The use of an HO-1 inhibitor in combination with resveratrol abrogated all protective effects [72].

Resveratrol has also been shown to induce the Nrf2/HO-1 axis, with consequent beneficial effects in experimental models of neurotoxicity [73], cerebral ischemic injury [74], and neointimal formation [75]. Further, Yu and colleagues described that the resveratrol-mediated protection, in a model of hepatic injury after traumainduced hemorrhage, was due to Akt-dependent upregulation of HO-1 [76]. In contrast, a recent report showed that resveratrol could also downregulate HO-1. In a lung adenocarcinoma model, the authors showed that the presence of resveratrol inhibited HO-1 expression with a subsequent decrease in cell migration and invasion and consequently reduced cancer metastasis [77].

## 8 Other Natural Compounds

In addition to the compounds discussed so far, recent published reports have shown that the list of substances that are natural inducers of HO-1 continues to expand. In a model of ethanol-induced liver disease, treatment with Ginkgo biloba extracts reduced liver damage in an HO-1-dependent manner [78]. Also, garlic-derived organosulfur compounds (diallyl sulfide, diallyl disulfide, and diallyl trisulfide) were able to upregulate HO-1, through an Nrf2-dependent pathway in human hepatoma cells [79] as well as in cardiomyocytes resulting in caspase-3 cleavage inhibition and decreased glucose-induced apoptosis [80].

Further, polyunsaturated fatty acids present in fish oil, especially eicosapentaenoic acid and docosahexaenoic acid (DHA), have been linked to favorable outcomes. Yang et al. showed that DHA treatment upregulates HO-1, increases Nrf2 nuclear translocation, and promotes higher antioxidant response element (ARE) activation measured by luciferase reporter activity. Inhibition of HO-1 in the majority of instances led to a reversal of these protective effects [81]. Using the same DHA compound, another group described that DHA-induced HO-1 expression promoted a decrease in the expression of matrix metalloproteinase 9 (MMP9), thereby reducing the metastatic capacity of cancer cells [82].

Perhaps the most popular beverage in the world is coffee. Scientifically speaking, it is a complex chemical mixture composed of several compounds. Part of it is formed by the caffeic acid esters, including caffeic acid phenethyl ester and caffeic acid ethyl ester. Such substances have been described as being able to promote a tremendous increase in HO-1 expression in macrophages. Moreover, the authors established that the ability of the caffeic acid esters to upregulate $\mathrm{HO}-1$ is dependent upon their chemical structures, rather than their reductive activity [83]. Furthermore, another compound present in coffee with known cytoprotective properties is kahweol. Hwang and Jeong showed in a model of neuronal disease that the use of kahweol reduced ROS production and apoptosis and, consequently, cell death. The authors showed that this protection was achieved by HO-1 induction in a mechanism dependent on PI3K/MAPK pathways, especially AKT and p38 [84].

## 9 Conclusions

Cultures worldwide have come to take advantage of the flora and fauna in their geographical locale and take advantage of the "fruit of the land" not only for sustenance but also for health applications. Aloe and witch hazel and the sap of tropical trees known as dragon's blood have been used for millennia for curing cuts, for itchiness, and as disinfectants. How these agents act in and on the body including the ones that are consumed, as described above, continues to be explored. There is no doubt that their effects on the body must change the expression of gene families including the ones that have come to be labeled as protective. The increased incidence of infectious and chronic diseases worldwide has raised the importance of searching for novel therapeutic targets. Since the 1960s, the HO-1 system has been widely studied and its cytoprotective mechanisms linked to better outcomes in a wide spectrum of diseases. Although most of the research focuses on purified elements for simplicity's sake, the alternative medicine approach has shown that natural elements can be terrific sources of innovative "laboratory substances" with highly specific biologic effects. In this chapter, we elucidated just a few of the powerfully protective effects when Nrf2/HO-1 pathway is activated and provided some examples of natural agents that clearly and simply function by taking advantage of the great healing power of HO-1 in the cell and tissue. In this era of industrialized food, perhaps the path of healing is to simply find a reliable farm and learn from the generations before us that have benefited off what the land has to offer.

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## Part III

Cancer and Cachexia

# Chapter 13 <br> Recent Developments in Treatment of Cachexia 

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## Abbreviations

ACE Angiotensin-converting enzyme
AIDS Acquired immunodeficiency syndrome
CHF Chronic heart failure
MPA Medroxyprogesterone
IL-1 Interleukin-1-b
IL-6 Interleukin-6
TNF- $\alpha$ TNF-alpha
IFN- $\boldsymbol{\gamma} \quad$ Interferon-gamma
IL-4 Interleukin-4
IL-10 Interleukin-10
IL-12 Interleukin-12
IL-15 Interleukin-15
MC4 Melanocortin
COX Cyclooxygenase
PUFA n-3 Polyunsaturated fatty acids
EPA Eicosapentaenoic acid
EPO Erythropoietin
CRF2R Corticotropin releasing factor 2 receptor

[^13]| SARM | Selective androgen receptor modulator |
| :--- | :--- |
| GH | Growth hormone |
| IGF-1 | Insulin-like growth factor-I |
| PIF | Proteolysis-inducing factor |
| TGF-beta | Transforming growth factor-beta |

## 1 Introduction

Cachexia is a syndrome associated with severe illnesses such as cancer, AIDS, chronic heart or kidney disease, chronic obstructive pulmonary disease, chronic infection, sepsis and cancer.

Following statistics, it is the main cause of death in $22 \%$ of cancer patients [1].
It is a complex metabolic syndrome, present in disease, characterised by loss of body weight (at least $5 \%$ ), loss of muscle and fat tissue, inflammation and anorexia. Cancer cachexia is also associated with metabolic alterations such as insulin resistance and increased muscle protein breakdown [2]. A recently published consensus states: "Cachexia, is a complex metabolic syndrome associated with underlying illness and characterised by loss of muscle with or without loss of fat mass. The prominent clinical feature of cachexia is weight loss in adults (corrected for fluid retention) or growth failure in children (excluding endocrine disorders). Anorexia, inflammation, insulin resistance and increased muscle protein breakdown are frequently associated with cachexia. Cachexia is distinct from starvation, age-related loss of muscle mass, primary depression, malabsorption and hyperthyroidism and is associated with increased morbidity" [3].

The loss of body weight is due to a combination of two main factors: on the one hand, anorexia, mediated by an increase in brain serotonergic activity, and on the other, metabolic disturbances in the host (increased energy inefficiency, insulin resistance, abnormal carbohydrate metabolism, adipose tissue dissolution, hypertriglyceridemia and muscle wasting) [2], and therefore, the use of total parenteral nutrition is not the solution to avoid loss of body weight.

With these two factors in mind, the therapeutic strategies have to focus on how to increase food intake and/or reversing catabolism and increasing anabolism in the cancer patient.

## 2 Latest Developments in Cachexia Drug Discovery

### 2.1 Appetite Stimulants

Megestrol acetate: Tomíska et al. [4] showed that an oral MEGACE suspension given to patients with far advanced cancer, suffering from anorexia and weight loss,
improved their appetite and their quality of life. In animals, megestrol acetate increases food intake, lean mass and improves physical performance [5].

Megestrol acetate and medroxyprogesterone (MPA) are synthetic, orally active derivatives of the naturally occurring progesterone. In humans these compounds stimulate appetite, caloric intake and nutritional status, as seen in several clinical trials (Table 13.1). In the case of megestrol acetate, the associated weight gain is probably partially mediated by the neuropeptide Y, a potent central appetite stimulant (Fig. 13.1). MPA has also shown to reduce the in vitro production of serotonin and cytokines (interleukin-1-b (IL-1), interleukin-6 (IL-6) and TNF- $\alpha$ ) by peripheral blood mononuclear cells of cancer patients. These humoral factors participate in the cachectic-anorexic response. Oral suspension of the progestational agent may be particularly useful in patients with far advanced disease, unable to take larger amount of pills.

Ghrelin: The orexigenic mediator ghrelin-an endogenous ligand for the growth hormone secretagogue receptor-has a key role in increasing appetite. In addition to increasing food intake, an experimental study [6] has shown that ghrelin improves cardiac structure and function, and diminishes the development of cardiac cachexia in CHF, suggesting that ghrelin has cardiovascular effects and regulates energy metabolism through growth hormone-dependent and -independent mechanisms (Fig. 13.1). Administration of ghrelin may be a new therapeutic strategy for the treatment of severe CHF. A phase II randomised, placebo-controlled, doubleblind study, using an oral ghrelin mimetic, showed an improvement in lean body mass, total body mass and hand grip strength in cachectic cancer patients [7]. Currently there are several clinical trials with ghrelin (Table 13.1). In particular, ANAMORELIN [7] (Helsinn Therapeutics), a ghrelin receptor agonist, administered orally, is on a phase II clinical trial for non-small-cell lung cancer patients (Table 13.1). Asubio Pharmaceuticals is involved in a phase II clinical trial with synthetic human ghrelin (SUN11031) in COPD patients (Table 13.1).

Other appetite stimulants present in clinical trials are PH 284 Pherin Pharmaceuticals (Phase II, end-stage cancer patients) (Table 13.1) and AEZS-130, an oral peptidomimetic growth hormone secretagogue (Aeterna Zentaris), now in phase I (Table 13.1). The exact target of PH 284 is not published yet.

MC4 receptor antagonists: Melanocortin (MC4) receptor is involved in the anorexigenic cascade, decreasing the neuropeptide Y and therefore food intake. The use of MC4 receptor antagonists has been proved to be effective in preventing anorexia, loss of lean body mass (Fig. 13.1) and basal energy expenditure in experimental animals suffering from cachexia [8]. Santhera pharmaceuticals have developed several orally active MC4 receptor antagonists for the treatment of cancer cachexia (Table 13.1). SNT207707 and SNT209858, two recently discovered, non-peptidic, orally active MC-4 receptor antagonists and BL-6020/979, an orally available, selective and potent MC-4R antagonist have all increased food intake and attenuated the reductions in body weight and muscle mass in mice bearing the C26 colon adenocarcinoma [9]. Unfortunately, only animal data are available.
Table 13.1 Selected promising cachexia treatment (adapted from [39])

|  | Company | Type | Pathological <br> condition | Clinical <br> Trial | Target |
| :--- | :--- | :--- | :--- | :--- | :--- |
| Drug | Acacia Pharma | Formoterol (beta-2agonist) <br> acetate | Phegestrol | Cancer | Phase IIa | | Anabolic/ |
| :---: |
| Anticatabolic |


| Phase IIb | Anticatabolic |
| :--- | :--- |
| Phase III | Anticatabolic |
| Phase II | Anabolic/ |
|  | Anticatabolic |
| Phase II/III | Anabolic/ |
|  | Anticatabolic |
| Phase II | Anticatabolic |
| Phase II | Unknown |
| Preclinical | Anabolic |
| Preclinical | Anticatabolic |
| Preclinical | Anticatabolic |
| Preclinical | Anticatabolic |

Cancer
Cancer (NSCLC)
Cancer (NSCLC)
Cancer
Cancer
Cancer
Cancer
Cancer
Cancer
Cancer
Peptide nucleic acid immunomodulator ACE inhibitor
Beta blocker
Nutraceutical
COX-2 inhibitor Unknown antibody
MC4 receptor antagonist
Soluble Activin receptor Type IIB
Cytokine
CRF2R A
CRF2R Agonist

## OHR Pharma <br> Ark Therapeutics

PsiOxus Therapeutics

## Nestle, Danone, Abbott,

Fresenius
Novartis
Santhera Pharmaceuticals
Amgen

OHR/AVR 118
Vitor
MT-102
EPA
Celecoxib (Celebrex)
BYM338
BL-6020/979
None
IL-15
PG-873637


Fig. 13.1 Anti-cachectic therapies based on stimulating appetite and interacting with both pro-cachectic and anti-cachectic cytokines

Cyproheptadine: anorexia may be mediated by an increased serotonergic activity in the brain (Fig. 13.1) [10]. Therefore, attempts to block serotonin activity during cancer cachexia have involved the use of cyproheptadine, a serotonin antagonist usually used for the treatment of allergies, and quite common as appetite stimulant. However, it did not prevent progressive weight loss in patients with advanced malignant disease [11]. A pilot study demonstrates that cyproheptadine treatment increases bodyweight in cachectic children [12].

### 2.2 Counteracting Pro-cachectic Cytokines

Cytokines act on multiple target sites such as bone marrow, myocytes, hepatocytes, adipocytes, endothelial cells and neurons, where they produce a complex cascade of biological responses accountable for the wasting in cachexia. The cytokines that have been implicated in this cachectic response are TNF- $\alpha$, IL-1, IL-6 and interferon-gamma (IFN- $\gamma$ ). They share the same metabolic effects and their activities are closely interrelated. In many cases, these cytokines exhibit synergic effects when administered together. Therefore, therapeutic strategies have been based on either blocking their synthesis or their action (Fig. 13.1).

Thalidomide ( $\alpha-N$-phthalimido glutarimide) is a drug that brings back sad memories. Indeed, its use for treatment against morning sickness in pregnant women in the late 50 s and early 60 s caused over 10,000 cases of severe malformations in newborn children. However, its use is being revised, since it has been demonstrated that it suppresses TNF- $\alpha$ production in monocytes in vitro [13] and normalises elevated TNF- $\alpha$ levels in vivo. A randomised placebo control trial showed that the drug was well tolerated and effective at attenuating loss of weight and lean body mass in patients with advanced pancreatic cancer [14] (Fig. 13.1). Lenalidomide is a thalidomide derivative developed by Celgene, approved for treating myelodysplastic syndromes, and is now being tested in a phase II clinical trial with advanced cancer patients (Table 13.1).

Other anti-cytokine strategies such as Etanercept (fusion protein directed against p75 TNF- $\alpha$ receptor) [15] showed, that patients with several advanced malignancies, treated with Etanercept, combined with an antitumor agent (Docetaxel) had more strength and tolerated better the antitumoural treatment (Fig. 13.1). There is also the work of Steffen et al. [16] showing that anti-TNF reduces rat skeletal muscle wasting in cardiac cachexia.

A humanised monoclonal anti-IL-6 antibody (Alder, Table 13.1) increases haemoglobin levels and prevents muscle wasting in cancer patients (Fig. 13.1). Targeting both TNF- $\alpha$ and IL-6 by means of a broad-spectrum peptide nucleic acid (OHR 118, OHR Pharma), resulted in increases in body weight and physical performance in patients with advanced cancer (Table 13.1).

The degree of the cachectic syndrome is dependent not only on the production of the catabolic pro-inflammatory cytokines but also on the so-called anti-inflammatory cytokines, such as interleukins-4, -10 and -12 (IL-4, IL-10, IL-12). Interleukin15 (IL-15) has been reported to be an anabolic factor for skeletal muscle [17], this cytokine is able to decrease protein degradation, the rate of DNA fragmentation and increase the mitochondrial uncoupling protein 3 expression in skeletal muscle, these being the most important trends associated with muscle wasting during cancer cachexia [17] (Fig. 13.1). In vitro experiments using isolated incubated muscles and muscle cells, support the in vivo observations and indicate that the action of the cytokine is direct upon skeletal muscle [18]. Although no clinical data are available, treatment of cachectic experimental animals with IL-15 leads to an improvement of muscle mass and performance (Table 13.1).

Cell growth may be controlled by the interaction of different types of prostaglandins: large amounts of these compounds are found both in tumour tissue and plasma from cancer patients. Several studies have examined the role of cyclooxygenase (COX) inhibitors on tumour growth and cachexia. The results obtained are not quite clear: Homem-De-Bittencourt et al. report that indomethacin, ibuprofen and aspirin markedly inhibit tumour growth and reduce anorexia in rats bearing the Walker-256 carcinosarcoma [19], McCarthy and Daun (using the same rat tumour model) also report a decrease in tumour weight but no reduction of anorexia or body weight loss [20]. Hussey and Tisdale have studied the effects of the COX-2 inhibitor meloxican on tumour growth and cachexia in the murine adenocarcinoma MAC16 [21]. The results suggest that the inhibitor is able to effectively attenuate
cachexia, possibly having a direct effect on skeletal muscle protein degradation (Fig. 13.1). Celecoxib, a COX-2 inhibitor developed by Pfizer (Table 13.1), has proved to be very efficient in a phase II study involving cachectic cancer patients. Treatment with the inhibitor increased not only lean body mass but also grip force and quality of life.

### 2.3 Other Drugs

The n-3 Polyunsaturated fatty acids (PUFA), as found in fish oil, have been proposed as very active in reducing either tumour growth or the associated wasting, especially of the adipose tissue. The interest in n-3 PUFA was originated from the observation that populations consuming a diet rich in PUFA's showed the lowest incidence of certain types of cancer. Most of the results with PUFA's showed a decrease of tumour cell proliferation and/or aggressivity. An improvement in lean body mass and in the quality of life was observed in a randomised double blind trial using a protein and energy dense N-3-fatty acid-enriched oral supplement [22], supplying 2.2 g or more of eicosapentaenoic acid (EPA)/day (Fig. 13.2). However, data arising from a large multicentre double-blind placebo-controlled trial indicate that EPA administration alone is not successful in the treatment of weight losing patients with advanced gastrointestinal or lung cancer [23]. A meta-analysis based on five trials concluded that there were insufficient data to establish whether oral EPA was better than placebo [24]. Comparisons of EPA combined with a protein energy supplementation versus a protein energy supplementation (without EPA) in the presence of an appetite stimulant (Megestrol Acetate) provided no evidence that EPA improves symptoms associated with the cachexia syndrome in patients with advanced cancer. But several recent trials suggest that EPA-enriched nutrition results in positive outcomes in cancer patients [25, 26]. In CHF, fish oils have anti-inflammatory effects by decreasing TNF- $\alpha$ production and increasing body weight.
$\beta 2$-adrenergic agonists have important effects on protein metabolism in skeletal muscle, improving protein deposition (Fig. 13.2). Apart from the older $\beta 2$-adrenergic agonists, such as clenbuterol, recently newer drugs such a formoterol have taken their place. Its use in experimental animals has proved to reverse muscle wasting associated with cancer [27], with the positive aspect of a lower toxicity. The anti-wasting effects of the drug were based on activation of the rate of protein synthesis and on the inhibition of the rate of muscle proteolysis. Northern blot analysis revealed that formoterol treatment resulted in a decrease in the mRNA content of ubiquitin and proteasome subunits in gastrocnemius muscles, together with a decreased proteasome activity. Probably the main antiproteolytic action of the drug is the inhibition of the ATP-ubiquitin-dependent proteolytic system [27]. The $\beta 2$-agonist also diminished the increased rate of muscle apoptosis present in tumour-bearing animals, and improved muscle regeneration by stimulating satellite cells. It seems as if formoterol has a selective, protective action on heart


Fig. 13.2 Anti-cachectic therapies based on counteracting inflammation and other anabolic and anticatabolic mechanisms
and skeletal muscle by antagonising the enhanced protein degradation present in cancer cachexia, being a potential therapeutic tool in pathologic states where muscle protein hypercatabolism is critical, such as in cancer cachexia or other wasting diseases [27]. Acacia Pharma has undertaken a phase IIa study investigating the effects of a combination of formoterol and megestrol acetate (APD 209) in 13 cachectic cancer patients. Six of the seven patients that completed the treatment had better muscle size and strength, and three patients had improved levels of daily physical activity (Table 13.1).

The administration of erythropoietin ( $E P O$ ) to cancer patients has a clinical benefit in patients with haemoglobin at or below normal levels. Kanzaki et al. [28] have shown that the positive therapeutic effects of EPO in cancer cachexia in tumour-bearing mice are due to an improvement in metabolic and exercise capacity via an increased erythrocyte count, and to the attenuation of cachectic manifestations by decreasing production of the cachexia-inducing cytokine, IL-6 (Fig. 13.2).

ACE-Inhibitors: angiotensin I and II induce directly protein degradation in skeletal muscle (Fig. 13.2). In CHF, inhibition of the angiotensin-converting enzyme (ACE) by administration of enalapril reduces the risk of weight loss and it is linked to improved survival. The results showed increased subcutaneous fat (increased skin fold thickness) and greater muscle bulk (increased mid-upper arm and tight circumferences), and increased plasma albumin and haematocrit. ACE inhibitors like captopril seem to act by decreasing the production of TNF-a by mononuclear cells, a mechanism to account for the beneficial effects (body weight)
in heart failure patients. The highly lipophilic ACE inhibitor imidapril attenuated the development of weight loss in mice bearing the MAC16 tumour, suggesting that angiotensin II may play a role in the development of cachexia in this model (Angiotensin II stimulates protein degradation through induction of the ubiquitinproteasome pathway). Ark Therapeutics is involved in a phase III study involving non-small-cell lung cancer patients, with VITOR, another type of ACE inhibitor (Table 13.1).

Beta-blockers can reduce body energy expenditure and improve efficiency of substrate utilisation (Fig. 13.2). CHF patients treated with $\beta$-blockers can increase total body fat mass and partially reverse cachexia [29]. A phase II clinical trial with non-small-cell lung cancer patients is now under development with MT-102 (PsiOxus Therapeutics) (Table 13.1).

The treatment with derivatives of gonadal steroids has important side effects such as masculinization, fluid retention and hepatic toxicity. Their benefits are that they promote nitrogen protein accumulation, counteracting the progressive nitrogen loss associated with cachexia (Fig. 13.2). A double-blind placebo-controlled trial suggests that nandrolone decanoate is effective in the treatment of cachectic AIDS patients, increasing lean body mass, quality of life and decreasing anti-AIDS treatment toxicity [30].

A recent clinical trial using a non-steroidal selective androgen receptor modulator (SARM) with the aim to increase lean body mass and improve physical performance in healthy elderly subjects was successful and therefore the potential activity of this class of drugs should be taken into consideration for cancer cachexia [31] (Fig. 13.2). Selective androgen receptor modulators are promising as a new function promoting anabolic therapy for several clinical conditions that manifest muscle wasting. Different SARMs are being developed and essayed in clinical trials at the present moment (Ostarine, GTx (Enobosarm), GLPG0492 (Galapagos) and PS178990 (Pharmacopeia)).

Administration of growth hormone (GH) results in an increase in whole body and skeletal muscle protein synthesis (Fig. 13.2). Animal studies have shown that administration of recombinant rat GH to methylcholanthrene-induced sarcomabearing rats resulted in considerable stimulation of protein synthesis without changing tumour growth, protein degradation or host composition [32]. On the other hand, Wolf et al. have reported improvements in whole body protein balance in cancer patients receiving GH [33]. The same research group has demonstrated that exogenous GH can attenuate weight loss and preserve host body composition in tumour-bearing rats undergoing chemotherapy with doxorubicin without stimulating tumour growth [34]. In untreated HIV patients, growth hormone deficiency contributes to loss of lean and fat mass. Administration of growth hormone successfully reverses this wasting process. GH treatment resulted in an improved nitrogen balance and attenuation of weight loss. Similarly, AIDS patients seem to suffer from GH resistance that can be reverted by either low dosages of recombinant insulin-like growth factor-I (IGF-1) or GH administration. O'Driscoll et al. [35], in a pilot study involving GH administration at the end stage of cardiac failure, suggest that GH has a beneficial effect in cardiac cachexia. However, the
results of these case studies must be interpreted with caution, since spontaneous improvement in functional and haemodynamic capacity cannot be ruled out. Theratechnologies has introduced a GH releasing factor analogue (ThGRF), and it is at present in different phase II trials in hip fracture and COPD patients (Table 13.1).

Myostatin, a transforming growth factor-beta (TGF-beta) super-family member, is a negative regulator of muscle growth and development; it is related to several forms of muscle wasting, such as the severe cachexia seen in AIDS and liver cirrhosis. McFarlane et al. [36] have demonstrated that myostatin induces cachexia through an NF-kB-independent mechanism by antagonising hypertrophy signalling through regulation of the AKT-FoxO1 pathway. Anti-myostatin strategies are therefore promising and should be considered in future clinical trials involving cachectic patients (Fig. 13.2). From this point of view, a phase II study in sarcopenic patients has been undertaken using AMG 745, a peptibody against myostatin (Table 13.1). Acceleron Pharma has performed a phase I study with ACE031, a soluble Activin receptor type IIB (Table 13.1).

The corticotropin releasing factor 2 receptor (CRF2R) has many biological activities, such as modulation of the stress response and the prevention of skeletal muscle wasting. Therefore, the use of CRF2R agonists has proved successfully in partially blocking muscle wasting in several models of experimental cachexia [37] (Fig. 13.2). There is, however, a lack of clinical data.

As previously stated, enhanced protein degradation in skeletal muscle during cachexia involves activation of the ubiquitin-proteasome system in muscle. Therefore, inhibitors of the ubiquitin-proteasome system such as peptide aldehyde, lactacystin and $\beta$-lactone-which effectively can block up to $90 \%$ of the degradation of normal proteins and short-lived proteins in the cells-are potential drugs for the treatment of muscle wasting (Fig. 13.2). The problem is the high toxicity of such compounds, since they are not specific inhibitors of the proteolytic system in muscle tissue. A substance that can specifically block myofibrillar protein degradation in skeletal muscle has not yet been discovered. From this point of view, the discovery of specific muscle ubiquitin ligases (Atrogin-1 and MuRF1) is particularly interesting since a tissue-specific inhibition of ubiquitin-proteasome proteolysis could be achieved if inhibitors of these ligases were discovered.

## 3 Summary

It remains quite clear that nutritional strategies are insufficient to reverse the cachexia syndrome. Therefore, if we want to increase food intake, we have to include pharmacological strategies [38].

Another very important factor is timing: in cancer patients, any therapy (nutritional/metabolic/pharmacological) has to be started at the earliest stage of the disease, before the weight loss reaches an irreversible state. Muscle mass and its loss is a keystone in cancer cachexia, due mainly to an increased degradation of the
myofibrillar protein (due to an activation of the ubiquitin-dependent proteolytic system), accompanied often by a decreased protein synthesis. Therapeutic approaches should aim for the neutralisation of the enhanced myofibrillar protein degradation. Unfortunately no definite mediators of cachexia have been identified, and it is difficult to apply a therapeutic approach based on the neutralisation of the potential mediators involved in muscle wasting (i.e. TNF- $\alpha$, IL-6, IFN-gamma, proteolysis-inducing factor (PIF)) because many of them are involved at the same time in promoting the metabolic alterations and the anorexia present in the cancer patients.

Before designing any strategy, the molecular mechanisms of these mediators have to be identified (Table 13.1). This is especially relevant because different mediators may be sharing the same signalling pathways. Both tumoural and humoral (mainly cytokines) factors-that trigger cachexia-may share common signalling pathways and, therefore, it is not very likely that a single drug will block the complex processes involved in cachexia. In addition, some of the mediators proposed for the wasting syndrome also play a role in the regulation of body weight in absolutely opposite states such as obesity. In conclusion, the future treatment of the cachectic syndrome will no doubt combine different pharmacological approaches.

Future treatments will combine anabolic and anticatabolic strategies, being ghrelin agonists and SARMS amongst the most promising ones. Also the blockage of myostatin could lead to the inhibition of muscle wasting.

## Key Terms and Definitions

Cachexia Physical wasting associated with loss of body weight and muscle mass, and often associated to severe diseases.
Muscle Loss of muscle mass caused either by disease or by lack of use, with wasting corresponding decreases in strength and mobility.
Myostatin Growth differentiation factor involved in the regulation of muscle size, being a potent inhibitor of muscle growth since embryonic development and throughout life.
Ghrelin Peptide hormone produced by epithelial cells lining the fundus of the stomach and epsilon cells of the pancreas that is a stimulant of appetite and feeding, and also a stimulator of growth hormone secretion.
Cytokines Regulatory proteins mainly released by immune cells and that act as intercellular mediators in the generation of the immune response, although some of them also have important metabolic effects.

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# Chapter 14 <br> Individualized Tumor Therapy: Biomarkers and Possibilities for Targeted Therapy with Natural Products 

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## 1 The Resistance Problem

Together with surgery and radiotherapy, chemotherapy is a main pillar of cancer treatment. Preoperative chemotherapy is used to reduce tumor size to enable either complete resection of an otherwise non-resectable tumor or the conservation of organs (e.g., breast) in patients with large tumors. Postoperative chemotherapy aims to kill residual tumor cells in the body that could not be removed by surgery and that could cause metastasis and refractory tumors. For decades, clinical trials have sought to optimize combination therapies for various tumor entities. Although many tumors respond to chemotherapy, not all patients benefit from anticancer drugs. Tumors often develop resistance to drugs and concentrations sufficient to eradicate all cancer cells in the body cannot be achieved due to the severe side effects of chemotherapy. Tumors may either be intrinsically resistant to drugs at the beginning of treatment (primary drug resistance) or initially be responsive to drugs and then develop resistance in the course of subsequent treatment cycles (secondary drug resistance). Some tumor types are sensitive to chemotherapy and have high rates success, e.g., childhood leukemia and testicle tumors. Other tumor types, such as brain tumors, pancreatic carcinoma, and kidney cancer, do not respond well to anticancer drugs. Even tumors of the same organ, but of different histology may respond differently. For instance, small-cell lung carcinoma (SCLC) is frequently sensitive to chemotherapy initially, but develops transient resistance after subsequent treatment cycles (secondary resistance), whereas non-small-cell lung carcinoma (NSCLC) is generally non-responsive to chemotherapy at the time of diagnosis (primary resistance). Other tumor types show mixed reactions with a good response in some patients and unresponsiveness in others, e.g., breast cancer. Our knowledge of which patients will benefit from chemotherapy is still

[^14]incomplete, although much progress has been made in cancer biology to better understand the mechanisms of drug resistance during the past three decades. Therefore, the remaining great challenge in clinical oncology is to predict which individuals will benefit from preoperative or postoperative chemotherapy. Because of the great heterogeneity between patients, tumors, and even between cells within the same tumor, the response rates of advanced cancers to chemotherapy can vary from 10 to $90 \%$ [1].

Significant efforts have been undertaken to identify prognostic markers that indicate the course of a disease and allow one to estimate patient outcomes and survival times [2]. Pathoclinical parameters have been developed that are clinically well established, e.g., tumor histology, tumor size, lymph node involvement, distant metastasis, age and comorbidity of the patient. These parameters are used to estimate the probability of progression-free survival (PFS) or overall survival (OS) of large cohorts of patients suffering from the same tumor type. These parameters indicate the survival likelihoods for subgroups of patients, but they do not predict the benefit of a specific therapy to any given individual patient.

In contrast to prognostic markers, predictive markers indicate the probability that an individual patient will to respond to a certain therapy [3]. Predictive markers may serve to select an anticancer agent for optimal response in a single tumor. Patients that are predicted not to respond to a specific chemotherapy could receive an alternative regimen with a greater probability of success. If predictive markers indicate that no possible successful treatments exist, a decision could be made not to treat a patient with chemotherapy, thus avoiding unnecessary toxicities and improving the patient's quality of life. Therefore, the aim of identifying and applying predictive markers is to improve effectiveness of tumor eradication and to avoid unnecessary toxicity to normal organs. Hence, predictive markers contribute not only to treatment success but also to improved quality of life for the patient. By avoiding ineffective treatment of unresponsive tumors, predictive markers may also help to decrease health-care costs-an aspect that is worth considering in the context of today's ever-growing health-care systems.

## 2 Chemosensitivity Testing

The idea of finding predictive markers for individualized cancer therapy was broached six decades ago [4]. Four decades ago, researchers investigated the use of assays to determine drug responsiveness of isolated tumor cells in vitro and to make predictions about the sensitivity or resistance of the patient [5-9]. Unfortunately, none of these first generation chemosensitivity assays were clinically established for routine diagnosis. Issues of practicality, e.g., long readout times, handling of radioactive material, necessitated new assays that were easier to perform. An important observation was that tumors resistant to one drug tend also to be resistant to other drugs [10]. At a time when the phenomenon of multidrug resistance was still unknown and most clinicians trusted combination
therapy to overcome resistance, some oncologists were reluctant to accept the resistance profiles ("oncogrammes") to a panel of drugs obtained by these chemosensitivity tests.

More recently, differential staining assays and ATP bioluminescence assays to measure tumor cell death have been developed. These assays are better accepted in the scientific community than the early chemosensitivity assays [11]. These newer assays have demonstrated high sensitivity and reproducibility and significant associations between in vitro results and drug response in patients for several tumor types [12-14].

Owing to the fact that suspensions of isolated tumor cells might not sufficiently reflect drug response in three-dimensional tumor issues, more sophisticated test models have been developed. For example, some assays use three-dimensional spheroid tumor cultures $[15,16]$ or measure in vitro metabolic activity of tumor biopsy pieces or slices, which retain the original tissue architecture of a tumor [17, 18].

## 3 Biomarkers

A plethora of investigations have focused on determining the prognostic value of cancer-related proteins or mRNA sequences. Drug resistance genes (ABC transporters, glutathione S-transferases, DNA topoisomerases, etc.), apoptosis genes (Bcl2-family members, survivin, Fas, caspases, etc.), DNA repair genes (MGMT, BRCA1/2, ERCC1), oncogenes (RAS, EGFR, HER2, MYC, etc.), tumor suppressor genes (TP53, RB1, etc.), metastasis genes (NM23 etc.), hormone receptors (ER, PR), and many others have been investigated [19-21]. Except for some examples (ER, HER2, EGFR, RAS) no reliable biomarkers are clinically available to predict the drug response of a tumor in an individual patient. One major difficulty in identifying biomarkers with prognostic value is the technical problems encountered.

Immunohistochemistry is a simple technique that is well suited for routine clinical application. However, it requires highly specific antibodies that do not cross-react with anything else in the sample, and the antibodies must be suitable for paraffin-embedded tissue. Guidelines exist for standardized semiquantitative evaluation of immunostainings. There is unexpectedly high variability between immunohistochemistry results obtained in different laboratories necessitating a standardized consensus on the assay protocol [22-24].

It turned out that multiple factors rather than single factors are involved in determining drug resistance and that single biomarkers are generally not sufficient to predict drug response. Investigations into the multifactorial nature of drug resistance take advantage of the development of the so-called "-omics" technologies (genomics and proteomics, which determine gene and protein expression levels at the proteome, transcriptome, and genome levels). The hope of such investigations is that obtaining a comprehensive picture of the gene and protein expression
levels will facilitate the identification of factors associated with drug sensitivity or resistance and improve biomarkers profiles for predictive diagnosis in individual patients.

In the following paragraphs, we report on our experiences and results in the quest for novel prognostic and predictive biomarkers.

### 3.1 DNA Biomarkers

### 3.1.1 Cytogenetic Aberrations

Chromosomal aberrations (amplifications, deletions, translocations, etc.) have been shown to contribute not only to the development of cancer but also to the survival time of patients and tumor response to therapy [25-27].

In 118 kidney carcinomas, classical cytogenetic staining techniques (DAPI staining and G-banding) revealed that a gain of band 31 to the end of the long arm of chromosome 5 (5q31qter) was significantly associated with better patient prognosis [28].

A more advanced technique is comparative genomic hybridization (CGH), which is able to detect unbalanced chromosomal aberrations (gains and losses of DNA). In preliminary investigations of sensitive and drug-resistant leukemia cell lines, we found that aberrations at the chromosomal loci $5 q 13,5 p 13 p 15.2,9 \mathrm{p} 21$, $9 q 31$ and 14 q21qter were associated with resistance to cytostatic drugs [29, 30]. We also investigated solid tumors by means of CGH in terms of prognostic value for survival time and predictive value for response to therapy. Among a total number of 35 oral squamous cell carcinomas, a gain at 7 p 12 was associated with decreased disease-free survival of patients in a subset of tumors [31]. The chromosomal locus 7 p 21 harbors the gene for the epidermal growth factor receptor ( $E G F R$ ), the abundance of which is a well known prognostic factor in oral squamous cell carcinoma and other tumor types [32-34].

### 3.1.2 Single Nucleotide Polymorphisms

Variations in the genetic code appear frequently in human genomes [35]. It was estimated that up to 250,000 single nucleotide polymorphisms (SNPs) are located within or close to coding regions of genes [36]. Therefore, the determination of SNPs and their relation to prognostic and predictive power has been a thriving field of research in the past years [37, 38].

Treatment of estrogen receptor-positive breast cancer by tamoxifen is a mainstay in the management of this disease. In the body, tamoxifen is metabolized to 4-OH-tamoxifen and 4-OH- N -desmethyltamoxifen (endoxifen), which binds to estrogen receptors with much higher affinity than tamoxifen itself. The conversion takes place in the liver by the drug-metabolizing enzyme cytochrome P450
monooxygenase isoform 2D6 (CYP2D6). Women with reduced CYP2D6 activity due to single nucleotide polymorphisms produce significantly less endoxifen and derive little benefit from tamoxifen therapy, despite positive estrogen receptor status in their tumors [39, 40]. Hence, CYP2D6 polymorphisms contribute to drug resistance and may serve as a predictive biomarker for therapy success.

Multidrug resistance is a severe obstacle to successfully curing cancer patients via chemotherapy. Drug efflux transporters, such as those of the ATP-binding cassette (ABC) transporter family, pump cancer drugs out of cancer cells and thereby increase survival of cancer cells. The best-known ABC transporter is P-glycoprotein, which is encoded by the $A B C B 1 / M D R 1$ gene [41, 42]. A total of 29 SNPs have been found in the human $A B C B 1 / M D R 1$ gene, some of which are thought to be of prognostic and predictive significance [43]. In colorectal carcinomas and acute lymphoblastic leukemias, we found that the C3435T polymorphism in the $A B C B 1 / M D R 1$ gene seemed to have no association with prognosis or response to therapy [44, 45]. These results are in accordance with the controversy surrounding the prognostic value of this particular SNP [46, 47].

### 3.1.3 DNA Methylation

One factor that influences gene expression epigenetically is the methylation of CpG islands in gene promoters. Among a panel of approximately $8,000 \mathrm{CpG}$ island fragments, 694 CpG island loci were identified to be hypermethylated in 14 colorectal tumors. One subpopulation showed high levels of hypermethylation, while the other one revealed little or no methylation [48]. This result pointed to a role for the known CpG island methylator phenotype (CIMP) in colorectal tumors [49]. It is a matter of ongoing discussion that the CIMP phenotype may be useful as a prognostic and predictive biomarker [50-52].

### 3.2 RNA Biomarkers

While in the past many investigations focused on the mRNA expression of single genes, the advent of microarray technology has revolutionized clinical oncology. Microarray analysis makes possible the identification of novel biomarkers and entire gene profiles with prognostic and predictive value at a transcriptome-wide level. Microarray technology led to the identification of novel tumors subtypes of otherwise histologically homogenous subgroups. It has also resulted in the association of gene expression profiles with response to chemotherapy and radiotherapy [53-56].

As mentioned above, ABC transporters translocate a diverse array of substrates. The human genome consists of 49 different ABC transporter genes, many of which have still not been well characterized. We have developed a low-density microarray with the human ABC transporters as a tool to investigate the role of known and
novel ABC transporters in drug resistance in cancer cell lines [57]. Applying this microarray to human biopsies, we found that acute myeloid leukemia (AML) cells overexpressed the $A B C A 2, A B C A 3, A B C B 2$, and $A B C C 10$ genes in comparison to healthy bone marrow samples [58]. Of them, $A B C A 3$ expression was three times higher in tumors of patients who did not achieve remission after chemotherapy than in patients in remission. In clinical samples of t -cell acute lymphoblastic leukemia, we also observed an overexpression of $A B C A 2$ and $A B C A 3$ mRNA [59].

### 3.3 Protein Biomarkers

For more than two decades, immunohistochemical analyses have been performed to identify prognostic and predictive biomarkers to complement established pathoclinical parameters (stage, grade, age, comorbidity, etc.). Over the years, it has become more and more clear that no single biomarker is sufficient to fulfill the requirements. One possibility to cope with the phenomenon of multifactorial drug resistance is to use a battery of selected known markers for prognostic and predictive evaluation in immunohistochemistry assays [60-62]. Another possibility is to apply proteomic methods and to analyze clinical samples in a comprehensive fashion [63-66]. Both approaches have advantages and disadvantages. Immunohistochemistry is easy to perform, but laborious and time-consuming. This technique can, however, be made considerably faster by the use of tissue arrays [67, 68]. Proteomic techniques need careful technical adjustment for reproducible measurements and may produce a high background of nonrelevant signals. On the other hand, novel markers can be identified via proteomics that might be overlooked by conventional techniques.

In NSCLC, we investigated the value of immunohistochemical markers for prognosis of survival, metastasis, and drug resistance. A total of 40 protein markers involved in drug resistance, proliferation, apoptosis, angiogenesis as well as oncoproteins and tumor suppressors were analyzed. The results of the immunohistochemistry were subjected to hierarchical cluster analysis to correlate them with the survival times of 216 patients. The expression profiles of FOS, TP53, RAS, ERBB1, JUN, PCNA, Cyclin A, FAS receptor, and HIF1B were significantly correlated with longer patient survival times [19]. This is of considerable clinical relevance, since patients with NSCLC usually have a poor prognosis for survival. The fact that a specific expression profile was associated with better long-term outcome in lung cancer patients indicates that this approach may be useful for the identification of lung cancer patients with differing prognoses who belong to otherwise histologically homogeneous subgroups. Such delineations would bring us one step away from generalized management of patients with NSCLC and one step closer to improved management of smaller subgroups of patients with this tumor type.

The prognosis of cancer patients is largely determined by the metastasis process. In view of this fact, we investigated 130 patients suffering from NSCLC by
immunohistochemistry and correlated the expression profiles identified by hierarchical cluster analysis with the metastatic status of the patients [20]. The expression of JUN, ERBB2, MYC, cyclin D, PCNA, BFGF, VEGF, and HSP70 were significantly correlated with lymph node metastasis, whereas the expression of Caspase3, FAS and FAS receptor, and PAI were inversely associated with lymph node involvement. Our result that specific expression profiles can be associated with metastasis for NSCLC is in accordance with reports for other tumor types [69-73].

The in vitro response of 94 patients with NSCLC to doxorubicin was determined to identify factors that indicate sensitivity or resistance to this cytostatic drug. By hierarchical cluster analysis of immunohistochemical data of 40 proteins, three different subgroups of tumors were identified [21], each with various expressions of P-glycoprotein, thymidylate synthase, glutathione S-transferase- $\pi$, metallothionein, $\mathrm{O}^{6}$-methylguanine-DNA-methyltransferase and major vault protein, VEGF, FLT1, ECGF1, PCNA, cyclin A and microvessel density. Hence, three different protein expression profiles correlating with doxorubicin resistance appeared in our analysis. This may be taken as a clue that different resistance phenotypes exist in NSCLC.

## 4 Targeted Therapy with Natural Products

### 4.1 Synthetic Lethality

One strategy is to take advantage of cancer-specific mutations that are not present in normal tissues to specifically target and kill cancer cells. If two parallel pathways both contribute to the same cellular process and one pathway is switched off in cancer cells by a specific mutation, the second pathway may be pharmacologically inhibited, which can lead to tumor cell death. Normal cells, which still have one intact pathway, can escape the detrimental effect of this pharmacological intervention [74, 75]. An illustrative example for cancer treatment by synthetic lethality is the inhibition of PARP-1, which specifically kills cancer with mutations in the BRCA1 or BRCA2 genes. Tumors with BRCA1/2 mutations have an impaired ability to repair double-strand breaks by homologous recombination, and are highly sensitive to inhibition of DNA-single strand break repair by PARP-1 inhibitors [76].

In addition to tumors harboring $B R C A 1$ or $B R C A 2$ mutations, PARP-1 inhibitors are also effective against tumors with mutations in other DNA repair genes such as ATM, NBS1, and genes involved in phosphatase and tensin homologue (PTEN) signaling [77].

In our own investigations, we focused on a tumor-specific chromosomal deletion, another class of mutation that provides opportunities for synthetic lethal treatment approaches. The short arm of band 21 of chromosome 8 is frequently deleted ( 9 p 21 del ) in many tumor types [78, 79]. The methylthioadenosine phosphorylase (MTAP) gene is located at 9 p 21 in addition to a cluster of interferon
genes and the tumor suppressor genes INK4A, INK4B, and ARF [80]. MTAP maintains the adenosine pool necessary for DNA synthesis. This enzyme represents a salvage mechanism for DNA synthesis inhibited by anticancer drugs such as methotrexate [81]. Tumors with the 9p21 deletion cannot use the MTAP salvage pathway and thus should respond well to methotrexate treatment. The use of methotrexate for 9p21del tumors represents a synthetic lethal approach comparable to PARP inhibitors for BRCAI/2-mutated tumors. However, tumors frequently develop resistance to methotrexate, e.g., by overexpression of the dihydrofolate reductase (DHFR) gene. Therefore, it would be valuable to identify other drugs that inhibit DNA synthesis in MTAP-deleted tumors without being involved in DHFRmediated drug resistance. L-alanosine is a chemotherapeutic amino acid analogue isolated from the bacterium Streptomyces alanosinicus. We found that methotrexate-resistant, DHFR-overexpressing tumor cells are not cross-resistant to L-alanosine [82, 83]. Therefore, L-alanosine may be more suitable than methotrexate for MTAP-mediated chemoselective treatment of tumors with 9p21 deletion.

### 4.2 Inhibitors of P-Glycoprotein

Inhibition of $A B C$ transporter-mediated drug efflux was suggested three decades ago [84]. However, synthetic compounds to inhibit P-glycoprotein-mediated efflux have not yet been clinically established. Although many synthetic compounds have been described as P-glycoprotein inhibitors [85], these compounds have demonstrated unacceptably high toxicities in clinical trials. The reasons for these adverse effects are as follows:

1. P-glycoprotein is also expressed in certain normal tissues, e.g., in the gastrointestinal tract, liver, kidney, blood-brain barrier. Hence, inhibition of P-glycoprotein may affect not only tumors but also normal tissues [86].
2. Some P-glycoprotein inhibitors were initially developed for other applications. If these drugs are used to block P-glycoprotein in tumors, their primary pharmacological activity may appear as a side effect. For instance, the cardiac activity of verapamil may cause cardiotoxicity when verapamil is used as a P-glycoprotein inhibitor in cancer therapy.
3. Some P-glycoprotein inhibitors induce Phase II liver enzymes (cytochrome P450 monooxygenases), which leads to metabolization and deactivation of established anticancer drugs more efficiently than in standard therapy without P-glycoprotein inhibitors. Hence, chemotherapy is more likely to fail with possibly fatal consequences for patients.
The fact that mdrla/lb $b^{-/-}$knockout mice are viable indicates that P-glycoprotein inhibition may not affect vital functions of organisms [87]. Therefore, continuing the search for P-glycoprotein inhibitors is merited. In particular, the use of natural products, which are generally considered to be less toxic than
synthetic drugs, should be considered. We initiated a search for P-glycoprotein inhibitors derived from natural sources [88]. Bufalin from the toad Bufo bufo inhibited P-glycoprotein efflux as well as transport of another ABC-transporter, MRP1 [89]. In multidrug-resistant cancer cells, dihydroevocarpine, evocarpine, evodiamine, rutaecarpine, and 1-methyl-2-undecyl-4-quinolone isolated from Evodia rutaecarpa as well as geranylated furocoumarins isolated from the fruits of Tetradium daniellii (Rutaceae) inhibited the export of calcein, a substrate of P-glycoprotein and can be used as a fluorescent probe to monitor P-glycoprotein activity [90, 91].

As P-glycoprotein is expressed not only in multidrug-resistant tumor cells but also in endothelial cells of brain blood vessels, P-glycoprotein-inhibiting phytochemicals may also compromise the integrity of the blood-brain barrier. Under physiological conditions, the blood-brain barrier protects the brain from toxic compounds taken up in food. However, it also inhibits drugs to treat brain cancer or neurodegenerative diseases from entering the brain. Therefore, delivery of such drugs can be improved by co-treatment with inhibitors of the blood-brain barrier. Investigating 57 compounds isolated from medicinal plants used in traditional Chinese medicine, we identified several P-glycoprotein inhibitors that increase calcein uptake of porcine brain capillary endothelial cells (PBCEC) (baicalein, bufalin, ent-16-atisen-19-oic acid, ent-15-antisen-19-oic acid, 4-methoxy[2,3-b] quinolone, glycomine A, glycomine B, deoxyserofendic acid, shogaol) [92]. Interestingly, two of these compounds inhibited P-glycoprotein-mediated calcein transport but were not cytotoxic (ent-16-atisen-19-oic acid and 4-methoxy[2,3-b] quinolone). Compounds that are nontoxic to PBCBC and human brain endothelial cells are needed to improve uptake of drugs for Alzheimer's or Parkinson's disease. Therefore, these two blood-brain barrier inhibitors may be interesting candidates for therapy of neurodegenerative diseases. Furthermore, four out of eight tested alkamides isolated from Echinacea angustifolia inhibited P-glycoprotein-mediated calcein transport in PBCEC [93].

### 4.3 Non-Cross-resistant Phytochemicals

Another strategy to overcome multidrug resistance is to use compounds that cannot be transported by P-glycoprotein. Such compounds should kill multidrug-resistant cells with similar efficacy as drug-sensitive ones. In the past, it has been shown that drugs from certain drug classes (e.g., alkylating agents and antimetabolites) are not recognized by P-glycoprotein as substrates and that multidrug-resistant cells are not cross-resistant to these drugs. This observation reinforces the relevance of combination regimens for cancer therapy. Application of drugs with different modes-ofaction can minimize the occurrence of cross-resistance and increase the success of chemotherapy. It is reasonable to search for novel cytotoxic phytochemicals that are not substrates of P-glycoprotein and whose modes of action are different from classical anticancer drugs. Among the phytochemicals that did not reveal cross-
resistance in P-glycoprotein-expressing cancer cells were maslinic acid, $N$ - $p$ coumaryl tyramine, and (E)-3-(4-hydroxyphenyl)-[2-(4-hydroxyphenyl)-ethyl]-prop-2-enamide, all of which were isolated from plants used in traditional Chinese medicine [94]. In addition, we have recently started to investigate phytochemicals isolated from medicinal plants from African folk medicines [95-99, 101]. We found that multidrug-resistant P-glycoprotein-positive tumor cells lacked crossresistance against several compounds from African plants, i.e., 6,8-diprenyleriodictyol from Dorstenia dinklagei, isoxanthochymol from Garcinia punctata, and guieranone A from Guiera senegalensis [98, 100-102].

### 4.4 Collateral Sensitivity of Multidrug-Resistant Cells

Remarkably, we found some phytochemicals that provoked hypersensitive responses in multidrug-resistant cells as compared to their parental drug-sensitive cell lines. This phenomenon, termed collateral sensitivity, is when a drug kills otherwise drug-resistant cells at lower concentrations than are needed to kill drugsensitive cells. The compounds that revealed collateral sensitivity in our investigations were cantharidin, tetracentronside, 3-(2-hydroxyethyl)-1H-indole-5-O- $\beta$-Dglucopyranoside, kaempferol, and gancaonin Q [10, 100]. Collateral sensitivity is a long-known phenomenon of drug-resistant tumors and many synthetic drugs have been described that invoke collateral sensitivity in resistant tumor cells [103, 104]. In fact, this phenomenon is not restricted to tumor cells and can also be found in drug-resistant E. coli or Saccharomyces cerevisiae [105, 106]. Collateral sensitivity of multidrug-resistant tumor cells towards natural products has not yet been analyzed in detail.

Collateral sensitivity to drugs may be used to specifically eradicate multidrugresistant cells and therefore has been compared to the concept of synthetic lethality [107, 108]. In synthetic lethality, normal cells are spared from the cytotoxicity of a small molecule since it specifically targets a mutation solely present in cancer cells (see above). In the context of multidrug resistance, collateral sensitivity to certain drugs results in specific eradication of P-glycoprotein-expressing cells while sparing normal cells.

### 4.5 Inhibitors of the Epidermal Growth Factor Receptor

The epidermal growth factor receptor (EGFR, c-ERBB1, HER1) is an important oncogene in many cancer types with prognostic relevance in estimating survival time and predictive value for the response of tumors to chemotherapy (see Sects. 3.1 and 3.3). Together with three other members of this gene family (HER2-4), EGFR regulates proliferation, differentiation, apoptosis, and metastasis [109].

In addition to its prognostic and predictive importance, EGFR is an exquisite target for the development of therapeutic antibodies and small molecules. Recently, it has clinically been recognized that EGFR-expressing tumors can develop resistance towards EGFR-directed drugs either by the emergence of point-mutated tumor subpopulations [110] or through mutations in downstream-signaling pathways. Thus, novel compounds are required for targeting EGFR-expressing tumors resistant to established EGFR inhibitors. The idea that novel EGFR inhibitors able to attack EGFR-mutated cancer cells has been recently validated in a highthroughput screening [111].

Starting from a library of 2,400 phytochemical compounds used in traditional Chinese medicine, a bioinformatical molecular docking approach was used to identify a panel of 20 candidate compounds that bind to EGFR's tyrosine kinase binding domain [33]. One of these compounds, dicentrine, is an aporphine-type isoquinoline alkaloid that preferentially killed EGFR-transfected cancer cells compared to the non-transfected control cells [112]. Using transcriptome-wide mRNA microarray analyses and bioinformatical pathway profiling, a number of signaling pathways were associated with the preferential cytotoxicity of dicentrine in EGFRtransfected cells including p53 signaling, BRCA1 damage response, G1/S and G2/M cell cycle regulation, and the aryl hydrocarbon receptor pathway [112].

Camptothecin derivatives were also found to exert preferential cytotoxicity in EGFR-transfected cells. Camptothecins are generally classified as DNA topoisomerase I inhibitors. Since the relationship between expression of DNA topoisomerase I and response of clinical tumors to camptothecins is rather weak, it has been suggested that other mechanisms may also play a role in camptothecin-mediated cytotoxicity. Our molecular docking results showed that camptothecin and its derivative, camptothecin 20- $\mathrm{N}, \mathrm{N}$-glycinate, bind to the same pharmacophore, albeit to partially different amino acids, as the clinically established EGFR-inhibitor, erlotinib [113]. Microarray analysis and pathway analysis revealed that G2/M DNA damage response, aryl hydrocarbon receptor signaling, and endoplasmic reticulum stress were among the pathways that might explain the preferential cytotoxicity of these camptothecins in EGFR-transfected cancer cells [113].

We also found one natural product derivative that targets signaling downstream of EGFR. Artesunate is a semisynthetic derivative of artemisinin, the active principle of Artemisia annиa L. Microarray-based gene expression profiles of the human kinome were correlated with the $\mathrm{IC}_{50}$ values for artesunate in 55 tumor cell lines. Significant relationships were found to genes of the RAS $>$ RAF $>$ MEK $>$ ERK pathway [114]. These associations were corroborated using cell lines transfected with cDNA for these signal transduction proteins. Transfected cell lines displayed more apoptosis upon artesunate treatment than non-transfected cell lines. The combination of erlotinib with artesunate resulted in synergistic cell killing in EGFR-transfected cancer cells but not in non-transfected control cells [115]. These results demonstrate that resistance of cancer cells to erlotinib may be modulated by phytochemicals or plant-based derivatives.

## 5 Conclusions and Perspectives

The search for prognostic and predictive markers over the past two decades has shown that tumor cell response to therapy cannot be explained sufficiently by any one single factor and that drug sensitivity and resistance is multifactorial in nature. The "-omics" technologies can help to identify novel biomarkers at the genomic, transcriptomic, and proteomic level. Various expression profiles are associated with subgroups of drug-resistant tumors and may aid in identifying novel subgroups with different therapy responses and survival times in otherwise histologically homogenous and non-distinguishable tumors [116-121].

On the other hand, we and others have often observed that the "omics"-based determination of thousands of genes is not necessarily superior to the measurement of a well-defined set of $10-50$ genes or proteins [125]. As a result, test systems focusing on a limited number of genes have been marketed as a robust clinical tool for estimating prognosis and treatment response in breast cancer [122-124].

As in the cases of MTAP, EGFR, and P-glycoprotein, the identification of molecular biomarkers also provides possible novel targets for developing tumorand target-specific treatment approaches [33]. Such targeted therapy opens an interesting domain for molecular diagnostics and individualized therapy. For example, overexpression of EGFR or HER2 indicates a worse prognosis if standard chemotherapy is applied, but indicates sensitivity to specific EGFR inhibitors (such as erlotinib or cetuximab) or HER2 inhibitors (such as trastuzumab). This raises the question of how knowledge of prognostic and predictive biomarkers can be integrated with inhibitors to specific cancer-related targets to develop a comprehensive concept of individualized therapy (Fig. 14.1).

Although the idea of personalized medicine is four decades old, it seems that its practical realization is still in its infancy. Given the diversity in the biology of different tumor types and the wealth of new information that can be expected from cancer genome projects, treatment schedules based on molecular diagnosis must be developed individually for each tumor type in order to realize the ultimate goal of personalized cancer therapy. Breast cancer may serve as a model cancer to exemplify how such a schedule for individualized drug treatment may be developed. A recent study of the Cancer Genome Atlas Network described mutations in 30-50 genes that together are sufficient for breast cancer development. This result is based on high-throughput screening of the tumor genomes of 825 breast cancer patients [126]. Breast cancer can be divided into three therapeutically relevant subgroups:

1. Estrogen receptor (ER) positive tumors, which can be treated with antihormonal therapy (tamoxifen) or aromatase inhibitors.
2. HER2 positive tumors, which can be treated with drugs directed against HER2 (trastuzumab).
3. ER-negative and HER2-negative tumors, which must be treated by standard chemotherapy.


Fig. 14.1 From standard chemotherapy to individualized therapy: A vision for the improvement of cancer therapy

Hence, immunohistochemical determination of the ER and HER2 status is decisive in the choice of treatment. The recent investigation of the Cancer Genome Atlas Network [126] based on whole genome sequencing classifies five subgroups of breast cancer tumors:

1. Luminal A tumors, which express estrogen and progesterone receptors, were the most common tumor type ( $44 \%$ ). They are sensitive towards hormonal therapy.
2. Luminal B tumors ( $24 \%$ ) show heightened proliferative activity compared to luminal A tumors. Classical chemotherapy plus hormonal therapy is recommended for this subgroup of tumors.
3. Basal-like tumors (19 \%) are resistant towards hormonal therapy and HER2directed drugs. They are known as triple negative breast cancers and are difficult to treat. There is some evidence that anti-angiogenic drugs might be suitable to manage triple-negative breast cancers.
4. HER2-expressing tumors (11 \%) are heterogeneous in their genetic profile. In general, they can be treated with HER2-directed drugs.
5. Normal-like breast cancers ( $2 \%$ ) are rare tumors. There are no clear options for providing treatment advantages.

This study provides an illustrative example of how genetic information can be translated and how treatment recommendations might be delineated. This is an important success for individualized tumor therapy. It can be expected that the Cancer Genome Atlas Project, which is currently undertaking sequencing of the 50 most important tumor types, will provide information on specific gene mutation profiles for each of these tumor types. This knowledge can then be used for the development of novel targeted therapies.

Considering that the majority of cancer drugs are derived from natural sources [127], natural products will also be an indispensable resource for the development
of new targeted drugs. Chemicals in plants and microorganisms have evolved over millions of years and their amazing bioactivity can be exploited for the development of novel drugs with improved features compared to the classical anticancer drugs. With this growing battery of biomarkers and corresponding targeted drugs, it is likely that custom-tailored combination treatments will soon become a reality and that each individual cancer patient will be treated based on his or her individual molecular tumor architecture.

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# Chapter 15 <br> Nutrition in Oncology: From Treating Cachexia to Targeting the Tumor 

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## 1 Introduction

The laws of physics dominate the universe and dictate that energy tends to equally distribute itself in every corner of the universe. This unavoidable evidence has major influence on life. Considering life as a highly specialized form of energy organization, the constant acquisition, storage, and disposal of energy is mandatory to preserve the energy-consuming intracellular compartments. In this light, it is no doubt that the most important step in the evolution of life was the acquisition of mitochondria by primordial cells, allowing for a more efficient extraction of energy from available nutrients [1].

Energy availability is key for survival. However, its constant acquisition may not be an efficient strategy to secure evolution. Particularly for multicellular organisms, devoting large proportion of available energy to the continuing search, acquisition, digestion, and absorption of nutrients may reduce the quota spendable by other and not less important functions, including reproduction and cognition. Also, nutrients are not constantly available due to seasonal changes, and periodical periods of famine may occur. Therefore, primordial organisms obtained substantial survival advantage when evolution selected biochemical strategies not only to store excess energy and reuse it when needed but also to switch off cellular pathways to save energy during famine [2]. It is no surprise that this biochemical package has been preserved in human genome up to now.

[^15]
## 2 Nutrition: A Key Target in Human Diseases

Human diseases can be defined and described from different points of view, including clinical, social, and economical perspectives. However, their intrinsic nature pertains to thermodynamic. In fact, the constellation of signs and symptoms reported by sick patients could be simply defined as the phenotypical expression of the genetically programmed failure of energy metabolism [3]. The metabolic program triggered by stress or trauma is complex and includes different manifestations [3]. Energy stores are rapidly wasted, whereas its acquisition is reduced due to anorexia. More importantly, available energy is not fully directed to enhance functionality (i.e., immune response, respiration, blood pressure, etc.); rather, it is largely tunneled toward futile cycles. It is acknowledged that such inefficient pathways create a protecting inner environment against invading microbes and traumatic injuries [3]. However, such defense strategy confers a survival advantage lasting few hours, maybe few days. Therefore, this programmed energy failure has minimal clinical impact, and actually it may become detrimental, in critically ill patients surviving their injuries thanks to critical care medicine as well as during chronic diseases, both now representing the vast majority of diseases affecting global population.

It is self-evident that the effective therapy of human diseases is the effective therapy of energy failure. Thus, it should include not only drugs (i.e., antibiotics, vasopressors, etc.) but nutrition as well, as a source of energy, proteins, and nutrients, reprogramming gene expression and redirecting deranged biochemistry.

## 3 Cancer Cachexia, Nutritional Support, and Clinical Outcome

The recently released results of the Global Burden of Disease Study 2010 reveal that, over the last 20 years, cancer maintained its top position among the most prevalent diseases across the world [4]. Consequently, it represented the focus of extraordinary and expensive efforts to develop effective therapeutic strategies, particularly aiming at improving the outcome of patients with advanced disease. Unfortunately, with limited results [5]. Interestingly, only recently nutrition has received attention and has been considered in the multimodal approach to cancer patients [6].

Among other pathological changes, the clinical journey of cancer patients is characterized by a metabolic switch, which directs energy metabolism from anabolism to catabolism [7]. The interest in food and appetite is reduced (i.e., cancer anorexia), thereby energy intake is limited. In the liver, protein synthesis is diverted from albumin to acute phase proteins, whereas gluconeogenesis increases. In muscles, insulin resistance occurs and protein catabolism is accelerated, which is not counteracted by the compensatory increase of anabolic pathways [8]. In the
adipose tissue, lipolysis increases leading to progressive wasting of adipose tissue. The constellation of symptoms and signs related to abnormal energy metabolism in cancer patients and ultimately leading to deterioration of nutritional status is defined as cancer cachexia. Its main feature is muscle mass loss, but the now widely accepted diagnostic criterion is simply based on the involuntary loss of body weight [9]. This syndrome encompasses an extraordinary variety of phenotypes, in which the specific contribution of reduced energy intake and abnormal metabolism to the clinical pictures may amply vary.

The onset of cancer cachexia is progressive. As a consequence, cachexia represents a continuum of symptoms and signs, ranging from pre-cachexia to refractory cachexia [9]. Pre-cachexia is the early stage of the syndrome and is characterized by sickness behavior (i.e., anorexia, increased levels of circulating inflammatory biomarkers) in the absence of significant weight loss. If not treated, pre-cachexia progresses to cachexia, in which weight loss becomes manifest, together with changes of body composition, muscle wasting being the most clinically relevant effect. It is important to avoid reaching the status of refractory cachexia, since attempts at reverting weight loss during this stage have generally failed. It should be acknowledged that the border between cachexia and refractory cachexia is not well defined since it is also related to the patients' genetic signature. Indeed, specific polymorphisms of key inflammatory mediators, including proinflammatory cytokines (i.e., TNF- $\alpha$, IL-1, IL-6), have been shown to significantly modify the phenotype of cancer cachexia. However, recent data suggest that cancer patients retain muscle anabolic potential up to 90 days from death, which may therefore be considered as the "door of no return" [10].

Cancer cachexia is a clinically relevant syndrome. Cachectic patients have shorter survival, worse quality of life, and increased number of complications associated with antitumor therapies [11]. Therefore, the investigation of the pathogenic mechanisms of cachexia is key to develop effective preventive and therapeutic strategies. The inflammatory response triggered by the tumor is the key pathogenic mechanism. Although the degree of inflammation in cancer cachexia is usually mild to moderate, its chronic impact on energy metabolism leads to progressive wasting. The organs targeted by inflammation include the brain, and in particular the hypothalamic area, where the physiological balance between prophagic and anorexigenic pathways is deranged to promote anorexia and reduced food intake [12]. Clearly, skeletal muscles and the adipose tissue are also involved in the programmed deterioration of nutritional status during tumor growth [8]. In fact, catabolic pathways have been found hyperexpressed in muscles and adipose tissue of cancer patients. Also the liver is targeted by cancer cachexia, since protein synthesis is diverted into energy-consuming futile cycles, leading to reduced albumin synthesis.

The clinical phenotype of cancer patients may largely vary. However, all tumor cells share specific biochemical properties. In particular, all cancer cells are not sensitive to growth inhibitors, can invade tissues and give metastasis, sustain angiogenesis, are self-sufficient in growth signals, have limitless replicative potential, and evade apoptosis [13].

Quite recently, the ability to create an inflammatory microenvironment has been added to the common features of cancer cells [14]. This biochemical feature appears to be key for tumor initiation, promotion and progression. The initial clone of mutating cells triggers an inflammatory response limited to the surrounding environment, which is sensed by neural afferents. This information is transported to the brain where it activates the physiological response, i.e., the vagal anti-inflammatory pathway [15]. The effect is to reduce inflammation and activate dendritic cells which hyperexpress the enzyme indoleamine-dioxygenase (IDO). The mechanistic role of IDO in tumor control is to deplete the microenvironment of the essential amino acid, tryptophan, and therefore to reduce cell replication [16]. However, cancer cells may fight back against immune surveillance, by expressing IDO by themselves to starve dendritic cells and reduce immune response.

It is now widely accepted that cancer cells replicate and tumors grow not despite but because of inflammation. Inflammation is used to stun immune surveillance and consequently may potently stimulate tumor growth. Supporting this view, many clinical trials have consistently demonstrated that the greater the inflammatory response measured in cancer patients by means of even gross markers, i.e., C-reactive protein, the lower the survival rate [17]. It is not surprising that inflammation is now being considered as a key target for anticancer therapies [18].

Considering that cancer cachexia results from the combination of reduced energy intake and increased catabolic drive, nutritional supplementation providing calories and proteins may have limited effects and only partially counteract the key feature of cachexia, i.e., muscle loss. In fact, although it has been demonstrated that the anabolic response to hyperaminoacidemia of cancer patients' muscles is not different from that of healthy volunteers [19], still the coexisting anabolic resistance impairs the complete utilization of exogenous nutrients. Therefore, the simultaneous use of anti-inflammatory agents has been advocated, i.e., cyclooxygenase inhibitors. Results from clinical trials demonstrated that this approach is effective in promoting preservation of muscle mass in cancer patients, particularly when integrated into a multimodal approach which includes nutritional support [20]. However, specific nutrients exert a similar anti-inflammatory activity when provided at larger doses than those provided by a regular diet. Among these nutrients, defined as nutraceuticals, omega-3 fatty acids, namely, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been extensively studied [21].

By replacing omega-6 fatty acids as substrates of cyclo-oxygenase and lipooxygenase, omega- 3 fatty acids lead to the production of metabolites whose pro-inflammatory effects are reduced when compared to those deriving from the metabolism of omega-6 fatty acids. The supplementation of EPA and DHA to cancer patients, alone or as part of energy-dense oral nutritional supplements, has been demonstrated to improve body composition and function, which translates into better clinical outcome [21]. In fact, based on the available evidence, it is now possible to demonstrate that nutritional therapy in cancer patients improves survival and quality of life by targeting cancer cachexia [22].

## 4 Nutrition and Treatment of Diseases

Nutrition support is a key component, together with pain control and psychological support, of palliative care. Within this context, the goal of nutrition is not to target directly tumor cells, but to support the host in order to allow him/her to withstand the side effects of anticancer therapy (i.e., surgical stress, toxicity, fatigue, psychological distress). It is important to note that in the definition of palliative care there is no mentioning of the timing of its delivery. However, in daily practice, palliative care is generally provided in patients with advanced disease for whom further therapies are not indicated. In contrast with this non-evidence based practice, a recent study showed that lung cancer patients receiving active treatment and concomitant palliative care, which included nutritional counseling, improved their mood and extended their survival when compared to patients receiving the standard of care [23]. Based on this evidence, the American Society for Clinical Oncology has issued a provisional clinical opinion urging the early integration of palliative care in the care of cancer patients [24].

Although it is difficult to ascertain the specific contribution, within palliative care, of nutrition support in improving mood and survival, it is tempting to suggest that nutrients may also have played a direct antitumor activity, beyond their impact on nutritional status. This hypothesis should not be surprising since animal behavior provides a large number of examples of self-medication as achieved by acute changes of the diet [25]. Therefore, it would not be surprising that Clinical Medicine is now discovering practices which are still found among living organisms, but have been abandoned by human beings hundreds of years ago.

During the last decades, the approach to diseases has been largely based on the introduction in the clinical practice of new drugs. Although it is acknowledged that drugs have redefined the global clinical scenario, on the other hand it cannot be denied that, at least in the oncology arena, the results obtained have been disappointing, in terms of limited response rate, increased toxicities, and impinged quality of life of patients [26]. This critical reappraisal led to strong recommendation requiring a thorough rethinking of the care delivered to cancer patients [5]. As a consequence, nutritional support has been recommended during radiotherapy and chemotherapy, in order to protect skeletal muscle mass and therefore reducing chemotherapy-induced toxicities. However, recent data seem to suggest that omega- 3 fatty acids may also directly act on the tumor itself and increase chemosensitivity [21]. This would not be surprising if we consider that human metabolism and genome have been primed by food for thousands of years, and therefore are likely to be modified by nutritional strategies while drugs have been "interacting" with human metabolism since a few decades.

## 5 Omega-3 Fatty Acids as Anticancer Agents

In general, the response rate achievable by chemotherapy in cancer patients with advanced disease ranges at around $30-40 \%$. Many factors may explain the limited benefits achievable in metastatic cancer patients. Although genetic plasticity of tumor cells allowing rapid adaptation to environmental changes is a key factor, recent data suggest that excess cancer treatment toxicity may occur if sarcopenia is not considered when dosing chemotherapy, leading to dose reduction and failure to complete the treatment schedule [27]. Therefore, prevention and/or treatment of muscle loss during cachexia may alleviate the side effects of antitumor therapies. However, beyond reducing toxicity, nutrition therapy may also favor response rate.

Omega-3 fatty acids are polyunsaturated fatty acids and therefore contain more than one double bond in the carbon chain of the molecule. Since double-bonds are preferential target for reactive oxygen species leading to peroxidation and cellular damage, omega- 3 fatty acids make cells more prone to oxidative stress-mediated damage when incorporated into cell membranes. Interestingly, many chemotherapeutic agents exert their antitumor effects by inducing oxidative stress. Therefore, it has been hypothesized that the supplementation with omega- 3 fatty acids could sensitize cancer cells to chemotherapy, resulting in enhanced response rate and possibly less side effects. This hypothesis has been extensively tested in experimental models [28], but only recently the results of preliminary clinical trials have been made available. Bougnoux et al. showed that in metastatic breast cancer patients, the supplementation of DHA during therapy increases omega- 3 fatty acids content in the cell membranes of a specific, high-incorporator subset of patients [29]. Interestingly, the high incorporators also showed a significant extension of survival. Murphy et al. investigated in patients with advanced lung cancer and receiving first-line chemotherapy whether the supplementation of fish oil containing EPA + DHA during chemotherapy could influence clinical outcome [30]. In patients receiving the standard of care, the response rate was approximately $26 \%$ but it rose to $60 \%$ in patients supplemented with fish oil. It is important to note that these exciting results should be considered as preliminary since the number of patients tested is very limited. Also, it remains to be elucidated how omega-3 fatty acids incorporation into cell membranes could be increased. However, these results represent a strong signal pointing to the possibility that combination of nutrition therapy and radiotherapy or chemotherapy may increase the efficacy and effectiveness of antitumor approaches, without increasing toxicity. This possibility is now being tested by ongoing clinical trials.

Fatty acids are energy sources but also precursors of bioactive compounds. Omega-6 and omega-3 fatty acids are substrates of lipo-oxygenase and cyclooxygenase, resulting in mediators of inflammation with more or less potent activity. Recently, considerable attention has been devoted to other metabolic pathways of fatty acids. Omega-3 and omega-6 fatty acids are also substrates of cytochrome P450 epoxygenases, which convert them to epoxy signaling lipids including epoxyeicosatrienoic acids derived from omega-6 arachidonic acid and
epoxydocosapentaenoic acids (EDPs) from omega-3 DHA [31]. Recent data show that DHA-deriving epoxy metabolites suppress angiogenesis and endothelial cell migration in vivo [31]. More interestingly, when EDPs are coadministered with a low-dose soluble epoxide hydrolase inhibitor, EDPs are stabilized in circulation, causing inhibition of primary tumor growth and metastasis in an experimental model of cancer [31]. These exciting results suggest that nutritional modulation of nutrient intake may offer an alternative strategy to increase the efficacy of drugbased approach to cancer patients.

## 6 Calorie Restriction and Disease Prevention: Is This the Solution?

During famine, a number of metabolic pathways are shut down and energy stores are preserved. Thus, the production of waste products of energy metabolism is reduced, including reactive oxygen species, which largely mediate oxidative stress. Considering that oxidative stress is implicated in ageing and disease, reducing energy intake without causing malnutrition may appear as an effective strategy to reduce oxidative stress, slow ageing process and prevent diseases [32]. The beneficial effects of calorie restriction (i.e., reduced energy intake by $20-40 \%$ below requirements) have been tested in cell cultures and invertebrates with positive results [32]. However, evidence in large and superior animals appears contradictory. Colman et al. showed in rhesus monkeys that calorie restriction results in reduced incidence of chronic diseases, including cancer, cardiovascular disease, and diabetes over a period of approximately 30 years [33]. In humans, compelling evidence for the beneficial effects on health of calorie restriction are not available, due to the complexity and financial implication of a long-term clinical trial. However, evidence have been produced showing that short-term calorie restriction, i.e., 6 months, results in improved surrogate markers of longevity, including core body temperature [34]. Subverting the then-flourishing concept of the key role of calorie restriction in slowing ageing, Mattison et al. have more recently demonstrated in the same animal model as Colman et al. that calorie restriction does not prevent the onset of chronic diseases [35]. Explanation of these conflicting results could lay in the diet the control animals were receiving in the two studies. In fact, in the Mattison et al.'s study, the control animals were receiving a healthier diet when compared to that used in the Colman et al.'s study, which contained an astonishing $28 \%$ of sucrose [36]. Therefore, it cannot be excluded that the results obtained by Colman et al. were more influenced by the toxicity of the control diet rather than by the beneficial effects of calorie restriction. Based on these results, it is now generally accepted that longevity is enhanced by factors other than only calorie restriction, and particularly by genetic background, healthy diet and active lifestyle [36]. Also, it is becoming clearer that a calorie is not a calorie and that the metabolic
effects of nutrients may be different even if providing the same amount of calories and nitrogen.

## 7 Fasting and Fasting-Mimicking Diet in Cancer

The metabolic effects of calorie restriction or even fasting may appear of clinical benefit in patients with cancer. In particular, fasting is a potent inducer of cellular protective responses, and therefore, short-term fasting has been proposed as a strategy to increase resistance of normal cells to the toxic effects of chemotherapy [37]. In contrast, cancer cells have lost the ability to protect themselves in the presence of limited availability of nutrients due to the activation of oncogenes. Therefore, short-term (i.e., 48-72 h) peri-chemotherapy fasting may reduce cancer therapy associated toxicity and increase antitumor effects [38]. This hypothesis has been tested in experimental models [39], but only anedoctal reports have been reported in cancer patients [40].

Particularly in cancer patients, fasting represents an extreme approach and compliance may be difficult. Considering the metabolic effects of specific nutrients, it is conceivable that specifically formulated diets may reproduce the same biological effects of fasting. Therefore, excluding or including specific nutrients in the diet may confer health benefits, by increasing sensitization of cancer cells to therapy, or increasing normal cell resistance to chemotherapy, or by directly targeting cancer and stromal cells. Many animal studies support this concept. Abdelwahab et al. showed that the ketogenic diet is effective as adjuvant treatment in rats receiving radiotherapy for malignant glioma [41]. Maddocks et al. demonstrated that selective serine starvation reduces tumor growth and extends survival in an experimental cancer model [42]. Peng et al. reported that increased resistance to surgical stress can be induced by single amino acid deprivation, i.e., tryptophan [43]. Whether fasting-mimicking diets could also benefit cancer patients remains to be addressed by adequately powered, prospective randomized currently ongoing trials.

## 8 Conclusion

Modulation of food intake robustly induces metabolic responses. Dietary habits are key factors in perturbating inner metabolic environment and favoring the onset of chronic diseases, including cancer, diabetes, and cardiovascular diseases. It is surprising to note that the impact of nutrition on health, and particularly on disease prevention, is widely recognized by doctors, politicians and lay people, but its role in favoring recovery from diseases or even in treatment of diseases is completely neglected. It is acknowledged that more statistically robust clinical trials addressing clinically relevant outcomes rather than simple nutritional variables are needed.

Also, it is acknowledged that translation into clinical practice of animal studies may not yield impressive and positive results since human diseases are far more complex than their laboratory models. Nevertheless, when the signal is separated by the noise, it is evident that nutrition remains an unexplored, cheap and already available opportunity to enhance global health by preventing and treating diseases. It would be extremely unwise to lose it.

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# Chapter 16 <br> Nutraceuticals in Preventive Oncology: Chemical Biology and Translational Pharmaceutical Science 

Ruiwen Zhang and Subhasree Nag

## 1 Introduction

Cancer is a leading cause of mortality and morbidity worldwide. The majority of human cancers are considered as a chronic disease resultant from genetic and epigenetic variations, mutations, and dysfunction that ultimately culminate into the cancer phenotype. The cancer phenotype is characterized by certain "hallmarks" such as sustained proliferative signaling, evasion of growth suppressors, resistance to cell death and replicative mortality, increased angiogenesis, and activation of invasion and metastasis [1]. Archeological and historical evidence suggest that there has never been a time in recorded human history when cancer was absent. Although not referred to specifically as cancer, ancient Egyptians called it the disease for which 'there is no treatment' [2-4]. While still controversial, cancer can be thought of as a chronic disease that usually goes through a precancerous stage prior to advancing to invasive and metastatic disease, providing an opportunity for early detection and prevention. Although the etiology for the majority of cancers is not fully understood, epidemiological, preclinical, and clinical investigations have identified several cancer risk factors, such as tobacco smoking, obesity, family history, sedentary lifestyle, infection and inflammation, and sun exposure, indicating that many cancers are preventable [5].

Epidemiological studies and cancer statistics indicate that the predominant forms of cancer and cancer-related deaths are those of the lung, breast, colorectal, and prostate [6], suggesting that the prevention of these leading cancers can reduce the total cancer burden, in terms of health, social, and economic impact. It is also noted that some of these cancers are more prevalent in the Western countries than Asian countries where a diet rich in vegetables and fruits with less fat/meat intake is often consumed [7, 8]. Many published studies demonstrate that dietary and

[^16]environmental factors greatly influence cellular function, metabolism, health, and diseases [9-21]. In addition to cancer chemopreventive agents, the evidence is mounting that relatively simple changes in lifestyle, especially in diet, can reap significant long-term health rewards [5]. Dietary supplements that provide physiological benefit or protection against chronic disease are generally defined as nutraceuticals [21]. This chapter primarily deals with the implications of nutraceuticals in chemoprevention and the mechanistic pathways that are modulated by nutraceuticals. We believe that more basic and translational researches on nutraceuticals as cancer preventive agents, including molecular targeting, effects on both tumor and tumor environment, and pharmacological characteristics, will ultimately demonstrate the use of nutraceuticals as an effective and safe approach to cancer prevention and therapy.

### 1.1 Carcinogenesis and Cancer Chemoprevention

Clinically, cancer may be considered to arise from interplay of inherited factors and environmental exposures, resulting in genomic instability, abnormal cellular/tissue growth and proliferation, and other cardinal features of cancer or so-called "hallmarks of cancer" [1, 22]. Inherited or germ-line factors include major defects in oncogenes such as Ras or tumor suppressor genes such as p53, APC (adenomatous polyposis coli)/BRCA (breast cancer) or subtle differences in gene expression, as exemplified by single nucleotide polymorphisms within key areas of the genome [22-28]. Both inherited mutations and mutations resultant from environmental exposure have the potential to be used as either molecular biomarker of cancer progression and/or targets for chemopreventive intervention.

Carcinogenesis, a continuous process involving the onset, development, growth, and progression of human malignancies, is experimentally categorized into three broad stages-initiation, promotion, and progression [22-27]. Accordingly, cancer chemoprevention is the inhibition or reversal of carcinogenesis at various stages [29]. The goals of primary, secondary, and tertiary chemoprevention are to prevent the development of precancerous lesions, to reverse prevalent lesions, and to suppress recurrent primary cancerous lesions, respectively [29-31]. Although cancer prevention generally is considered to be a relatively new field of medicine [29], the linkage between cancer and environmental factors has been observed and recorded for at least the past three hundred years. For example, in 1727, Le Clerc suggested cutting out swellings, polyps, and tumefactions before they became cancerous, and in 1775, Percival Pott, an English physician, reported a causal relationship between soot exposure and cancer of the scrotum (later identified as squamous cell carcinoma) in chimney sweeps [2,3]. This increased incidence of cancer was seen to be obliterated in sweeps who wore protective clothing. In early 20th century, Lathrop and Loeb reported a linkage between mammary cancer and ovarian hormones in mice; the result was in agreement with earlier findings by Beatson in 1896 that oophorectomy in a patient with breast cancer resulted in
disease recession [2]. In 1925, Wolbach and Howe reported that epithelial tissues of rats on vitamin A-deficient diets acquired neoplastic characteristics which were readily reversed upon incorporation of the deprived vitamin back into diet. The seminal work in 1950s provided critical evidences supporting systemic cancer prevention, when the linkage between cancer development and environmental insults such as smoking and exposure to tar was firmly established. In the 1960s, several groups demonstrated the relationships between metabolic enzyme activity and carcinogenesis [2, 3, 29]. Talalay helped extend this work in late-1970s studies he called "chemoprotection," linking basic molecular studies with preventive effects of the food preservatives BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) and dietary approaches involving vegetables such as broccoli. He hypothesized that these antioxidant moieties not only would enhance potential therapeutic and preventive effects of natural vitamin A but also would reduce its well-known severe toxicity (hypervitaminosis A) [32, 33]. In 1976, Sporn introduced the term "chemoprevention" for describing such preventive studies. Subsequently, several preclinical and clinical studies have demonstrated the inverse association of dietary factor intake such as vitamins and trace micronutrients with cancer [16-19, 29].

### 1.2 Nutraceuticals

"Nutraceutical" is a portmanteau word first coined by Stephen DeFelice in 1989 from "nutrition" and "pharmaceutical" [34]. It may refer to any dietary components that provide medical and health benefits. A number of nutraceuticals have been identified during the past decades; several studies have demonstrated the association between consumption of vegetable/fruit-rich diets and decreased risk of cancer [16-19, 21, 35, 36]. These observations have led to the development and usage of various phytochemicals for cancer chemoprevention. An ideally effective nutraceutical should be able to induce a quantifiable degree of change in tumor dynamics at a low, nontoxic dose. Thus, a dietary component must be efficacious and acting at a low dose to qualify as a nutraceutical. If anticancer effects are achieved slowly, several issues, such as maintenance of a tolerable dose, reaching effective levels in the plasma/tumor site, compound stability and bioavailability, may become serious challenges. In addition, there is increasing evidence supporting the use of a combinatorial approach to cancer therapy, i.e., combining a nutraceutical with either an effective synthetic drug or another nutraceutical, and the development of novel delivery systems for nutraceuticals and combined therapy, including nanoparticles [37-42]. On the other hand, cancer researchers and oncologists should be aware of the fact that the use of dietary supplements for promoting health and/or treatment diseases, including cancer, has been becoming a common practice. The potential drug-nutraceutical, food-nutraceutical, nutraceutical-nutraceutical interactions may affect the outcomes of cancer prevention and treatment, especially when the nutraceuticals of interest have inhibitory or inducible effects on drug metabolism,
such as P450 enzymes. Therefore, future evidence-based cancer chemoprevention should rely on a better understanding of mechanisms of action, especially at gene and molecular levels.

## 2 Mechanisms of Action of Natural Product Anticancer Agents

As aforementioned, cancer is a class of complex and chronic diseases that develop over an extended period of time. Research indicates that the process from initiation to cancer to clinically detectable disease can take as long as 10 to 20 years. Carcinogenesis is initiated when a normal cell is transformed through various mechanisms such as the activation of proto-oncogenes and the suppression of tumor suppressor genes. Such transformed cells gain the ability of uncontrolled proliferation through "self-sufficiency in growth signals and insensitivity to antigrowth signals" [1], and, at the same time, they evade apoptosis, resulting in tumor growth. The development of new blood vessels (angiogenesis), which provide nutrients and growth factors to the tumor, enables tumor sustainability and tissue invasion, leading to metastasis that is ultimately lethal. During this entire process, cells often accumulate alterations in multiple cellular signaling pathways. Additionally, gene mutations can occur after conventional cancer therapy, resulting in decreased therapeutic response, drug resistance and tumor reoccurrence. Considering that cancer is a multifactorial disease with the causative cellular genomic instability resulting in pleiotropic effects [1, 22-27], mono-modal therapy is not very effective in several cancer types [43], and combination therapy has become a mainstay in the treatment of human cancer. Similarly, targeting multiple steps of carcinogenesis is needed to develop effective cancer prevention approaches.

There is an increasing interest in developing multi-modal disease prevention strategy, including healthy diet and changes in life style [16-19, 21, 4446]. Nutraceuticals have been shown to target one or more of the various targets in carcinogenesis, including inflammation, cell proliferation, apoptosis, invasion, and angiogenesis. Over two hundred nutraceuticals have been identified and some of these nutraceuticals have been demonstrated to have anticancer activities, through downregulation of transcription factors, e.g., $\mathrm{NF} \mathrm{\kappa B}$ (nuclear factor $\kappa \mathrm{B}$ ), anti-apoptotic proteins, e.g., Bcl-2 (B-cell lymphoma 2) and Bcl-xL (B-cell lym-phoma-extra large), cell proliferation promoting proteins, e.g., cyclin D1, c-Myc (cellular myelocytomatosis oncogene), and invasive/metastatic genes, e.g., matrix metalloproteinases (MMPs), intracellular adhesion molecule-1 (ICAM-1), and vascular endothelial growth factor (VEGF) [47, 48]. Figure 16.1 depicts the exemplary nutraceuticals that have been reported to have anticancer activities, representing a variety of naturally occurring compounds with diverse, complex chemical structures [48, 49]. Natural compounds, owing to their intricate chemical structures, can serve as prototype compounds, allowing the rational design of new drugs, biomimetic synthesis development, and the discovery of new therapeutic compounds


Cholecalciferol


Epigallocatechin 3-gallate


D-Limonene


Beta carotene


Allicin

indole-3-carbinol


Ascorbic acid


Phenethy lisothiocyanate

sempervirine

quercetin


Gingerol


3,30-diindolylmethane


Sulforaphane


25-OCH3-PPD


25-OH-PPT


25-OH-PPD


Resveratrol

Fig. 16.1 Chemical structures of selected nutraceuticals that have anticancer activity
[50-52]. Intensive investigations on the structure-activity relationships (SAR) of natural compounds, using combinatorial techniques, often lead to the generation of vast libraries of analogous molecules and the selection and development of novel, more potent, and less toxic compounds. Additionally, combinatorial biosynthesis allows complex metabolic pathways to be explored, making it possible to improve the production of a given natural compound and its metabolites [50-52]. These synthetic analogs and/or metabolites would further aid the SAR studies. It is generally believed that natural products have multiple molecular targets that can be linked to both therapeutic effects and possible side effects. The newly developed genomic and proteomic technologies help identify novel pharmacological targets, permitting the validation of novel targets and the generation of novel lead compounds directed to these targets. An integrated approach that combines the use of structural chemical databases with databases on target genes and proteins should facilitate the creation of new chemical entities for better efficacy and less toxicity [53].

One of the best examples in this research area is the intensive investigations on curcumin, a flavonoid obtained from the dried rhizomes of Curcuma longa, a spicy Indian food, which has gained immense interest due to its antioxidant, antiproliferative, antiangiogenic, and anti-tumorigenic properties [54-68]. However, owing to its poor bioavailability, it has not been successfully developed as an effective therapeutic drug [69]. The significant anticancer activity of curcumin in various cancer models, along with its drug-like properties such as low molecular weight and lack of severe toxicity, makes this molecule a good natural lead compound for the development of chemotherapeutic derivatives or analogs. Consequently, several curcumin analogs have been prepared; one widely used structural modification truncates the central conjugated beta-diketone in curcumin to a monocarbonyl dienone [70]. These compounds exhibit remarkable cytotoxic activity against various cancer cell lines and anti-angiogenic activity in cell cultures and, more importantly, retain toxicity profiles comparable to that of the parent compound, with some of them exhibiting good oral bioavailability and good pharmacokinetic profiles [70].

As discussed earlier, naturally derived compounds often possess more than one biological target. This is particularly true for curcumin. To date, it has been demonstrated to affect more than 50 different cellular factors that have a role in regulation of various cellular pathways such as cell cycle, apoptosis, and cell division (mitosis), in inflammatory response and in cancer metastasis [54-68]. In comparison to rationally designed synthetic molecules which are often designed to act on a particular molecular target, natural compounds act on a "molecular network" of cellular signaling factors, possessing multi-path, multi-target, and multi-system actions. Research on such complex drug-target interaction networks is imperative for the discovery of novel targets and for repurposing a known drug or nutraceutical for cancer prevention and treatment.

To develop an effective and safe cancer chemoprevention approach, one cannot overlook the fact that some nutraceuticals, such as ginseng saponins, significantly modulate the activity of drug-metabolizing enzymes (notably the cytochrome P450 family) and/or the drug transporter P-glycoprotein [71]. For example, the ginsenoside Rd (from ginseng) shows potent inhibitory effects on CYP2C9 and CYP3A4 activities in human liver microsomes [72]. To complicate matters more, the ginsenoside Rb1 exhibits complex site-specific metabolism. The decomposition mode of Rb 1 in rat stomach differs from that of Rg 1 in rat large intestine. Hydroperoxidation of Rb 1 occurs in rat stomach, which is identified as the 25-hydroperoxy-23-ene derivative of Rb1 (gypenoside VIII). But in rat large intestine, five decomposition products of Rb 1 are observed, which are identified as gypenoside XVII, ginsenoside Rd, ginsenoside F2, compound K and VIII [73, 74]. Compound K, an oral metabolite of the protopanaxadiol and protopanaxatriol ginsenosides, exhibits moderate inhibition of the CYP2C9 activity, while ginsenosides PPD and PPT exhibit potent competitive inhibition against CYP3A4 activity [75-77]. These studies have clear implication in the use of natural product anticancer agents in the clinic. First, their effects on drug metabolizing enzymes may be better used in modulating the metabolism of carcinogens and/or anticancer
agents. Second, the differential effects of these natural compounds on different members of the drug metabolizing enzymes may affect both therapeutic response and toxicity profiles when chemotherapeutic agents are used in combination with nutraceuticals, as a result from physician directed use or patient self-medication. Finally, since these dug metabolizing enzymes often have genetic variations in humans, the effects of nutraceuticals on these enzymes may have different impact on individual patients. These considerations should be given when nutraceuticals are used alone or in combination with conventional therapeutic or preventive agents.

## 3 Chemical Biology and Translational Pharmacology of Nutraceuticals

In a diet, the entire plant/edible part of the plant is often used. Traditional medical practice such as Traditional Chinese Medicine (TCM) and Ayurveda advocates the use of the entire plant for treatment regimens [78]. It is now generally recognized that only one or few particular components are responsible for the observed therapeutic activities and the entire plant or its crude extracts might not be needed for the treatment. For example, the ginseng saponins are more effective than the whole plant, in relation to several suggested actions as diverse as antiinflammatory, immunomodulatory, antidiabetic, cardioprotective, and anticancer effects [72].

Investigations using modern technologies in molecular pharmacology and chemical biology would facilitate the identification of the actual bioactive components and their corresponding molecular targets. Once the presence of a certain molecular target is correlated to the disease state, it may also serve as a biomarker for that disease [79]. The validation of molecular targets can be complicated and the value of such target in drug discovery and development should be examined on a case-bycase basis. For example, oncogenes, as opposed to tumor suppressor genes, may present as a more attractive therapeutic target since it is relatively easier to inhibit the increased activity of a gene/protein than to restore one which is lost in cancer development and progression. Oncogene addiction has been suggested as one of the characteristics of cancer. Several natural product anticancer agents have been recently found to inhibit oncogene expression and/or function [72].

Presently, cutting-edge technologies incorporating multidisciplinary sciences such as bioinformatics, molecular biology, molecular pharmacology, and clinical medicine are used for target identification and validation. Cancer models (both in vitro cell lines and in vivo models) with tailored expression levels of the putative drug target are often used in new drug development and efficacy studies of nutraceuticals for cancer prevention and treatment. Preclinical studies identify lead compounds and evaluate them for "drug-like" properties such as minimal host toxicity, sufficient bioavailability, and therapeutic efficacy in well-
characterized tumor models. Finally, the candidate with maximum optimum therapeutic characteristics may proceed to clinical trials [53, 80, 81]. It should be noted that, thus far, there are limited clinical successful cases of nutraceuticals as cancer preventive agents.

### 3.1 Lessons from the SELECT Trial

The Selenium and Vitamin E Cancer Prevention Trial (SELECT) was one of the biggest controlled chemoprevention trials in history (with more than 35,000 male participants) [82-94]. The SELECT trial was conducted to assess the effectiveness of selenium and vitamin E alone, and in combination, on the incidence of prostate cancer, and employed a methodology superior to the traditional correlation-based trials that tested the association between ingestion of a certain dietary component and incidence of disease. The basis of the SELECT trial was the preclinical animal studies and correlational population studies. The randomized, double-blind, pla-cebo-controlled clinical trial, surprisingly, found that neither selenium nor vitamin E reduced the incidence of prostate cancer and that vitamin $E$ alone was associated with a $17 \%$ increased risk of prostate cancer compared to placebo [82-87].

The potential role of selenium in chemoprevention was initially suggested by a large randomized trials conducted in the selenium-deficient regions of Qidong and Linxian in China [90]. The Qidong/Linxian trials assessed the cancer preventive activities of selenium against cancers of liver, stomach, and esophagus, but not prostate, by supplementation of dietary common salt with sodium selenite, which provided $50-80 \mu \mathrm{~g}$ of selenium per day [90]. The primary hypothesis-generating trial prompting inclusion of selenium in SELECT was the randomized double blind Nutritional Prevention of Cancer (NPC) Trial with 1312 American participants who had a history of skin cancer. Another trial, the ATBC ( $\alpha$-Tocopherol/ $\beta$-carotene) trial gave the inspiration for inclusion of vitamin E in the SELECT trial [82-87, 90].

Retrospectively, the SELECT trial was initiated on the basis of sound evidence suggesting the potential of selenium and vitamin $E$ in reducing the risk of prostate cancer. However, the trial was terminated early on account of both safety concerns and negative data for the formulations and doses given. It is suggested that the use of L-selenomethionine rather than high-selenium brewer's yeast as used in the NPC trial in SELECT did not possess good cytotoxic activity in vivo [68]. Since, the oral doses or formulations required to deliver selenium metabolites to prostate cells in vivo were not yet established, this could explain why SELECT did not duplicate earlier results. Additionally, participants in the earlier NPC study who benefited from selenium administration were recruited from eastern USA, a traditionally seleno-deficient region, had lower baseline selenium levels. Thus, it might have been more beneficial to conduct a trial such as SELECT in a seleno-deficient region, to better replicate the conditions under which selenized yeast provided chemopreventive benefit in the earlier NPC trial. A better understanding of selenium biology would have also helped design a more effective and well-balanced
trial. Thus, the major lesson from the SELECT trial is that thorough understanding of molecular mechanism of action, strong preclinical evidence in different diseaserelevant models, careful pharmacological and pharmaceutical design are necessary before advancing to large-scale, time-consuming, and costly clinical trials, especially for prevention trials.

### 3.2 Development of Anticancer Nutraceuticals

As discussed above, understanding the chemical biology of a dietary compound provides important insights as to its perceived therapeutic effects and molecular mechanisms of action. In this discussion, we will use the example of inhibitors of the $m d m 2$ (mouse double minute 2 homolog) oncogene. The $m d m 2$ overexpression is associated with poor prognosis, drug resistance, and lower overall survival in cancer patients [95-103]. As a negative regulator of the tumor suppressor p53, MDM2 has been proven to be an important oncogenic molecule with several cancer-promoting activities [95]. Many of its oncogenic activities even occur, independent of p53 [95, 104, 105]. Advances in the understanding of the conformation and structure of MDM2 have sparked the discovery and development of MDM2 inhibitors as anticancer agents [106-115]. Molecular modeling approaches including pharmacophore-based, molecular docking studies have helped in structure-based drug design [106]. In a high-throughput electrochemiluminescent screen of more than 144,000 natural product extracts, sempervirine, a natural indole alkaloid was identified as an inhibitor of MDM2's E3 ligase. Further studies have shown that sempervirine can activate p53 through inhibiting the MDM2-mediated ubiquitination and degradation of p53. These effects have now led to further studies on the anticancer potential of Sempervirine [116].

Extensive studies on the biology of carcinogenesis prove that all malignant cell transformation and subsequent tumor growth cause concomitant alterations in multiple cellular signaling pathways. Thus, synthetic chemotherapeutic agents attempting to treat neoplastic tumors via the so-called "one gene-one target" approach, often result in abject failure. Natural compounds, on the other hand, show the capability to have multiple targets. Ji et al.[117] explain this paradigm, using the example of the flavonoid quercetin, which occurs naturally in tea and grapes. Quercetin can inhibit structurally diverse enzymes in different biosynthetic pathways, possibly due to the fact that quercetin, with its complex carbon framework, has multiple structurally diverse binding groups, a subset of which is sufficient to bind to the enzyme binding cavity. Core structures of natural compounds with diverse ligand binding groups are able to bind more easily with the enzyme; what with both the cavity as well as the ligand being flexible entities allowing them to adapt their configuration for optimal binding [118, 119]. This diversity and flexibility thus allows them to interact with their therapeutic target(s). Pharmacokinetic studies in humans indicate that the disposition of quercetin is largely dependent on the sugar moiety attached to it (quite similar to the ginsenoside
saponins) and may contribute to its cancer protective effects [120]. Similar results are also seen in the ginseng metabolite Compound K which also exhibits potent antitumor activity [72, 77].

It must also be appreciated that natural compounds often have several drawbacks, such as toxicity, complex structure, poor water solubility, low bioavailability, and low content of actual therapeutic component in natural sources [5053]. Therefore, most of the candidate natural compounds need structural modifications and SAR research to develop next-generation compounds with better anticancer activity and less host toxicity. At the same time, the series of compounds derived through structural modification are useful in studies aiming at a better understanding of the mechanisms of action. Artemisinin is a fitting example of a successfully modified natural compound [121-125]. Artemisinin is an antimalarial drug with low water solubility and consequently low bioavailability. Upon reduction to dihydroarteannuin, a notable increase is seen in its antimalarial activity [123-125]. The methyl ether derivative of artemisinin, artemether, is soluble in oil and can be formulated as an oil-based injection with a concomitant increase in bioavailability. In the 1990s, artemisinin was rediscovered as antitumoral compound with excellent cytotoxic activities, with even the structural analogs of the artemisinin parent nucleus showing impressive antitumor effects. Several groups have elucidated the molecular mechanisms of action for artemisinin as an anticancer agent, including apoptosis through a caspase-dependent mitochondrial pathway, $\mathrm{G}_{1}$-phase cell cycle arrest in a p53 independent manner, and cellular iron depletion via a nonclassical endocytic pathway [126-128]. In addition, dihydroartemisinin also increased the efficacy of the chemotherapeutic agent gemcitabine. Thus, artemisinin presents a classical example of development natural compound into an effective drug for use in the clinic. Similar approaches can be used in discovering and developing anticancer nutraceuticals, including validating anticancer properties, identifying molecular targets linked to anticancer effects, performing chemical structure modifications and/or synthesis or semi-synthesis of derivatives, and testing novel derivatives with better physicochemical properties for therapeutic efficacy and safety in preclinical and clinical studies.

### 3.3 Identification of Molecular Targets of Anticancer Natural Products

As indicated above, natural products often possess more than one biological target, and a better understanding of the "molecular network" of targeted signaling pathways is imperative in designing better prevention and therapies incorporating one or more nutraceuticals/dietary agents which affect different targets, thus providing a synergistic mechanism. In this section, we discuss this multi-target concept, using a few exemplary phytochemicals that have been widely investigated for their chemopreventive activities (Fig. 16.2).


Fig. 16.2 Exemplary biochemical pathways affected by selected dietary chemopreventive agents

### 3.3.1 Genistein

Several population studies have demonstrated that people with cultures adhering to a soy-rich diet show lower incidence of breast cancer [7, 8]. The isoflavones in a soy-based diet are majorly responsible for conferring the anticarcinogenic properties [8]. Among isoflavones, the major constituents that are majorly involved in cancer prevention and therapy include genistein and diadzein [129]. Genistein influences multiple biochemical functions in living cells, including activation of PPARs (peroxisome proliferator-activated receptors), inhibition of tyrosine kinases, inhibition of topoisomerases, and induction of autophagy [130-136]. Evidence of the anti-proliferative activity of genistein in vitro stems from its ability to inhibit the tyrosine kinase enzyme that is most often upregulated in cancer cells [130]. As a chemopreventive agent, genistein affects the differentiation process of mammary tissue, causing early differentiation of rat mammary tissue into terminal buds while making them less susceptible to carcinogens or estrogen. Many aggressive cancers present altered epidermal growth factor (EGF) receptors on their cell surface activating the downstream cell division signaling pathway. When EGF binds to its receptors, the tyrosine kinase activation results in the phosphorylation of tyrosine residues of proteins involved in downstream cell signaling pathways that trigger cell division [21, 130]. Studies indicate that genistein increases the EGF transcription early in development of breast tissue, driving differentiation and faster
development. However, over the course of time, this prevents breast lesion formation in ducts. Another mechanism by which genistein may contribute to chemoprevention is downregulation of the MDM2 oncogenes. We have demonstrated that genistein downregulates MDM2 at both the transcriptional and posttranslational levels [133], regardless of the p53 status of the cells. These activities are also independent of the tyrosine kinase inhibitory activity of the compound, implying that there is no cross-talk between these two mechanisms. Genistein also increases p21 expression levels, thereby inhibiting uncontrolled proliferation [133]. Encouraged by the excellent anticancer activity of genistein, several semisynthetic analogs such as synthetic genistein glycosides and 7-O-modified genistein derivatives are being developed as anticancer agents [136]. One of the biggest problems with development of genistein as an effective chemopreventive agent is its low oral bioavailability, which may also be responsible for its unclear therapeutic effects and large inter-individual variations in clinical trials.

### 3.3.2 Ginsenosides

Ginseng, a common and widely used ingredient in traditional Chinese medicine, is a highly popular dietary supplement in the USA. In fact, the sale of ginseng products in the USA alone was estimated to reach US\$83 million in 2010. Ginseng has been used traditionally for the treatment of fatigue, to stop bleeding, and for maintaining cardiovascular health. The Shen Nong's Herbal Classic attributes life enhancing and health boosting properties to ginseng and states that it is good for "enlightening the mind and increasing the wisdom" [72]. It is now known that ginsenosides, the steroidal active components present in the Panax species, contribute to the diverse pharmacological activities of Panax plants, including their anti-inflammatory, immunomodulatory, antidiabetic, cardioprotective, and anticancer effects [137]. To date, more than 40 different saponins have been isolated and most of them show excellent cytotoxic profiles. Several reports have elucidated the anticancer properties of these ginsenosides [72].

Ginsenoside saponins exhibit diverse molecular mechanisms of action, regulating most known modulators of carcinogenesis, including regulation of cell proliferation, growth factors, tumor suppressors, oncogenes, cell death mediators, inflammatory response molecules, and protein kinases. The anticancer activities of ginseng saponins follow a well-defined structure-activity relationship with the aglycone moieties possessing more cytotoxicity than compounds with attached sugar molecules [72]. Recently, we have identified several new ginsenosides, including $20(S)$-25-methoxyl-dammarane-3 $\beta, 12 \beta, 20$-triol ( $25-\mathrm{OCH}_{3}-\mathrm{PPD}$ ), a protopanaxadiol from Panax notoginseng, which exhibits excellent anticancer activity against various human cancers, including prostate, pancreatic, lung, and breast cancers. In fact, it may be the most potent anticancer ginsenoside discovered to date. It downregulates MDM2 expression in the different cancer types in vitro and in vivo [72, 137-140]. Another two ginsenosides, $20(R)$-dammarane$3 \beta, 6 \alpha, 12 \beta, 20,25-$ pentol (25-OH-PPT) and $20(R)$-dammarane- $3 \beta, 12 \beta, 20,25$-tetrol
(25-OH-PPD) from $P$. notoginseng also show greater cytotoxic effects against several cancer cell lines than Rg 3 , which is marketed as an anticancer agent in China [137]. As seen with $25-\mathrm{OCH}_{3}-\mathrm{PPD}$, MDM2 protein levels are decreased after exposure to both $25-\mathrm{OH}-\mathrm{PPD}$ and $25-\mathrm{OH}-\mathrm{PPT}$. Preclinical studies on ginsenosides reveal that they are substrates for the CYP enzymes, and are absorbed well from the small intestine. Ginsenosides are, typically, metabolized to active metabolites in vivo, the most noteworthy being Compound K [77].

Because of the pleiotropic actions of the ginsenosides, drug resistance to these compounds may not be readily developed. Furthermore, the selective ability to kill tumor cells with little or no toxicity to normal cells makes ginsenosides attractive anticancer drug candidates. However, there still are several challenges. Different species of ginseng have different saponin profiles, and the mode of isolation from the plant or subsequent treatment of the plant extracts affects the quantity of its active anticancer principles. For example, heat treated ginseng extracts possess higher quantity of Rg 3 , Rh2, and PPD [72]. As a result, it is often difficult to standardize studies and varying results are seen. Also, sometimes conflicting results in clinical trials involving ginsenosides have been noticed. Both a large casecontrol study as well as a small cohort study has suggested that ginseng use is associated with a significant (more than $60 \%$ ) reduction in gastric cancer risk in Korean populations [72]. However, when this study was attempted to be duplicated in a large prospective cohort study in a Chinese women population, no association between ginseng intake and gastric cancer risk was found. Whether these differences were due to the mode of preparation of the plant extracts (since no isolated compound was used) has not yet been proven.

### 3.3.3 Curcumin

Curcumin has multimodal properties and affects simultaneously numerous molecular and biochemical signaling cascades, including the inflammation-related TNF- $\alpha$ (tumor necrosis factor- $\alpha$ )/NFкB survival pathway [54-68]. The anticancer potential of curcumin stems from its ability to suppress proliferation of different types of tumor cells, to downregulate transcription factors $\mathrm{NF} \mathrm{KB}, \mathrm{AP}-1$ (activating protein1), and Egr-1 (early growth response protein 1), to decrease the expression of COX2 (PTGS2, prostaglandin-endoperoxide synthase 2), LOX (lysyl oxidase), iNOS (Inducible nitric oxide synthase), MMP-9, TNF, chemokines, cell surface adhesion molecules, and cyclin D1 [48, 141]. It is well known that chronic inflammatory conditions caused by genetic mutations, autoimmune diseases, and exposure to environmental factors such as bacteria (H.Pylori) increase the risk of cancer. In fact, epidemiological studies attribute up to $25 \%$ of cancer deaths worldwide to chronic inflammation [141-144]. Surprisingly, cancer tissues without any history of precancerous inflammation also exhibit inflammatory markers and morphology. It is suggested that the inflammatory state helps maintain and promote cancer progression and achieve the complete malignant phenotype, including tumor tissue remodeling, angiogenesis, metastasis, and the suppression of the innate anticancer
immune response [142]. Oncogenic signaling pathways such as Ras-Raf or NFкB mediate inflammation to facilitate cell transformation. The NFкB signaling plays crucial roles in both precancerous chronic inflammation as well as cancer induced inflammation [142-144]. Activation of NFкB induces expression of inflammatory cytokines, adhesion molecules, enzymes in the prostaglandin-synthesis pathway (such as COX2), inducible nitric oxide synthase (iNOS), angiogenic factors (VEGF), and anti-apoptotic genes (such as Bcl-2). COX2 and PGE2 (prostaglandin E2) synthase regulate various aspects of tumor progression, angiogenesis, and metastasis. Similar to NFкB, the STAT3 (signal transducer and activator of transcription 3)/ TGF $\beta$ (transforming growth factor $\beta$ ) signaling pathway is constitutively activated in tumor cells and is involved in oncogenesis and inhibition of apoptosis. The activation of STAT3 in tumor cells has also been implicated in immune evasion via inhibition of dendritic cell maturation and the subsequent immune response [142, 145-148].

Curcumin also downregulates growth factor receptors (such as EGFR (epidermal growth factor receptor) and HER2 (human epidermal growth factor receptor 2)) and inhibits the activity of c -Jun N -terminal kinase, protein tyrosine kinases and protein serine/threonine kinases. It inhibits the growth of LNCaP xenografts in nude mice by inducing apoptosis and inhibiting proliferation and sensitizing these tumors to undergo apoptosis by TRAIL (NF-related apoptosis-inducing ligand) [48, 141]. In xenograft tumors, curcumin upregulates the expression of TRAIL-R1/DR4, TRAIL-R2/DR5, Bax (bcl-2-associated X protein), Bak (bcl-2 homologous antagonist/killer) p21/WAF1, and p27/KIP1, and inhibits the activation of NFкB and its gene products. Curcumin treatment with TRAIL in combination with genistein sensitizes TRAIL-resistant AGS gastric adenocarcinoma cells to TRAIL-mediated apoptosis. Curcumin has also been reported to induce apoptosis in human melanoma cells through a Fas receptor/caspase-8 pathway independent of p53. Another property ascribed to curcumin is that of inhibition of c-jun/AP-1 (activator protein 1) function and JNK (c-Jun N-terminal kinase) activation [141].

One of the likely mechanisms underlying inhibition of NFкB activation by curcumin involves suppressing the degradation of the inhibitory unit of I kappa B alpha ( $\mathrm{I} \kappa \mathrm{B} \alpha$ ) which prevents subsequent nuclear translocation of the functionally active subunit of NFкB. Curcumin inhibits tumor formation in several chemically induced carcinogenesis models such as benzopyrene induced forestomach carcinogenesis, $N$-ethyl- $N$ '-nitro- $N$-nitrosoguanidine (ENNG)-induced duodenal carcinogenesis, and azoxymethane (AOM)-induced colon carcinogenesis. Recently, we discover another important mechanism by which curcumin exerts its anticancer activity, i.e., downregulation of MDM2 transcription through the PI3K (phosphatidylinositol 3-kinase)/mTOR (mammalian target of rapamycin)/ETS2 pathway [55]. We further demonstrate that curcumin can sensitize human cancer cells to chemotherapy and radiation by the MDM2-knockdown. However, curcumin is notorious for its poor bioavailability in vivo. This has prompted medicinal chemists to synthesize curcumin derivatives with better pharmacokinetic properties, which has led to several potent new candidate anticancer agents [48, 56, 141].
Table 16.1 Major classes of nutraceuticals and their mechanisms of action and clinical statuses.

| Phytochemical class | Bioactive compounds | Chemopreventive mechanism | Dietary source | Experimental model | Clinical studies/evidence | Ref. |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Vitamins | Vitamin A | Causes G1 cell-cycle arrest; represses expression of a cassette of genes on chromosome 12 p in these cells; induces differentiation in carcinoma cells | Carrots | Human embryonic carcinoma cells | Yes (negative results seen) | [149-152] |
|  | Vitamin B | Deficiency in folate, methi onine, and choline leads to hepatocellular carci noma via miR-122 downregulation; Folic acid also blocks ethanol-induced terato genesis in fetal mouse brain through miR-10a downregulation | Meat, spinach | Male fisher rats, human (colon, hepatoma) cancer cell lines | No | [153, 154] |
|  | Ascorbic acid | Induces caspaseindependent cell death associated with autophagy, peroxide formation and ROS. | Citrus fruits | Pancreatic cancer cell line | No | [155, 156] |
|  | $\begin{array}{r} \text { Vitamin } \mathrm{D}- \\ \text { calciferol } \end{array}$ | Inhibits tumor angiogene sis; stimulates mutual adherence of cells; enhances contact inhibi tion; Vitamin D metab olites help maintain a normal calcium gradient that induces terminal differentiation and apoptosis | Milk | Rats, human cancer cell lines | Yes (breast cancer, thyroid cancer, early stage prostate cancer ${ }^{\text {a }}$ ) | [157-166] |

Table 16.1 (continued)

| Phytochemical class | Bioactive compounds | Chemopreventive mechanism | Dietary source | Experimental model | Clinical studies/evidence | Ref. |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | Vitamin ETocotrienol | Downregulates inflamma tory cytokines (IL-1, IL-6, IL-8, IL-12, IFN- $\gamma$ ), telomerase HTERT, EGFR, antiapoptotic proteins (Bcl-xl, Bcl-2);causes S-phase arrest in hepa toma cells and downregulates cdks; induces apoptosis through TGF- $\beta$ /FAS/ JNK signaling pathway (breast); TRAIL medi ated apoptosis (colon); Bax/Bid mediated apo ptosis (liver); inhibits angiogenesis by suppressing HIF-1 $\alpha$; causes chemosensi tization in hormone refractory prostate cancer | Nuts | Breast, colon, liver, lung, pancreas, prostate, stomach cancer cell lines; +SA murine mammary tumor cell line; Chick chorioallan toic membrane | Yes (Part of the SELECT trial), colorectal cancer patients undergoing surgery ${ }^{\text {a }}$ | [167] |
| Minerals | Zinc | Stabilizes p53 | Sesame, nuts | Rats, human cancer cell lines | Yes (IRX-2 RegimenPhase II) ${ }^{\text {a }}$ | [168] |


|  | Selenium | Shows pro-oxidant (induc ing ROS and causing apoptosis), antiproli feration (growth inhibi tion, cell cycle arrest), anti-angiogenesis, antiinflammation, immunomodulator | Brazil nuts, tuna, crab, and lobster | Rats, human cancer cell lines | SELECT trial, SELEBAT trial, bladder cancer ${ }^{\text {a }}$, colorectal cancer ${ }^{\text {a }}$ | 169-177] |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Alkaloids | Piperine | Inhibits matrix metalloproteinase pro duction and TNF- $\alpha$ / NFкB pathways | Pepper | Melanoma cells | In combination with res veratrol to increase the latter's bioavailability ${ }^{\text {a }}$ | [178] |
| Monoterpenes | Limonene | Inhibits the LPS-induced inflammation including cell migration and pro duction of NO; inhibits IFN- $\gamma$ and IL-4 produc tion in mouse model; upregulates caspases 3 and 9, Bax, p53 and downregulates $\mathrm{Bcl}-2$ | Citrus oils from orange, lemon, lime, grapefruit etc. | Mouse model, human cancer cell lines | Breast cancer, no study results yet posted | [179-181] |
| Organosulfides | Allicin | Inhibits spontaneous and TNF- $\alpha$ induced secre tion of IL- $1 \beta$, IL-8; sup presses IL-8 and IL-1 $\beta$ mRNA levels; sup presses degradation of IкB; upregulates caspases 3, 8, and 9; cleaves PARP; inhibits TNF- $\alpha$ induced ICAM-1 expression | Garlic | Intestinal epithelial cells; HUVECs | Dietary intervention study in patients with Follicu lar Lymphoma (FL) Stage III/IV; alli cin (garlic extract) one of the components | [182-184] |

Table 16.1 (continued)

| Phytochemical class | Bioactive compounds | Chemopreventive mechanism | Dietary source | Experimental model | Clinical studies/evidence | Ref. |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | Indole-3-carbinol | Selectively inhibits CYP450 enzymes involved in carcinogen metabolic activation; inactivates Akt, inhibits Sp1 promoter activity; inhibits ER- $\alpha$ transcrip tion; downregulates AR transcription | Cabbage | Rats, human cancer cell lines | Prostate cancer, primary preventive studies (Phase I) | [185, 186] |
|  | Isothiocyanates (PEITC/ BITC) | Induces cytoprotective pro teins through the Keap1/ Nrf2/ARE pathway; inhibits proinflammatory responses through the NFкB pathway; induces cell cycle arrest by downregulation of cyclin D1; causes apo ptosis by caspase dependent pathway; inhibits angiogenesis and metastasis | Broccoli | Azoxymethane-induced colon carcinogenesis rat model, human cancer cell lines | Lung cancer ${ }^{\text {a }}$ | [187] |
|  | Sulforaphane | Upregulates FAS ligand, caspases 3,8 , and 9 , cytochrome c; downregulates $\mathrm{Bcl}-2$; cleaves PARP; inhibits NFкB transcriptional activity, nuclear trans location of p65 subunit; inhibits expression of VEGF, cyclin D1, cdk4, cdk6 and Bcl-XL | Broccoli | Ovarian cancer cell line, prostate cancer cell line | Prostate cancer, breast cancer ${ }^{\text {a }}$ | [188-191] |

[192-194]
[195, 196]
Table 16.1 (continued)

| Phytochemical class | Bioactive compounds | Chemopreventive mechanism | Dietary source | Experimental model | Clinical studies/evidence | Ref. |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | Capsaicin | Causes cell cycle arrest at G2/M phase; induces disruption of the mito chondrial membrane potential; activates caspases 9, 3 and cleaves PARP; downregulates expres sion of phospho-PI3 Kinase p85 (Tyr458) and phospho-Akt (Ser473); reduces Trx expression and dissoci ates Trx-ASK1 complex and induces apoptosis via activation of ASK1; induces ROS generation via mitochondrial electron transport systems I and III | Chili peppers | Human KB cancer cells, pancreatic cancer cell line, orthotopic pancreatic cancer model in nu/nu mouse | No results for chemoprevention | [197-200] |
|  | Ellagic acid | Causes G0/G1 arrest; upregulates p21 and p53; leads to caspase mediated apoptosis; inhibits ABCG2 (Breast cancer resistance pro tein) -mediated transport | Black berries, raspberry | Human cancer cell lines | Dietary intervention in follicular lymphoma | [201, 202] |

[203, 204]

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| Ginger root | Human cancer <br> cell lines | No |
| :---: | :---: | :--- |
| Milk thistle | Human cancer <br> cell lines | ALL |
| Brewed black <br> tea | Rats, human cancer <br> cell lines | Prostate (with genistein) |
|  |  |  |
| Turmeric | Mouse melanoma, <br> pancreatic, breast, <br> prostate, etc. human <br> cancer cell lines | Colon cancer ${ }^{\text {a }}$, breast |
| cancer, head and |  |  |
| neck cancer ${ }^{\text {a }}$ |  |  |

Induces caspase 3 depen
dent apoptosis and
autophagy in cancer
cells; activates p53
Inhibits the invasion via
JNK/AP-1/MMP-2
modulation; inhibits
Wnt/ß-catenin signaling
Causes G1 phase arrest;
upregulates p21, p27,
and p53;suppresses pro
tein kinases; inhibits
NFkB activation;
inhibits telomerase
Downregulates cyclin D1,
NFкB, COX-2;
downregulates MDM2
through the PI3K/
mTOR/ETS2 pathway;
suppresses c-jun/c-fos
expression; suppresses
EGFR tyrosine kinase
activity; blocks the chy
motrypsin like activity
of the proteasome
Induces G2/M phase
cell cycle arrest;
downregulates levels
of cyclin A, cyclin B,
phosphorylated forms
of cdc2 and cdc25
of cdc2 and cdc25
Gingerol
Silymarin/
Silibinin
Quercetin
䔍
Apigenin
Table 16.1 (continued)

| Phytochemical class | Bioactive compounds | Chemopreventive mechanism | Dietary source | Experimental model | Clinical studies/evidence | Ref. |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | Daidzein | Downregulates MMPs and inhibits cell invasion | Soy | Breast, colon, liver, lung, pancreas, prostate, stomach cancer cell lines; | Human postmenopausal women; Men with Prostate neoplasia | [226, 227] |
|  | Genistein | Causes activation of PPARs, inhibition of tyrosine kinases, inhibition of topoisomerases, stimu lation of autophagy | Soy | Breast, colon, liver, lung, pancreas, prostate, stomach cancer cell lines; | Human postmenopausal women; Breast cancer patients | $\begin{gathered} {[131-136,} \\ 228] \end{gathered}$ |
| Triterpenoids | Ginsenosides | Inhibit oncogenes c-myc, c-fos; downregulates nucleophosmin; increase levels of p53 and p 2 ; regulate mitochondrial cytochrome C, PARP, and C9; inhibit MMP-2 and 9 ; inhibits adhesion of metastatic cells to basement membrane. | Panax ginseng, Panax quinefolium, Panax notoginseng | Breast, colon, liver, lung, pancreas, prostate, stomach cancer cell lines; | Gastric cancer (Korea/China), Breast cancer | $\begin{gathered} {[72,137-} \\ 140] \end{gathered}$ |
| Anthocyanins/ Anthocyanidins | Cyanidin | Inhibits TNF- $\alpha$ inflamma tion pathway; induces JNK dependent apopto sis via increase in intra cellular ROS levels | Red cabbage, purple potatoes, Grape seed extracts, wine | Breast, colon, liver, lung, pancreas, prostate, stomach cancer cell lines; | n-3 fatty acids and antho cyanins for improving cognition in breast can cer treated patients | [229] |

[230-235]

IRX-2 Regimen: The IRX-2 regimen is the combination of a 2-week course of IRX-2 itself, an initial dose of cyclophosphamide, and a 3-week course of indomethacin and zinc supplementation
ATBC trial: Alpha-Tocopherol Beta carotene trial
${ }^{\text {a }}$ Active clinical studies; not yet completed or still recruiting volunteers

In Table 16.1, we further summarize the chemopreventive mechanisms of action of major phytochemical classes, along with additional information as to their clinical status.

### 3.3.4 Epigallocatechin-3-Gallate (EGCG)

Epigallocatechin-3-Gallate (EGCG) is one of the major food-derived phytochemical constituents that are extensively studied for their chemopreventive and chemotherapeutic use. It is polyphenol tannin present in green tea. EGCG exerts its anticancer properties via several mechanisms such as inhibiting angiogenesis by affecting VEGF transcription, inhibiting growth promoting signal transduction pathways via PI3K/Akt/NFkB, inhibiting EGFR, inhibiting HER2 receptor phosphorylation in breast carcinoma cells that constitutively expresses HER2/neu receptor [21, 236-238], inducing apoptosis in estrogen receptor-(ER-) independent breast cancer cells, preventing metastasis [21]. However, high doses of EGCG can induce hypoxia-inducible factor 1 (HIF-1) which can lead to tumor cell proliferation through alternate survival pathway mechanisms [21]. Most studies indicate that anticancer properties of EGCG are shown at higher doses which may be physiologically unachievable through dietary consumption. Also, at higher doses EGCG may mediate pro-proliferative effects. Therefore, clinical trials aimed at achieving desired antitumor effects at much lower doses combine EGCG with chemotherapeutics such as taxol [21]. Thus, EGCG can be exploited as a chemopreventive agent if it prevents neoplastic lesions from appearing at low concentrations and for prolonged periods of time. In vitro studies that use relatively high doses of EGCG may actually translate into tumor promoting than preventive effects, when administered over longer exposure periods. Over time, EGCG can also induce drug resistance via NFкB mediated pathway. The above observations emphasize the importance of a dual-drug treatment approach for cancer therapy. In a recent review, Saldanha et al. [21] postulate that EGCG will be more effective if administered early in combination treatment, followed by other phytochemicals or drugs, so that can further potentiate the efficacy of the treatment regimen.

## 4 Perspective and Future Directions

It is now well accepted that nutrition/diet plays an important role in the prevention and treatment of chronic diseases such as cancer. Epidemiological studies have demonstrated that vegetable and fruit consumption is constantly associated with a reduced risk of a variety of cancers [7-9], and that dietary intake of vitamins, minerals, and antioxidants such as carotenoids from these sources is similarly correlated with a reduced cancer risk [16-19]. The very nature of cancer that includes progressively accumulating genetic and molecular alterations offers multiple targets for putative chemopreventive strategies. The lengthy process of carcinogenesis and cancer progression also permits screening of high-risk individuals
and early detection of cancers. Effective early stage cancer screening identifies not only malignancies that are more responsive to therapy, but also premalignant lesions that can be removed and/or prevented from progressing to invasive disease stages. Thus, a successful chemoprevention program requires an in-depth understanding of the entire carcinogenesis process. Basic and translational researches capitalizing on these cutting-edge technologies are necessary to facilitate the development of chemopreventive agents. The identification and validation of molecular and genomic biomarkers will be necessary to identify and quantify risk in prospective cohorts as well as finding use as surrogate end points in clinical studies. Another direction is to generate new animal models of carcinogenesis that mimic human disease (including transgenic and gene knockout mice) which can be used to validate surrogate end points. Besides epidemiological studies, basic researches to determine the mechanisms of action and evaluate the chemopreventive efficacy of dietary components are indispensable.

As we progressively inch towards a better scientific understanding of the mechanisms of the carcinogenic process, a prudent strategy to reduce the risk of cancer incidence and mortality would include increased consumption of vegetables and fruits as a part of a healthy, balanced diet. This would include eating between five to nine servings of fruits and vegetables every day. At the same time, in order to lend further credibility to chemoprevention studies, a standardized evidence-based approach to the development of nutrition-related guidelines is needed. For this purpose, one needs to follow the principles of evidence-based medicine such as GRADE (Grading of Recommendations Assessment, Development and Evaluation) which are based on randomized clinical trials. However, in chemoprevention, one has to rely on trials with risk factor or surrogate end-points, since studies sufficiently powered for clinical end-points or mortality, the usual expectation of evidence-based medicine, might not be feasible. Defined criteria must be developed for determining whether a risk factor may be deemed to be an appropriate end-point.

Many of the natural products do not possess "drug like" qualities and present disadvantages like poor bioavailability and inability to reach site of action. Therefore, it is essential to further develop these compounds by structural modification and/or better delivery. In contrast to the classical way of cancer treatment studies, where research starts from the disease followed by gene identification and concludes with specific cancer gene targeting and drug development, in chemoprevention trials, natural products are at the top of the pyramid: based on epidemiological and experimental studies, these agents are isolated from their natural sources, purified and assayed to investigate their ability to kill precancerous and cancerous cells. Since the end point for both studies is cancer eradication, a concerted effort must be made to complement each other in order to discovery novel ways to fight cancer. Diverse dietary constituents such as vitamins A and D, genistein, EGCG, sulforaphane, curcumin, piperine, theanine, and choline have also been shown to modify self-renewal properties of cancer stem cells, further indicating the importance of these dietary factors in cancer prevention.

In the past three decades, the approach to cancer prevention research has evolved to a comprehensive process, from population and epidemiological studies, to
molecular targeting and immunological intervention, and to identifying high-risk precancerous lesions in individuals using emerging early detection technologies, to controlled, randomized clinical trials. Therefore, the future of chemoprevention lies on the better identification of risk cohorts who are more likely to benefit and to be more tolerant of risks through improved risk models, more insights into molecular carcinogenesis and validation of molecular targets, the identification and development of more specific and highly targeted agents that can achieve dramatic improvements in efficacy with fewer side effects, and the testing of agent combinations that appear to offer another path to greater efficacy with fewer, more tolerable risks in chemoprevention. The future of molecular prevention is highly promising due to a wealth of promising new natural compounds for clinical development as preventive agents and the greater appreciation of the need to balance risks and benefits. Additional strategies should specifically be focused on interrupting the late-stage, but still premalignant, processes that lead to in situ cancers, with the goal of averting progression to invasive cancer. It is hoped that the global implementation of these scientifically sound lifestyle-based and evidence-based cancer prevention strategies has the potential to reduce cancer incidence, prevalence, and mortality and social economic impact of cancer worldwide.

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## Part IV Allergy

# Chapter 17 <br> The Onset of Atopic Dermatitis: Underlying Mechanisms 

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## 1 Introduction

Eczema or atopic dermatitis (AD) is the most common chronic skin disease in children with an increasing prevalence worldwide. The incidence has increased during the last 30 years, with $20 \%$ of infants and children experiencing symptoms [6]. The wide range in the prevalence and the more frequent occurrence in urban areas suggest that environmental factors play a role in the development of atopic dermatitis. Atopic dermatitis often precedes asthma and allergic disorders, and more than $50 \%$ of children with AD will develop asthma and/or allergies [34]. Several epidemiological studies provide evidence for the so-called "The Atopic March" from AD , suggesting that AD is the starting point for subsequent allergic diseases [61]. Most of the children develop AD before the age of 2 years, and usually AD starts during the first months of life [38], however, AD is not present at birth. The etiology and pathophysiology of AD is not completely defined, though it is unlikely that allergic reactions are involved in the initiation of $A D$. This review aims to highlight recent insights into the pathophysiology of the onset of AD , focusing on the interplay between skin barrier abnormalities, inflammation, and skin microbiota and to discuss potential strategies for prevention and treatment.

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## 2 The Normal Skin at Birth

An intact, healthy skin barrier is important in the first line defense against environmental substances, including various pathogens and allergens. Although infants are born with a competent skin barrier, their skin is still developing through the first year of life and is different from adult skin (reviewed by [65]). The differences between infant and adult skin can be divided into structural differences, compositional differences and functional differences. Neonatal skin is drier and has a higher pH compared to that of adults, although both features are rapidly changing during the first months of life. In addition to structural and functional changes, the composition of commensal bacteria residing the skin surface evolves during the first year of life [13].

AD is not present at birth, but starts during the first weeks or months of life, especially in children who are born with a dry skin. Alterations in skin barrier properties that are observed in AD in older subjects include increased transepidermal water loss (TEWL), changes in skin surface pH , increased skin permeability, altered bacterial colonization and compromised skin barrier integrity. Whether these skin barrier abnormalities are primary in the pathophysiology of AD or whether it is merely a reflection of downstream consequences of intrinsic inflammatory disease remains to be fully elucidated.

## 3 Atopic Dermatitis in Young Children: Epidemiological and Clinical Features

The prevalence of allergic diseases is increasing in most countries, although the latest data from the ISAAC showed that in some countries the prevalence of atopic dermatitis seems to be leveling or decreasing [68]. For other countries, mainly formerly low-allergy prevalence developing countries, a substantial increase has been reported, especially in the younger age group [68]. It is not possible to attribute the change in atopic dermatitis prevalence to a singular environmental or genetic risk factor and it is likely that different risk factors are playing a role in specific types of atopic dermatitis. For example, mutations in the skin barrier protein, filaggrin, have been defined as a strong risk factor for atopic dermatitis in selected populations, with a reported filaggrin mutation carrier frequency between 14 and $56 \%$ in patients with atopic dermatitis [16].

The distribution of AD is largely age dependent. In the first 2 years of life (the infantile phase), the clinical features of AD are being characterized by lesions mainly localized to the head, the scalp and extensor areas of the limbs, and are made up by edema, erythema, oozing, and crusting (Fig. 17.1). The exposure to saliva and food exacerbates the clinical lesions. Secondary infection and xerosis are common [61]. The childhood phase (from 2 years to puberty), is characterized by lichenification and dryness, primarily affecting hands, feet, and flexural areas of


Fig. 17.1 The clinical features of AD in children. The face, especially the cheeks and the chin, and flexural areas such as the elbows often show edema and erythema
elbows and knees, the wrists, neck, and face. For the clinical evaluation of the severity of AD, the SCORAD index is being used [2]. Infants and young children with AD have distinctive intensity items of the SCORAD index and it has been postulated that different immune mechanisms are involved. The acute inflammation characteristic of the infantile phase shows marked infiltration with Th2 cells, while during the chronic childhood phase a switch to Th1 response has been reported [42].

## 4 The Role of Allergy in Atopic Dermatitis

There is a continuing controversy whether allergic sensitization is an essential feature of AD and the exact link remains to be elucidated. Different contrasting hypotheses have been postulated. Some suggest that allergic reactions can cause AD [57], while others suggest that allergy is a consequence of chronic AD, caused by percutaneous allergen penetration through a defective skin barrier [47]. A recent study suggested that allergic sensitization occurs through the skin resulting in specific allergen-specific cutaneous T cell responses [15].

Although most patients with AD show high levels of IgE, some do not show clinical features of allergy differentiating an IgE-associated extrinsic type from a nonallergic or intrinsic type of atopic dermatitis [54], although new insights may reveal more types of atopic dermatitis. In infancy, intrinsic AD is more prevalent than extrinsic AD . Moreover, in infants AD lesions occur before allergic symptoms, and therefore, it is questionable whether allergic reactions are responsible for the initiation of AD.

The causative role of food allergy in the initiation of AD has been a subject of debate and controversial results have been reported [22]. Studies have demonstrated
that a low food allergen diet is associated with a significant reduction in the prevalence of AD in infancy, and using double-blind placebo-controlled food challenges (DBPCFC), food has been demonstrated to induce AD [9], although the occurrence of true food-induced AD is rare [1]. The difficulty to provide conclusive evidence that food allergy can induce AD is associated to the complicated diagnosis of food allergy. The presence of food-specific IgE support sensitization, but does not always correlates with clinical allergy. Recently, the diagnostic value of specific IgE have been determined and may offer a better positive predictive value [26]. In addition, there are reports suggesting that the use of the atopy patch test in combination with IgE testing increases the positive predictive value of the diagnosis of food allergy [1], although others have reported only a small added benefit of atopy patch test [19]. Moreover, food allergy can also be non-IgE mediated which further complicates diagnosing the interplay between food allergy and AD.

Recent research indicates the role of house dust mites (HDM) as a cause of AD [22]. Different mechanisms have been suggested, including the inherent proteolytic enzyme activity that can contribute to skin barrier impairment, activation of proteinase-activated receptors-2 (PAR-2) inducing epidermal barrier dysfunction, the superantigenic function, and IgE-mediated inflammation, resulting in tissue damage. However, the causative role of HDM-allergy in AD is still controversial as it was suggested that HDM allergy is a consequence of the impaired skin barrier function.

## 5 Differences Between Normal and Eczematous Skin

The skin barrier function resides primarily within the stratum corneum (SC), the top layer of the epidermis, and has until recently relatively been ignored as an important factor in AD [29]. Differences between the skin barrier properties between normal and eczematous skin involve at least four elements (Fig. 17.2): (1) the physical barrier, characterized by increased transepidermal water loss (TEWL) and compromised skin permeability barrier integrity, (2) the chemical barrier reflected by changes in skin surface pH and alterations in expression of antimicrobial peptides (AMPs), (3) the microbiological barrier reflected by altered bacterial colonization, and (4) the immunological barrier, illustrated by reduced expression of TLRs. However, the question remains whether these defects are cause or consequence of AD .

TEWL measurement is performed by a noninvasive method that can be used to monitor changes in SC barrier function. High TEWL is suggestive of incomplete skin barrier function. In patients with AD, TEWL was found to be increased in both dry non-eczematous skin and clinically normal skin [67]. Recent studies indicated that the expression of thymic stromal lymphopoietin (TSLP) is increased in the SC of AD patients and correlates with SC hydration and SCORAD [58]. The expression of TSLP can be induced by different stimuli, including allergen-driven


Fig. 17.2 Complex interplay between impaired skin barrier, altered skin microbiome, and dysfunctional immune response in pathophysiology of $A D$. The onset of $A D$ may start with an impaired skin barrier, either genetically programmed or due to specific environmental factors that via epigenetic mechanisms impair the skin barrier. The alterations in the skin barrier, physically and or chemically, may lead to altered skin colonization and impaired skin immune defense (e.g., reduced expression of IL-1). Moreover, disruption of the skin barrier may enable the uptake of allergens, irritants, and microbes by dendritic cells, thereby triggering inflammatory immune responses involving immune components like TSLP. The inflammatory immune reaction subsequently can further impair the skin barrier functionality by decreasing the expression of skin barrier proteins like FLG, contributing to the onset of AD
proteases, as well as endogenous proteases, such as kallikrein 5, which is overexpressed in patients with AD [37], and by specific TLRs [39].

Moreover, the antimicrobial barrier is compromised in AD due to an impaired expression of antimicrobial proteins (AMPs) that play a key role in the innate immune defense system of the skin. AMPs are produced by keratinocytes and the expression of some of these peptides is constitutive, whereas the expression of others is triggered by inflammation. Several studies showed a deficiency in the expression of AMPs in subjects with AD [51]. The reduced expression of AMPs
together with the observed higher pH in the skin of AD patients [59] is most likely playing a role in bacterial colonization, including colonization with Staphylococcus aureus, although other abnormalities could be involved. Approximately $90 \%$ of AD patients are colonized with $S$ aureus that can trigger multiple inflammatory cascades [41]. Toxins produced by $S$ aureus can act as superantigens and thereby activate T cells contributing to Th2-mediated inflammatory reactions [40]. The question remains whether this is primary or secondary.

## 6 The Role of Skin Barrier Proteins in Atopic Dermatitis

Different proteins of the epidermis playing an important role in the barrier function are impaired in the skin of patients with AD (Table 17.1). Filaggrin (FLG) has been the most studied and its gene has the highest association with AD [52], subsequent allergic sensitization, and allergic disorders [8]. Next to FLG, abnormalities in other proteins expressed in the uppermost part of the epidermis have been identified to be associated with AD. These include impaired expression in tight junction proteins, such as claudins [17], and scaffolding proteins such as loricrin and involucrin [35],. Importantly, the expression of these proteins can be influenced by ongoing inflammatory processes in the skin [32] and therefore could be cause or consequence of AD . Another important factor in the maintenance of the barrier function of the epidermis is the control of skin proteases by skin protease inhibitors, such as SPINK, a gene absent in Netherton's syndrome, which is characterized by an extreme severe type of AD [10]. Increased protease functioning also occurs in AD patients, including an increase in stratum corneum chymotryptic enzyme [66] and mast cell chymase [4]. Allergens such as house dust mite and cockroach themselves and Staphylococcus aureus can be proteolytically active, thereby decreasing the skin barrier function [46]. A recent review [37] describes the role of epidermal innate receptors in regulating the skin barrier and that defects in these innate receptors play a role in the pathogenesis of AD.

Whether skin barrier dysfunction precedes skin inflammation that initiates the development of AD remains to be fully elucidated.

## 7 Early Skin Colonization: Its Role in Atopic Dermatitis

The composition of the human skin microbiome is dynamic and has been demonstrated to evolve over the first year of life along with the structural and functional development of the skin [13]. The skin microbiome depends on the local environment of the skin area and differences have been reported between moist and dry sites [25]. Capone et al. showed relative instability of the infant skin microbiome that may provide the opportunity for aberrant skin development. Interestingly, the newborn skin microbiome is extremely dynamic and initial differences, for

Table 17.1 Summary of skin proteins associated with AD

| Component | Protein | Reference |
| :--- | :--- | :--- |
| Skin barrier proteins | Filaggrin | $[52]$ |
|  | Filaggrin2 | $[55]$ |
|  | Hornerin | $[55]$ |
| Scaffolding proteins | S100/A11 | $[31]$ |
|  | Involucrin | $[35]$ |
| Tight junctions | Loricrin | $[35]$ |
| Skin protease inhibitors | Claudins | $[17]$ |
|  | SPINK | $[10]$ |

example due to difference in mode of delivery, e.g., caesarean section vs. vaginal delivery [20], disappear within a month of life.

The composition of the skin microbiota of AD patients differs from healthy controls [18] and it was shown that the reduction in microbial diversity precedes worsening of AD disease severity in children [36]. Interestingly, AD treatments with topical steroids or oral antibiotics have been demonstrated to modify microbial diversity preceding improvements in disease severity. However, from current data it is not possible to determine whether the composition of the microbiome can be the cause or result of AD . The skin microbiome is suggested to play an important role in the development of the skin immune [48] and barrier function [27]. Aberrant development of the skin immune system may be linked to the development of AD , allergy [61] or even asthma [5].

## 8 Pro-Inflammatory Status of the Skin in the Onset of Atopic Dermatitis

Although a number of immune abnormalities have been described in AD, including increased TSLP and Th2 activation and decreased expression of AMPs, it remains to be elucidated whether skin barrier dysfunction precedes immune dysregulation ("outside-in" hypothesis) or immune dysregulation precedes barrier changes ("inside-out" hypothesis) initiating the onset of AD [21]. Moreover, the increased susceptibility to microbial colonization and infections in AD patients indicates the complexity underlying the pathogenesis of AD.

Available data supports both hypotheses. Enhanced penetration of allergens and pathogens due to impaired skin barrier can lead to inflammatory and allergic immune responses. Experimental evidence demonstrated the elicitation of a Th2-response after (mechanical) disruption of the skin barrier accompanied by an increased expression of TSLP. TSLP plays a key role in the allergic inflammation and activates different effector cells, including mast cells and basophils (reviewed in [69]). Perturbations of the SC barrier also trigger the activation of Langerhans
cells [12]. Antigens can be taken up by Langerhans cells, subsequently migrating to the draining lymph nodes and activate Th 2 responses.

On the other hand, inflammation itself can also alter skin barrier integrity. Th2 cytokines have been reported to down regulate the expression of skin barrier proteins, including filaggrin [32], involucrin, and loricrin [35]. Therefore, patients with AD may have acquired skin barrier defects, which may explain why not all AD patients are carriers of skin barrier mutations. The role of the microbial colonization adds another layer of complexity to the underlying mechanisms in the onset of AD [37].

## 9 Are There Epigenetic Mechanisms Involved in Atopic Dermatitis?

The development of AD is influenced by multiple factors. Genetic as well as early life environmental factors, including allergen environmental exposures [33], infections [7], autoimmunity [63] and alcohol intake during pregnancy [14, 44] are all involved. Epigenetic mechanisms provide new insights on how the environment is implicated in the development of genetically determined immune-mediated diseases, including allergy, and how environmental changes drive the epidemics of allergic diseases (reviewed by [45]). Although there is clear evidence that immune development in under epigenetic regulation and that alterations in epigenetic programming in allergy-prone infants are involved, little is known about epigenetic mechanisms in AD. A recent study from Liang et al. demonstrated a role of epigenetic changes in the pathogenesis of AD [43]. Their results indicated demethylation of specific regulatory elements within the FCER1G gene leading to overexpression of the high affinity $\operatorname{IgE}$ receptor (FceRI) on monocytes from patients with AD. However, further research will be required to determine the cause leading to the epigenetic changes and its potential role in the onset of AD.

## 10 A Model of Onset of Atopic Dermatitis in Early Infancy

Although progress has been made in the pathophysiology of AD , it remains a complex disorder with a complex interplay between skin barrier, immune system, skin microbiome, and epigenetics (Fig. 17.2). Environmental factor-induced changes in gene expression may be key in the etiology of AD . It remains to be elucidated whether epigenetic changes are causative in skin barrier impairments, leading to altered skin microbial colonization and immune alterations or whether immune alterations are the consequence of epigenetic changes causing skin barrier dysfunction, causing increased allergen and pathogen penetration leading to

AD. New insights into mechanisms underlying the onset of AD are pivotal to develop early intervention strategies to prevent the development of $A D$.

## 11 Nutritional Intervention in Prevention and Treatment of Atopic Dermatitis

Understanding how environmental changes modify gene expression and thereby contribute to allergic diseases may provide an opportunity to strategies to prevent allergic disease. Emerging evidence indicates that nutrition can influence epigenetics. In particular, recent research has focused on the impact of prenatal nutritional factors. One of the notable observations in an animal study is that maternal supplementation with folate led to differential methylation and development of allergic diseases in the offspring [30]. Nutritional intervention during pregnancy or postnatally may reprogram gene expression and thereby prevent disease. Nutritional factors may also directly interact with microbiota and with the immune system, thereby modulating disease. Different nutritional factors are now under active study for both prevention and treatment of AD , including probiotics, prebiotics, long-chain polyunsaturated fatty acids (LCPUFAs), and vitamin D.

In the context of prevention of AD with probiotics, a plethora of literature has been published. Certain strains of probiotics are effective in the prevention of $A D$, especially if administered prenatally [62]. However, there is evidence that probiotics may increase the development of atopic sensitization [50, 64], thereby questioning the effectiveness of probiotics in preventing AD . In addition, to date there is one published study that addresses the capacity of oral supplementation with probiotics to control microbial colonization of the skin in which no differences on skin colonization was observed [49]. The use of probiotics for the treatment of AD seems less promising despite some positive results [11].

Prebiotics are nondigestible oligosaccharides that reach the colon intact and are known for their ability to selectively stimulate the growth and activity of bacteria that exert positive health effects [23]. Beneficial effects have been observed on prevention of AD in clinical trials with specific mixtures of oligosaccharides [62], although more studies are required. To date, there are limited data to support the therapeutic use of prebiotics in AD. There is one study that reported beneficial effects of prebiotics in the treatment of AD [60].

Essential fatty acids must be acquired from the diet and are the precursors for LCPUFAs that have been implicated as being important for the development of the immune system. LCPUFAs have been shown to influence the immune system via different mechanisms [24]. Although epidemiological studies support the hypothesis of a relationship between higher intake of n-6 PUFA and increased prevalence of allergic disease [28], clinical beneficial effects from intervention studies are more conflicting and the protective effect is likely to be greatest in pregnancy [53].

Vitamin D has important effects on the immune system and in particular has been demonstrated to influence antimicrobial defenses and skin barrier function and in dampening inflammatory responses, all important features of AD. However, the role of vitamin D in prevention of AD has been controversial. Although available evidence suggests a positive correlation between serum vitamin D levels and AD [56], studies with oral supplementation with vitamin D showed an increased risk of AD [3].

Further research to provide conclusive evidence on the effects of nutrient supplementation as well as elucidating the underlying mechanisms is necessary.

## 12 Conclusion

In patients with AD , impairments in skin barrier and alterations in skin microbiological colonization and the immune system are described. However, which of these defects initiate AD remains to be established (Fig. 17.2). Insights in this complex interplay will be important in the development of targeted preventive and therapeutic strategies for AD that may also interrupt the progress to other allergic disorders. Although there are different hypothesis on the onset of AD, it is becoming increasingly clear that the skin barrier plays a key role in the process. New techniques including high-throughput expression profiling and proteinomics may facilitate the identification of relevant components in the onset of AD.

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# Chapter 18 <br> Cow's Milk Allergy: Protein Hydrolysates or Amino Acid Formula? 

Christophe Dupont

## 1 Introduction

Cows' milk protein allergy (CMPA) manifests by clinical symptoms related to the abnormal immune response of the host after ingestion of these proteins and affects $2-7 \%$ of children [1]. Symptoms that may affect the skin (urticaria, atopic dermatitis), the digestive tract (vomiting, diarrhoea) as well as the respiratory tract (rhinitis, asthma) are often combined and may be associated with failure to thrive and anaemia, according to various syndromes. The immune mechanism may be IgE mediated, non-IgE mediated or both. The diagnosis of CMPA, suggested clinically, aided by skin tests (prick tests), specific IgE assays and/or patch tests, requires the elimination-challenge procedure. CMPA has been subject to extensive reviews and position papers [2-9]. When confirmed, CMPA requires the elimination of cows' milk proteins (CMP) from the patient's diet. In breastfed infants, pursuing breastfeeding as long as possible is the best option. If mother does not want to or cannot breastfeed, cows' milk formulas are replaced by adapted ones, based on cows' milk hydrolysates, rice hydrolysates or amino acid mixtures in order to avoid any protein of mammal or vegetal origin and thus further reduce the risk of intolerance. Feeding this child always needs taking into account the potential nutritional issues related to manipulating the child's diet at an age of maximal growth and nutrient/energy requirements. This chapter reviews the pros and cons of the different nutritional options chosen.

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## 2 Nutritional Issues During CMPA

The nutritional impact of CMPA (see [7] for a review) varies considerably in expression and intensity and should be systematically evaluated. Intestinal mucosal inflammation may induce malabsorption and/or protein-losing enteropathy, but also skin protein losses may be the consequence of atopic dermatitis. The elimination diet prevents the deleterious effects of allergic inflammation but may impair the adequate intake of essential nutrients: undernutrition may be the consequence of an uncontrolled elimination diet [10-13]. This is all the more important in case of multiple food allergies when exclusion of foods such as wheat or egg renders the child's menu very difficult to settle and nutritional and growth deficiencies more likely to occur $[14,15,13]$ : the nutritional risk increases in case of multiple food allergies, since the elimination diet results in multiple exclusions. Children with at least two food allergies might be slightly shorter than those with a single food allergy $(p<0.05$ ) [14]. A low Ca intake is especially marked in children with CMPA or multiple allergies. Children with CMPA and asthma, for more than 4 years, and who were treated with corticosteroids, ingested only $25 \%$ of the DRI of calcium [16]. Iron deficiency, the most common nutritional deficiency associated with CMPA, has been rarely investigated. In an Italian study, $25 \%$ of patients with CMPA were iron deficient [17]. Isolated iron-deficiency anaemia can reveal CMPA [18]. Some cases of infant CMPA manifest themselves in a failure to thrive. The long-term consequences of these nutritional deficiencies are unknown.

## 3 The Need for Appropriate Replacement Formulas

Breastfeeding, if still possible, is the first choice for CMPA patients. When not possible or not desired, breastfeeding is replaced by a substitute, which has to provide normal growth and development. Most of the time, it is an extensively hydrolysed formula (eHF), based on a source protein, usually from milk, which has been "extensively hydrolysed" to considerably reduce allergenicity. eHFs are distinct from partially hydrolysed formulas ( pHFs ), referred to as "hypoallergenic" or "HA" in some countries, to be used only in non-breastfed infants considered at risk for allergy [19]. In documented CMPA, the only suitable formulas are eHFs, but there are no physical, chemical or immunological criteria that allow any regulatory distinction between eHF and pHF [20]. eHFs may not be tolerated in a certain number of children with CMPA, hence the need for amino acid-based formulas (AAFs).

## 4 Cows' Milk Protein-Based Hydrolysates

eHFs used in replacement of cows' milk formulas, cows' milk and dairy products have been analysed by the Committee of Nutrition of the French Society of Pediatrics [20, 19, 7]. Previously, in 1993, the European Society for Paediatric Gastroenterology and Nutrition (ESPGHAN) recommended to use formulas containing proteins with a molecular weight (MW) <1,300 Daltons (Da) [21]. This requirement is relevant in terms of quality control (reproducibility among manufacturing processes) but does not allow predicting the degree of immunogenicity or potential reaction in a given child [22].
eHFs also comply with the European Union Commission Directive 1999/21/EC of 25 March 1999 on dietary foods for special medical purposes [23]. The European Commission has set limits to the content of immunoreactive proteins in hydrolysates to $<1 \%$ of the total content of nitrogen-containing substances and determines the adequacy and safety of a hydrolysate on (1) experimental studies (oral administration should not induce sensitisation, in animals, to the intact proteins from which the hydrolysate is manufactured) and (2) on clinical trials, showing that the hydrolysate is tolerated by more than $90 \%$ of infants presenting with hypersensitivity to the proteins from which the hydrolysate is manufactured [24]. This $1 \%$ threshold of immunoreactive proteins in hydrolysates dates back to the ESPGHAN recommendation of 1999 [22] and does not rely on any clinical trials of good scientific quality. Some authors indicate that suitable thresholds of reaction might be closer to $1 / 1,000$ [25]. Recommendations for adapted clinical trials have been made in 2004 [26].
eHFs available in many European countries are almost all lactose free, and the protein portion consists of either cows' milk casein hydrolysates or cows' milk whey protein hydrolysates.

Comparison of the molecular weights of peptides and residual allergenicity of cows' milk hydrolysates is difficult, because manufacturing processes change and are not always communicated by manufacturers. Early published data (from the 1990s) indicated the lowest residual allergenicity with Nutramigen ${ }^{\circledR}$, lower than that of Alfaré ${ }^{\circledR}$, Peptijunior ${ }^{\circledR}$ and Nutrilon Pepti ${ }^{\circledR}$, in that order [27-32]. Recent modifications of the whey hydrolysate used in Alfaré led to a reduction of its molecular weight profile, similar to that of the newly launched Althéra ${ }^{\circledR}$, characterised by a median peptide size of 362 Da , with $99.7 \%$ of peptides $<2,400 \mathrm{Da}$ [33].

Diagnostic tests, RAST specific and/or skin prick tests (SPT) of the abovementioned eHFs, were performed in the same period in children presenting with IgE-mediated CMPA. With Nutramigen ${ }^{\circledR}$, the SPT was positive in $0 / 10$ [34], $4 / 10$ [35] and $1 / 42$ [36] children. Comparative studies showed positivity in $0 / 15$ with Nutramigen ${ }^{\circledR}$ and $1 / 15$ with Alfare ${ }^{\circledR}{ }^{\circledR}$ [37], in $0 / 17$ with Nutramigen ${ }^{\circledR}$ and $2 / 17$ with Alfare ${ }^{\circledR}$ [29] and in $6 / 31$ with Nutrilon Pepti ${ }^{\circledR}$ [38]. RAST was more frequently positive, in $2 / 15$ children with Nutramigen ${ }^{\circledR}$ and $7 / 15$ with Peptijunior ${ }^{\circledR}$ [39], 4/10 with Nutramigen ${ }^{\circledR}$ and $5 / 10$ with Alfare ${ }^{\circledR}$ [29].

Clinical studies confirming the efficacy of eHFs in the treatment of CMPA have been rare, performed with a small number of children usually older than 6 months with IgE-mediated allergies. Nutramigen ${ }^{\circledR}$ has been the more extensively investigated, being frequently the reference formula in controlled trials. In a total of 97 children, its efficacy ranged from 93.8 to $100 \%$ [29, 34, 35, 40-42]. The efficacy of Nutrilon Pepti ${ }^{\circledR}$ in 75 children reached $79.5 \%$ [43] and $98 \%$ [38]. The efficacy of Peptijunior ${ }^{\circledR}$ in 29 children, all less than 3 months of age, was $79.3 \%$ [43]. The efficacy of Alfare ${ }^{\circledR}$ amounted to six cases out of eight children [29]. These older studies have been carried out using product formulations frequently different from those of currently marketed products, evolutions being however always towards higher hydrolysis. Based on clinical practice, these products are well tolerated by most allergic children.

Newly launched formulas are now tested much more extensively as recommended. The recently launched eHF Althéra ${ }^{\circledR}$, with a low peptide molecular weight, identical to the reformulated Alfare ${ }^{\circledR}$, induced no reaction, similarly to the AAF reference product in 34 infants with CMPA demonstrated by placebocontrolled food challenge [33]. This whey eHF proved equally effective to Nutramigen ${ }^{\circledR}$, when both were enriched with different probiotic strains [44]. A study conducted on Frisolac Allergycare ${ }^{\circledR}$ (also Allernova ${ }^{\circledR}$ and Allernova AR $^{\circledR}$ when thickened) that included 28 children over the age of 1.5 years showed an efficacy of $100 \%$ [42]. It was successfully used to feed 119 infants with CMA [45].

## 5 Rice Protein Hydrolysates

Protein hydrolysates not originating from cows' milk have become available. A prospective tolerance study of a rice eHF supplemented with lysine and threonine enrolled 99 children with CMPA and a mean age of 3 years [46]. Patients often developed serum anti-rice protein IgE (RAST: 21/91; immunoblotting: 70/96), but only six reacted to the rice eHF, which makes the formula suitable for children with CMPA. A rice eHF supplemented with lysine, threonine and tryptophan is available in several European countries: a study showed that it was well tolerated by $90 \%$ of infants (mean age: 4.4 months) presenting with CMPA [47].

## 6 eHF Added with Lactose

Lactose is rarely used in eHFs. It is not, in theory, contra-indicated in the diets of children with CMPA. However, lactose used in the food industry may, depending on its degree of purification, contain significant traces of CMP, sometimes responsible for allergic reactions, which has led some authors to consider it to be inappropriate when used in food to be consumed by children with CMPA. The reaction to CMP traces (up to $2 \%$ ) in "drug"-grade lactose is also possible [48].

## 7 eHF Added with Probiotics

The putative interest of some probiotics has been suggested, but there is currently no evidence that probiotics can be helpful in the treatment of a child with CMPA [49]. A study argues against the efficacy of probiotics (Lactobacillus casei and Bifidobacterium lactis Bb CRL431-12) in the process of tolerance acquisition [45]. In contrast, a recent randomised trial showed that when incorporated into Nutramigen ${ }^{\circledR}$, Lactobacillus GG speeds up tolerance acquisition in infants with CMPA [50].

The use of compounds presumed to be active on the immuno-allergic reaction, in addition to the milk substitute, should be considered with great caution in the current state of evidence. Similarly, allergic individuals may react to CMP contaminants after the ingestion of probiotics raised on lactose or milk [51, 52].

## 8 Allergy to eHF and Its Association with Multiple Food Allergy

Calculating that 90-95 \% of children allergic to CMP respond to eHFs [35, 21, 53] implies that $5-10 \%$ still react to them.

Numerous reports described hypersensitivity reactions to eHFs in infants with CMPA both with immediate and delayed reactions [54-58]. Other options are needed for children allergic to eHFs [59]. The availability of an AAF (Neocate ${ }^{\circledR}$, SHS International) [57, 60] provided one treatment option and offered the ability to refine the diagnosis of eHF allergy [58, 61-63]. Sixteen children with slowly evolving symptoms suggesting CMPA that persisted on an eHF diet [61] were switched to this AAF. The response was good in 13 cases, with a decrease in symptoms and an increase in weight gain. The intestinal permeability decreased, probably due to a decrease in local inflammation. These children relapsed on subsequent challenge with an eHF, confirming their allergy to eHF. In a similar study [62], 28 children with CMPA and no response to eHF were fed the same AAF for 2 weeks, with symptom resolution in 25 cases. When later challenged with an eHF, 8 responders showed tolerance and 17 relapsed, confirming eHF allergy in nearly half of the patients.

Allergy to eHF may be part of a more severe syndrome, multiple food allergy, as described by Hill et al. [80]: 18 infants (median age 7 months) with suspected multiple food allergy were given the AAF Neocate ${ }^{\circledR}$ for 2 months followed by a 7-day double-blind, placebo-controlled food challenge with the infant formula best tolerated previously, e.g. whey hydrolysate, casein hydrolysate or soy. Twelve infants experienced irritability, vomiting, diarrhoea and/or eczema during the challenge. In infants more than 12 months old, parents also reported adverse
reactions with a median of six from a panel of ten foods. This means that during CMPA, allergy to eHF is associated with allergy to a number of other foods, from which comes the wording "multiple food allergy".

The time course of allergy to eHF may differ according to the presence or the absence of multiple food allergy [63]. When allergy involves eHFs and several other foods, tolerance of eHFs and of CMP occurs later and a restricted diet based on AAF feeding is required for a longer duration. In the study of another AAF EleCare ${ }^{\circledR}$ [81] in 31 consecutive children, 29 had multiple food allergy and 13 had allergy to eHFs: enrolment occurred at a median age of 23.3 months, and the AAF was given for a median of 21 months. In contrast, a lower age at diagnosis might be beneficial, with a lesser duration of symptoms, a decreased number of foods involved and a dominance of digestive symptoms [63, 80]).

## 9 Free Amino Acid-Based Formulas

Allergy to eHFs is one of the major clinical conditions leading to the use of AAF. Being based on free amino acids, formulas are almost devoid of intact proteins and peptides. The only traces that may occur in these formulae would come from contaminants in the starch and lipid parts. AAFs available in the market are limited: Neocate ${ }^{\circledR}$, Neocate Advance ${ }^{\circledR}$ after 1 year (Nutricia) and Nutramigen $A^{\circledR}{ }^{\circledR}$ (MeadJohnson). Two companies are carrying out clinical trials with an AAF, Nestlé and Novalac (thickened AAF) [68].

A systematic review of 20 studies on the use of an AAF (Neocate ${ }^{\circledR}$ ) in patients presenting with CMPA concluded as to its efficacy, tolerance and safety [64]. This formula proved particularly efficient in IgE-mediated gastro-enteroproctitis with a failure to thrive or a severe atopic eczema [60, 65]. A study shows that Nutramigen $\mathrm{AA}^{\circledR}$ is efficient and allows normal growth in infants with CMPA [66]. A recent study shows that the addition of a symbiotic to Neocate ${ }^{\circledR}$ allows hypolallergenicity and normal growth in infants with CMPA [67]. A recent randomised controlled trial was performed with a new, thickened, AAF (Novalac) in comparison with a reference one, in infants with allergy to milk and to eHFs, showing efficacy and safety for both formulas [68].

## 10 Growth of Children with CMPA Fed a Substitute Formula

The ESPGHAN published in 2001 recommendations and comments on the nutritional and safety assessment of breast milk substitutes and other dietary products for infants for long- and short-term outcomes and encouraged health-care providers to
promote the incorporation of these principles into their national regulatory processes [69]. Only a few formulae marketed in Europe have been subject to studies evaluating adequately their nutritional efficacy.

Healthy infants fed with casein eHF (including Nutramigen ${ }^{\circledR}$ ) had a poorer iron status and an excessive amino acid intake, resulting in a rise in blood urea nitrogen and plasma amino acids, compared to infants fed with a standard formula, warranting both reducing and balancing the amino acid composition of some formulae [70]. The growth pattern feeding with hydrolysates has been nicely investigated in the GINI study [71, 72]: feeding with a casein eHF (Nutramigen ${ }^{\circledR}$ ) induced a transient reduction in weight gain during the first year of life, without long-term consequences on body mass index (BMI) [71, 72]. In children with CMPA fed Althéra ${ }^{\circledR}$ for 6 months, length and head circumference were similar to Euro growth standards, but weight gain was slightly lower, similarly to the comparator Neocate ${ }^{\circledR}$ [33]. In Finnish children with proven CMPA, and fed from 7.5 months with a soy protein follow-on formula or with a soluble protein eHF (PeptidiTutteli ${ }^{\circledR}$, Valio Ltd, Finland), often supplemented with Ca and vitamin D, growth and nutritional status were adequate [73].

Several rice eHFs have been evaluated. Infants with confirmed CMPA-related atopic dermatitis were given openly a rice eHF supplemented with lysine and threonine, a soy protein IF or a casein eHF and compared with an unrestricted diet in the absence of CMPA [74]. The mean weight/age Z score at 2 years of age was similar in the three CMPA groups, but lower with the rice eHF diet than with the unrestricted diet during the periods between 9 and 12 months and 12-18 months, i.e. after the start of complementary feeding. Healthy infants fed for 16 weeks with a rice eHF diet supplemented with lysine and threonine or with a cows' milk IF had comparable normal growth and biochemical parameters [75]. Infants breastfed for at least 4 months and suffering from CMPA were either breastfed until 12 months or randomly weaned at 5-6 months of age to a soy protein IF, a casein eHF or a rice eHF [76]. Weight/age and height/age Z scores were below the mean at 6 months of age in all groups, probably due to CMPA. With the rice eHF, the height/age Z score was identical to that of the soy group and the breastfed group at 9 and 12 months. A rice eHF enriched with lysine, threonine and tryptophan was compared to a casein eHF in infants with CMPA and a mean age of 4 months [47]. Infants with a baseline weight lower than average normalised their weight by age 12 months using the rice eHF versus 18 months using the casein eHF.

Several clinical trials have shown that Neocate ${ }^{\circledR}$ ensured normal growth in the case of an allergy to eHF and in the case of multiple food allergies as well as a growth pattern identical to that obtained with eHFs when they are well tolerated [60, 65, 64]. Another study also showed that growth obtained with Nutramigen $\mathrm{AA}^{\circledR}$ in children with CMPA is comparable to that obtained with Nutramigen ${ }^{\circledR}$, a casein-based eHF [66]. Recent data with a thickened AAF showed appropriate growth during the first 1-month follow-up period [68].

## 11 Social Conditions of Use: Reimbursement

The cost of formulae to be used during CMPA is as follows: AAFs $>$ milk eHFs $>$ rice eHFs $>$ soy protein formulae. Cows' milk and rice eHFs (where available) are usually sold in pharmacies. On a family point of view, the cost largely depends upon the reimbursement rate by the health-care system, with great variations from one country to another within the European Union (EU).

## 12 Using eHF or AAF During Cow's Milk Allergy: Recommendations by Expert Working Parties

Recommendations for the use of eHFs in CMPA are limited. The choice of a hydrolysate varies from one country to another or within the same country. An international working group has released draft recommendations for the management of CMPA in infants, whether breastfed or not. In case of suspicion of CMPA of moderate severity, the working group recommends using an eHF based on soluble protein or casein; in severe cases (where there is either a life-threatening risk or a severe failure to thrive), the recommendation is to readily use an AAF [2]. An Australian expert's panel suggests eHFs as the first choice in infants less than 6 months of age, where a current allergy exists, concerning gastrointestinal symptoms and atopic eczema. The group recommends soy protein IF in infants over 6 months with presently existing reactions to CMP and in the case of gastrointestinal symptoms or atopic dermatitis with normal growth. In the case of anaphylaxis, the group recommends AAFs as a first choice, until allergic tests have been performed, in order to avoid any severe reaction to an eHF [77, 3].

In 2006, the ESPGHAN Committee on Nutrition recommended using eHFs in cases of proven CMPA in infants and avoiding soy protein infant formulas before the age of 6 months. Above this age, they have been proposed because of their lower cost and greater acceptability, but a test of clinical tolerance to soy protein has to be performed first [78]. In 2008, the American Academy of Pediatrics recommended the use of eHFs as a first choice in the case of proven CMPA and of AAFs in the case of a failure of eHFs [79].

The committee on Nutrition of the French Society of Pediatrics [7] made several recommendations: (1) If the infant is not breastfed or if the mother cannot or no longer wishes to breastfeed, the first choice is an extensive hydrolysate (eHF) of CMP. (2) If the eHF fails to achieve the desired result, an AAF is warranted. (3) In the case of anaphylaxis, eosinophilic oeso-gastro-enteropathy, failure to thrive or severe colitis, the use in first intention of either an eHF or an AAF is a valid option. (4) Rice protein eHFs offer an alternative to eHFs from animal origin. (5) Soy protein infant formulas can be used after the age of 6 months, after ensuring a good clinical tolerance to soy.

## 13 Comments on the Respective Use of eHF or AAF During CMPA

Several considerations can be made. Basically, we lack scientific data concerning the use of these different formulas during CMPA. More data are available on the efficacy and the safety of the new formulas coming on the market.

However, questions still not or partially answered are numerous. Is it possible to use a formula based on rice hydrolysates in children allergic to milk-based ones? What is the long-term outcome of the use of these different formulas in children diagnosed with CMPA in terms of acquisition of tolerance? One might consider that keeping a certain amount of milk peptides, using a milk-based eHF, might help the child acquire tolerance to milk. However, nothing is proven on that matter. In children with CMPA, the choice is actually between completely removing the milk allergens, using rice-based hydrolysates or an AAF and providing small amounts of milk peptides. No study to date has investigated the outcome of these different options.

Another question relates to the diagnosis of allergy to hydrolysates: What criteria should lead to consider this diagnosis? It is the experience of the author that this condition is largely underrecognised, in infants with, e.g., inconsolable crying resisting to eHF feeding and thus labelled non-allergic in its origin and in children with atopic dermatitis or gastroesophageal reflux remitting only partially with eHF feeding. Only a widely recognised definition of allergy to eHF may help clinicians in deciding which formulas seem more appropriate.

On a general point of view, the recommendations made by several scientific bodies, favouring first an eHF and then an AAF, seem the best approach. Recognising rapidly the lack of effect of a hydrolysate is the key and requires a close follow-up of children whose diet has been modified. Noticeably, such a close follow-up is recommended by all working parties, with the goal of avoiding any risk of nutritional problem in the child.

At last, the respective costs and reimbursement rates by the health-care system of cows' milk and rice protein eHFs, AAF and soy formulas are of considerable weight in the physician decision, and it appears that variation is numerous from one member state to another one in the European Union.

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# Chapter 19 <br> Allergen Avoidance Versus Tolerance Induction 

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## 1 Introduction

The number of allergic individuals is still increasing and, to date, allergic diseases cannot be effectively prevented or treated by the current management. Moreover, the definition of prevention, treatment, or management is varying throughout literature. This confusion is, at least partly, caused by the atopic march. The atopic march "refers to the natural history of atopic manifestations, which is characterized by a typical sequence of IgE antibody responses and clinical symptoms that appear early in life, persist over years or decades, and often remit spontaneous with age" [1]. This means that treatment of an early event like Atopic Dermatitis (AD) caused by a cow's milk allergy may at the same time prevent the onset of subsequent allergic diseases, e.g., seasonal rhinitis. A graphical representation of the atopic march is depicted in Fig. 19.1. Atopic sensitization is mostly determined by the presence of specific IgE antibodies at different ages, amongst which the prenatal period [2]. Whether these prenatal IgE antibodies in cord blood are originating from the fetus or the mother remains inconclusive. In addition, the effect of maternal exposure to allergens and its effect on the infants' immune system are currently extensively studied.

For now, management is limited to the identification and avoidance of allergens that induce the allergies and the use of medical substances to ameliorate the symptoms. In general, three different subtypes of IgE-mediated (food) allergy can be distinguished: transient, persistent, and oral allergy syndrome [3]. Although the

[^19]

Relative prevalence of symptoms according to age
(symptoms can occur simultaneously).
Fig. 19.1 Relative prevalence of common atopic childhood diseases according to age. The different symptoms can occur simultaneously
distinction between transient and persistent is not always clear, patients with transient food allergy usually outgrow their allergy over time, supporting a food avoidance strategy as recommended treatment [3]. However, the need to actively prevent or treat allergies is currently resulting in several therapeutic approaches like the use of biological [4, 5], allergen-specific immunotherapy [3, 6, 7], or the use of dietary compounds like herbal formulations, probiotics, or non-digestible oligosaccharides [8-10]. Furthermore, it is still debated whether avoidance of allergenic foods is actually necessary as also reviewed by Du Toit et al. [11]. The same review also highlighted another essential aspect when aiming for general conclusions from all clinical trials investigating the protective effect of interventions on food allergy: methodological differences that complicate the interpretation of the study outcomes [11].

## 2 Therapeutic Approaches

### 2.1 The Use of Biologicals

A number of reviews provided a recent overview of the work done with biologicals. Guttman et al. gave an extensive overview of all current targets for AD treatment with biologicals [4], whereas Yang et al. focused on food allergy [5]. Although these atopic diseases have a common ground, effectiveness of biologicals can differ
per outcome parameter. An example of such biologics is the humanized monoclonal anti-IgE antibody, which binds to an allergic epitope in order to prevent binding to mast cells and basophils. By blocking the binding of $\operatorname{IgE}$ to the IgE-receptor, crosslinking of allergen-specific IgE antibodies, and subsequent degranulation of the mast cell or basophil is omitted $[4,5]$. Different types of anti-IgE antibodies have shown similar results: anti-IgE not only reduces the amount of free IgE but also markedly reduces the expression of the FceRI on mast cells and basophils [5]. Therefore, this biological is used for individuals that are already sensitized and already display allergen-specific antibodies. It is thus considered as treatment and not as prevention. Another biological that is used for the prevention of allergic responses is the anti-interleukin (IL)-5 monoclonal antibody. Although an anti-IL-5 antibody (mepolizumab) has shown to be effective in reducing the peripheral and tissue eosinophilia in eosinophilic esophagitis, there was only a limited effect on the symptoms. In addition, moderate to severe AD patients on anti-IL-5 treatment also showed less eosinophilia but no improvement of the clinical symptoms. Therefore this approach needs to be tested in larger controlled trials in order to draw conclusions about its efficacy [4, 5]. Rachid and Umetsu have written an extensive review about the immunological mechanisms for desensitization and tolerance in food allergy, showing the complexity of different immune cells involved [12]. This complexity is one of the reasons why it is so difficult to fit the right type of immunotherapy to the right type of patient.

### 2.2 The Use of Immunotherapy

In case of oral allergy syndrome, treatment with subcutaneous immunotherapy or sublingual immunotherapy (SLIT) has displayed variable beneficial effects leaving treatment recommendations controversial [3, 13]. However, the use of SLIT with food allergens shows to be successful up to maximal dose limit of the tolerated food. Recently, combination of SLIT with oral immunotherapy (OIT) seems to provide a potential to increase the tolerated dosage of food allergens [3]. Also in the case of anti-IgE treatment, a combination with allergen-specific immunotherapy has shown promising results in patients with allergic rhinitis [5]. However, further studies are needed to assess safety and to standardize the methods [3, 5, 12]. A Cochrane report on the effectiveness of OIT for cow's milk allergy has shown that milk OIT is an effective method to induce desensitization in patients with IgE-mediated cow's milk allergy [7]. An additional percentage of patients only had a partial desensitization, still leading to a larger safety margin in case of accidental exposures. In any case, it is essential to realize that, although OIT is successful in case of cow's milk allergy, ingestion of tolerated amounts of cow's milk after the desensitization is crucial for the maintenance of the induced tolerance. A similar conclusion was drawn for peanut-specific OIT: it is a promising therapeutic approach for the management of IgE-mediated peanut allergy, but there
is insufficient evidence for long-term effectiveness, safety, and cost-effectiveness to recommend its routine use in clinical practice [6].

### 2.3 The Use of Dietary Compounds

Several dietary compounds have been investigated for their impact on (food) allergy. Lepski and Brockmeyer provide an overview of dietary compounds with more details about the mechanistics of action. One example is the role of retinoic acid (metabolite of vitamin A) in the promotion of secretory $\operatorname{Ig} A$-switch of B cells and the development of regulatory T cells in mesenteric lymph nodes [8]. Also dietary fibers and prebiotics have been investigated for their immunomodulating role in the onset and management of allergies. It has been shown that these fibers affect the gut microbiota and that the subsequently produced short chain fatty acids can directly signal to immune cells [8]. Rijnierse et al. reviewed the influence of dietary fibers on different allergic disorders, also highlighting the direct effects on immune cells [9]. The underlying mechanism of the specific oligosaccharide mixture was further unraveled by de Kivit et al., who described the upregulation of the soluble lectin galectin- 9 by intestinal epithelial cells exposed to a mixture of short chain galacto-oligosaccharides (sc)GOS, long chain fructo-oligosaccharides (lc)FOS, and TLR9 ligands [14]. Subsequently, they showed that an intervention with scGOS/lcFOS and a beneficial microbe Bifidobacterium breve M-16 V enhances serum galectin-9 levels, which is associated with the prevention of allergic symptoms in food allergic mice and infants [15]. Although most investigated compounds are (scarcely) present in the European/Western diet, some compounds present in traditional Brazilian and Asian diets have also shown an effective reduction of allergic diseases. An example of such compound is Arctium lappa L., which has shown to be effective in allergy-symptom reduction through the inhibition of mast cell degranulation and cys-leukotriene release [16]. Considering the effectiveness of this example, a whole range of possibly immunomodulatory exotic and/or traditional dietary opportunities are ready to be further explored. Another possibility to interfere with allergic symptoms is to reduce the recruitment of leucocytes from the circulation to the sites of allergic inflammation. Dietary compounds that have shown such activity are sphingolipids. Sphingolipids are present in various amounts in foods like fruits, dairy products, eggs, and soybean, and are hydrolyzed throughout the gastrointestinal tract into metabolites that are used in various cellular functions [17, 18].

To better understand which approaches will gain the most promising results, a better understanding of the early immune development seems to be essential. An obvious factor associated with early immune development is the effect of human breast milk on the development of the newborn. Although multiple meta-analyses have been performed to investigate the hypothesis that breastfeeding protects against the development of allergic diseases [19, 20], conclusions from the metaanalyses are contradictory [21]. However, despite this controversy, breastfeeding
should still be promoted considering its psychological, nutritional, and immunological benefits [22]. In addition, the effects of the maternal diet on the composition of human milk are investigated to substantiate the hypothesis that the susceptibility of infants towards allergic diseases can be reduced by maternal imprinting or epigenetic modification [10]. Or, in other words, the dietary habits of the mother can influence the atopic susceptibility of the offspring. For example, maternal cow's milk avoidance is associated with lower levels of mucosal specific IgA and the development of CMA in infants. As human milk IgA might play a role in preventing excessive, uncontrolled food antigen uptake in the gut lumen, Järvinen et al. propose that high specific IgA levels in human milk have a protective effect against food allergy [23].

## 3 The Influence of the Maternal Diet

Modern "western" diets are characterized by a lower intake of omega-3 ( $\omega-3$ ) polyunsaturated fatty acids (PUFAs) and a higher intake of $\omega-6$ PUFAs. This change in consumption has led to the assumption that postnatal intervention with fish oil would reduce the development of allergic diseases [24]. For example, dietary $\omega$-3 PUFA supplementation in a murine model for cow's milk allergy largely prevented allergic sensitization by suppressing the humoral response, enhancing local intestinal and systemic regulatory T cells, and reducing acute allergic symptoms [25]. In addition, the same group has shown direct effects of $\omega-3$ PUFA on a key player in the allergic response, the mast cell [26]. Although this hypothesis does not seem applicable to all postnatal administration studies, multiple observational and intervention studies suggest the effectiveness of fish oil or nourishment with fatty fish like salmon when applied during pregnancy [27, 28].

Besides PUFAs, antioxidants have been suggested as relevant dietary compounds that can influence the allergic susceptibility. Patelarou et al. have published a systematic review about the negative association of the antioxidant status of the mother during pregnancy or the antioxidant intake of young infants and the allergic susceptibility of the infants [29]. In addition, several studies have shown that a Mediterranean diet of high antioxidant-containing foods like whole grain cereals, fruit, vegetables, legumes, and nuts has been associated with reduced risk of asthma, wheezing, and Allergic Rhinitis in Mexico, Spain, and Greece [30]. The role of antioxidants in the onset of allergic diseases is, however, ambivalent. For example, vitamin D has been described as both beneficial and counteractive in the reduction of allergic susceptibility. This bivalent activity seems dependent on the timing of vitamin D exposure [31]. Nevertheless, the antioxidants vitamin C , vitamin $\mathrm{E}, \beta$-carotene, and selenium have shown a protective capacity [21, 27]. Another dietary factor that has shown beneficial effects on the allergic susceptibility is the use of probiotics. Although the use of general terms like "probiotics" is debated (reviewed by [32]), prenatal and postnatal probiotic supplementation has shown to reduce the allergic susceptibility of infants at risk [33]. In these studies,
mostly combinations of different probiotics are administered, e.g., Lactobacillus (L.) rhamnosus LPR and Bifidobacterium (B.) longum BL999 [33], L. paracasei ST11 and B. longum BL999 [33], L. rhamnosus GG, L. acidophilus La-5 and B. animalis subsp. lactis $\mathrm{Bb}-12$ [34], and B. bifidum, B. lactis, and Lactococcus lactis [35]. However, strategies for allergy prevention that can restore the favorable patterns and diversity of enteric microbiota require knowledge on both strainspecific effects and the timing of administration [36].

### 3.1 Allergens in the Maternal Diet

Even though part of the maternal diet can reduce the allergic susceptibility of her offspring, the question whether a mother should omit the intake of allergens during pregnancy or lactation is extensively debated in literature. One major concern of this allergen avoidance is the nutritional welfare of both mother and infant [37]. In addition, there are at least three possible routes for allergen exposure of a newly born: (1) via ingestion of allergen-containing amniotic fluid into the gastrointestinal (GI) tract of the fetus [37-39], (2) via direct transfer of allergens across the placenta [37, 40, 41], and (3) via allergen-containing breast milk [41-43].

It is known that a fetus at week $19-20$ of gestation already has detectable IgM-positive B cells in the circulation, indicating that an already full functioning sensitization process is in place [37, 39]. In addition, at 22 weeks of gestation, allergen-specific immune responses are detected in cord blood mononuclear cells [44]. Maternal dietary allergens are detected in both maternal and infants' blood directly after birth and there are indications that the fetus may be exposed to dietary allergens from the second trimester of pregnancy onwards through both transamniotic and trans-placental routes [37]. Furthermore, even when mothers are following a strict dietary avoidance regime, e.g., eggs, this is still not a guarantee that the infant will not be exposed to that allergen. This might be due to rare unintended ingestion of the protein by the mother, but might also be the result of environmental exposure through the mother's skin as was shown in severe eczema subjects [37].

In addition, the debate about whether parental atopy is a "guarantee" for atopic offspring is still ongoing. Especially in mouse models, there seems to be a difference in how tolerance is induced. Hansen et al. directly compared the mechanism by which tolerance was induced during pregnancy, early and late immunization. They showed that both maternal allergen exposure and postnatal mucosal allergen exposure of the offspring reduced allergen-specific IgE levels, albeit through presumably different mechanisms that do not all rely on the maternal immune response [45]. Also others suggested that early exposure to allergens like cow's milk protein and ovalbumin (OVA) conferred protection against allergy independently of the parental atopy [45, 46]. Furthermore, Ellertsen et al. demonstrated that maternal Th2-type immune responses are associated with a stronger reduction in allergen-specific IgE levels in the offspring than maternal Th1-type immune
responses. Although they used a limited set of microbial adjuvants, their findings do support the concept of allergy prevention through maternal immunization [47].

### 3.1.1 Allergen Exposure During Pregnancy

In case of allergen exposure via the GI tract, it is necessary to highlight that significant quantities of allergens like the House Dust Mite (HDM) allergen Der p-1 and OVA from chicken egg have been detected in amniotic fluid [37-39]. Furthermore, nutritive allergens such as OVA and beta-lactoglobulin (BLG) are also capable of passing the placenta, and inhalant allergens like HDM and cat allergens have been detected in umbilical cord blood [37, 40, 41]. However, it is not clear whether these allergens will reach the placenta in an intact manner in an in vivo situation, as diverse enzymes expressed in saliva, stomach, and intestine are able to break down allergenic dietary proteins into peptides without antigenic capacity [48]. Although it is good to keep this in mind, multiple research groups have shown that manipulation of the murine maternal immune response by allergen immunization during pregnancy reduces the allergen-specific IgE responses in their offspring after immunization [45, 49]. It has been shown that these circulating maternal antibodies in the offspring may diminish allergen processing and presentation by antigen presenting cells to T cells, thereby preventing neonatal sensitization [50, 51]. Furthermore, maternal immunization up-regulates the inhibitory IgG receptor Fc $\gamma$ RIIb on neonatal B cells in early life [52]. If thereafter maternal antibodies and the specific allergen form a complex, these complexes will cross-link the Fc $\gamma$ RIIb, leading to B cell inhibition [52].

Although these studies do not exclude that the effects are solely described to the prenatal or the postnatal period, it is suggested that postnatal mucosal allergen exposure could induce allergen-specific tolerance as the newborns' immune system is still maturing. However, there is still much debate about this early life introduction of allergens to prevent allergy [41, 45, 53-56]. Following the American Academy of Peadiatrics in 2008 [57], Kramer and Kakuma performed another systematic review in 2012 on the evidence for maternal allergen avoidance during pregnancy, laction or both to reduce atopic diseases in their offspring. Similar to the outcome in 2008, they conclude that allergen avoidance during pregnancy is unlikely to reduce atopic disease in the offspring, whereas avoidance during lactation might reduce atopic eczema if the mother is classified as high risk [58].

### 3.1.2 Allergen Exposure During Lactation

Daily consumption of one egg per day leads to higher OVA concentrations in human breast milk than the concentrations found in egg-avoiding mothers [41]. A similar finding has been reported in mice, where maternal exposure of OVA led to antigen transfer to the offspring via breast milk, eventually leading to antigenspecific tolerance [43]. This OVA-specific tolerance induction is attributed to the
combined exposure to allergen and transforming growth factor- $\beta$ (TGF- $\beta$ ) present in breast milk, leading to the allergy-preventing development of regulatory T cells (Tregs) in the offspring [42]. Since these findings were done in healthy, nonallergic mice, the same group investigated whether similar results could be found in allergic murine mothers. They showed that breastfeeding by antigen-exposed sensitized mothers abolished asthma development in the offspring. However, in contrast to the allergy-preventing development of Tregs in nonallergic mice, protection conferred by sensitized mothers was more effective, and did not require the presence of TGF- $\beta$ in the milk [56]. When investigating the mechanism further, they discovered that antigen-IgG complexes present in milk were effectively transferred to the breastfed offspring through a specific receptor called FcRn which subsequently induced active tolerance [56].

Fusaro et al. showed that prenatal murine exposure to allergens can both lead to tolerance and sensitization of the offspring, depending on the timing and amount of allergen administration [49]. Only little information is available about the presence of allergens in human breast milk. This scarcity of data is partly due to the difficulties in detecting allergenic proteins that have homologs in human milk, as shown by Bertino et al. They investigated the presence of major cow's milk allergens called BLG and casein and described how human milk proteins interfere in the detection method of bovine milk proteins [59]. Later on, this same group identified bovine alpha-S1-casein in human colostrum from both preterm and term births [60]. As mentioned before, also other dietary allergens like OVA are transported to the breast milk. However, up to $25 \%$ of the egg-consuming mothers had a delayed or even absent excretion of OVA to the breast milk [41, 61]. It is, however, unclear whether this is a specific phenomenon for this particular allergen, or whether this is the case for more allergens in these "non-transporting" mothers. Only a few groups are working on the mechanism by which proteins are transported. One of these showed that dephosphorylation of OVA reduces the passages through an intestinal epithelial Caco-2 cell monolayer [62]. Hopefully in the future more of these mechanistic approaches will give a better insight into the ways allergic proteins are recognized and/or processed differently by atopic compared to non-atopic individuals.

## 4 Allergen Exposure Beyond Breastfeeding

Human milk is considered the best nutrition for newborn infants because it contains optimal ingredients for healthy growth and development. Amongst others, breastfeeding confers protection against allergic diseases [57, 63]. The protective role of human milk seems to be the consequence of a synergistic action between a wide range of health-promoting components, such as carbohydrates, nucleotides, fatty acids, immunoglobulins, cytokines, immune cells, lysozyme, and lactoferrin [64-67]. Recently, other immunomodulatory factors like exosomes and microRNAs (miRNAs) have been found in human milk [68, 69]. However, to
date, not much is known about their function or mechanism of action regarding their role in the development of the infant's immune system. Moreover, breast milk content changes over time to ensure optimal passive and active protection and growth for the child [70-72]. If a mother, for whatever reason, ceases breastfeeding, an alternative nutritional source needs to be selected. Dependent on the geographical location, multiple infant formulae are available containing cow's milk proteins casein and/or whey or soybean proteins (mainly USA and UK). All infant formulae available on the market should be produced according to the guidelines on Global Standard for the Composition of Infant Formula, published by the ESPGHAN coordinated international Expert Group [73] and set worldwide by the CODEX. Whenever a child is susceptible for allergic diseases or already has an allergy, multiple hydrolyzed formulae or an amino acid-based formula can be used to prevent or reduce sensitization or elicitation by an allergen, respectively.

### 4.1 Hydrolyzed Formula

Especially in small infants, the options for allergen avoidance within nutrition are limited. It is therefore of great importance to have a clear understanding of the nutritional requirements and combine this with the current possibilities to reduce allergic responses. In general, infant formulae nowadays contain whole proteins (standard formula), partially or extensively hydrolyzed proteins or single amino acids (AA). However, definitions of partial or extensively hydrolyzed proteins are lacking, which make discussions on the suitability of such products on the prevention or management of allergic diseases very complicated. Based on the degree of hydrolysis and the length of the remaining peptides, hydrolyzed proteins are categorized as partial or extensive hydrolyzates [74-76]. Extensively hydrolyzed formulae contain only small peptides and are mainly used as a replacement for cow's milk-containing formulae in allergic children [57, 75]. In contrast, the partial hydrolyzates may contain larger protein fragments and are used in infants at risk for cow's milk allergy. They were initially developed to reduce the allergen load and improve the taste of hydrolyzates [77]. Later, it was hypothesized that less hydrolyzed proteins are more immunogenic and therefore may prevent cow's milk allergy by inducing tolerance towards cow's milk proteins [74, 78]. However, larger protein fragments also result in increased allergenicity, and therefore these hydrolyzates are not suitable for the treatment of cow's milk allergic children [75, 76, 79].

The efficacy and safety of every partially and extensively hydrolyzed formula needs to be established as each manufacturer uses different protein sources, hydrolyzation methods, and hydrolyzation degree. These compositions should meet the requirements of the Commission Directive 1999/21/EC on dietary foods for special medical purposes. In addition, cow's milk allergic children can react to different proteins in the milk. Although cow's milk contains over 25 proteins, the two major allergenic classes are caseins (ca. $30 \mathrm{~g} / \mathrm{L}$ ) and whey proteins (ca. $5 \mathrm{~g} / \mathrm{L}$ ).

The major whey protein allergens are $\beta$-lactoglobulin ( $\beta$-LG) and $\alpha$-lactoglobulin (ALA), whereas the main casein allergens are $\alpha$ S1-, $\alpha$ S2-, and $\beta$-caseins [80, 81]. The concentration of the allergenic proteins in cow's milk is amongst others dependent on the housing and feeding conditions of the cows [82].

The nutritional requirements of infants are amongst others dependent on the availability of specific amino acids present in the protein fraction [73], which normally are derived from unprocessed proteins that are cleaved by enzymes in the digestive tract. It is known that hydrolyzed proteins are more rapidly digested and absorbed when compared with intact proteins [83]. This may result in an increased oxidation of essential AA and, subsequently, in a lower yield of essential AA for protein synthesis [84]. The latter is one of the reasons why the "safe" protein-energy ratio to go to market without clinical evidence on growth is higher in hydrolyzed formula ( $2.25 \mathrm{~g} / 100 \mathrm{kcal}$ ) when compared with intact protein formula ( $2.0 \mathrm{~g} / 100 \mathrm{kcal}$ ) [73].

Partially hydrolyzed whey formula have been safely and lawfully marketed in Europe and the United States, but are not considered to be hypoallergenic as they might cause allergic reactions in one-third to half of the cow's milk allergic infants [85]. The FDA therefore concludes that "partially hydrolyzed formula should not be fed to infants who are allergic to milk or to infants with existing milk allergy symptoms" [85]. Greer et al. state that there is no proof that the use of hydrolyzed formula is any better than human milk in the prevention of atopic diseases [57]. Especially since little is known about the allergens present in breast milk and their role in the allergic susceptibility, the comparison between breast milk and infant formula remains difficult. However, as mentioned before, Coscia et al. showed the presence of intact bovine $\alpha$-S1-casein in both term and preterm colostrums [60], suggesting that also human milk can expose an infant to allergens derived from cow's milk. For this reason, mothers that exclusively breast feed and have an infant with clinical allergic symptoms are advised to eliminate dairy products from their diet for approximately $2-3$ weeks after which the child's symptoms should rapidly disappear [86].

Whereas animal studies indicated that only partial hydrolyzates induce tolerance [87, 88], both partial and extensive hydrolyzates seem to prevent cow's milk allergy and atopic dermatitis in high-risk children [57, 75, 89-94]. Alexander et al. performed a systematic review on the capacity of $100 \%$ partial whey hydrolyzates to reduce the cumulative incidence of atopic outcomes including AD and concluded that these formulae reduce the risk of AD compared to infants fed an intact protein cow's milk formula [95, 96]. In line with Greer et al. and the WHO, they do also state that breastfeeding is the standard for infant nutrition, but if breastfeeding cannot be utilized, a $100 \%$ partial whey hydrolyzate may reduce the risk of AD. However, evidence for this beneficial effect in humans is limited due to methodological problems and inconsistent findings [57, 75, 85, 91, 97, 98]. As mentioned above, the lack of specification of partial and extensively hydrolyzed proteins and knowledge of which peptide size is required for sensitization in different individuals, the discussion between suitability of partial compared to extensively hydrolyzed formula to prevent sensitization remains to be elucidated.

Up till now, this debate within the hydrolyzate field has led to the differential availability of partial and/or extensive hydrolyzates in even different European countries. For example, Germany, the Netherlands, and Austria have partial hydrolyzates on the market, whereas in the UK these products are not available. However, the very limited clinical data to support the concept of allergy prevention by hydrolyzed formula has recently led to an extensive debate within the scientific literature on whether the used approach is the right one. It is under discussion that allergy avoidance is the wrong way to go and that active tolerance induction is the future route to go. Others state that if a formula should be used, an extensively hydrolyzed formula is recommended [86]. One large ( $n=2,252$ high-risk infants) randomized controlled trial designed to test the relative efficacy of cow's milk, partially hydrolyzed whey formula, extensively hydrolyzed whey formula, and extensively hydrolyzed casein formula in preventing the cumulative incidence of AD in the first 10 years of life was conducted. The conclusion of this GINI study was that there was no effect by any of the hydrolyzates on the prevention of asthma, allergic rhinitis, or allergen sensitization, and avoidance of cow's milk proteins is not the only factor that needs to be taken into account [99]. Statements that researchers investigating allergies need to realize that hardly any applicable effective prevention strategies have been developed in the past 25 years, and that novel ideas are essential to win the battle against the epidemic of the twenty-first century, are emerging [100]. The fact that interest groups like the EAACI are writing a position paper and introduce their efforts on systematic review protocols [101] are indicative for the need of alternative measures to aid allergic individuals in their battle against allergies.

### 4.2 Tolerogenic Peptides and Peptide Immunotherapy

Ideally, both a preventive and curative therapy for cow's milk allergy should induce tolerance without activating mast cells and basophils. To induce T cell anergy or Tregs, T cells should be activated via their T cell receptor without co-stimulation or in the presence of specific cytokines such as IL-10 and TGF- $\beta$ [102-104]. The T cell receptor recognizes peptides of $9-12$ amino acids long, which are much smaller than the peptides that are needed to cross-link IgE (minimal 35 amino acids) [105108]. Therefore, it has been suggested that using peptides that are too small to crosslink IgE but long enough to induce T cell activation may be a safe alternative for conventional immunotherapy. The potential of peptide immunotherapy has mainly been investigated for inhalation allergies.

Preventive and curative treatment with peptides reduced T cell responses, antibody production, and/or allergic symptoms in mice. Moreover, curative peptide immunotherapy was effective in cat and bee venom-allergic patients [109-111]. In these studies, a mixture of peptides ( $10-17$ amino acids long) was administered intradermally or subcutaneously. The treatment significantly reduced the allergic symptoms and no acute allergic side effects were observed. In cat-allergic patients,
the peptides did induce late allergic symptoms, but these side effects decreased during treatment [112, 113]. Interestingly, Patel et al. showed that four injections of a peptide mixture were already effective and decreased allergic symptoms even 9 months after the therapy was stopped [111]. To date, a limited number of studies have investigated the potential of peptide immunotherapy for food allergy. For example, Rupa et al. showed that oral treatment with a peptide of ovomucoid in a curative setting significantly decreased allergic symptoms in a mouse model for egg allergy [114]. For cow's milk allergy, only the efficacy of preventive treatment has been investigated. Hirahara et al. showed that preventive intradermal treatment with a peptide of $\alpha-\mathrm{S} 1$ casein reduced T cell and antibody responses to the intact protein in mice [115]. Moreover, previous studies have shown that prophylactic treatment with partial whey hydrolyzates reduced allergic symptoms in mouse models for cow's milk allergy [87, 88]. In addition, Bøgh et al. showed that co-immunization of intact $\beta$-lactoglobulin with digested $\beta$-lactoglobulin reduces the sensitizing capacity of intact BLG, which could result from tolerogenic mechanisms induced by the digestion products [116]. Interestingly, Knipping et al. have indicated that during the hydrolysis of whey proteins there is a certain time point at which the formed peptides are too small to induce basophil activation but long enough to induce T cell activation [117]. However, whether these peptides are able to induce tolerance is unclear.

Recently, Meulenbroek et al. showed that prior exposure to specific peptides of $\beta$-lactoglobulin reduces the allergic response to whey [118]. They showed that regulatory dendritic and T cells might be involved in this and that the combination of peptides with a scGOS/lcFOS/pAOS-containing diet enhances this allergy reducing effect. Although this approach seems to direct towards tolerance induction, still many questions regarding the tolerogenic peptides need to be validated. For example, it is known that there are variations between individuals in the sequences that are recognized as immunogenic or allergenic. Texier et al. showed that each HLA-DR allele expressed a unique binding pattern of Api m1 peptides (a major allergen of bee venom) [119]. Individuals that express different MHC molecules on the antigen presenting cells may bind/present different peptides and thus recognize different T cell epitopes. This may also explain the diverse T cell response that were observed by Meulenbroek et al., who describe that none of the T cell epitopes was recognized by all patients in their epitope-mapping experiments with whey-derived peptides [118, 120]. Based on these data, it seems necessary to not only look at the different allergens that cause a variety of allergic disease (e.g., asthma, food allergy, or eczema) but also at ethnic backgrounds, dietary habits, and exposure to immune activating triggers like viruses, bacteria, and parasites [10].

## 5 Summary

The prevalence of allergies is still increasing and standard management is not sufficient to stop this ascent. Over the last decades, more and more animal studies and clinical trial are investigating the safety and clinical and immunological


Fig. 19.2 Evolution of allergy management strategies
efficacy of biologics, immunotherapy, and other pharmaceuticals on the treatment of the different allergies. It seems to become logic that there is no golden bullet that will serve all allergic diseases and more specialized solutions are required. It is likely that the way forward lies in the combination of life style management, nutritional support (e.g., prebiotics, probiotics, PUFAs) to preset the immune system towards tolerance induction and allergen-specific immunotherapies (e.g., SLIT, OIT) either based on whole proteins or tolerance inducing peptides (see Fig. 19.2). It is clear that the future direction in allergy management is shifting away from the classical allergen avoidance into active tolerance induction.

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## Part V Brain and Neuroimmunity

# Chapter 20 <br> Nutrition and Cognitive Decline in Older Persons: Bridging the Gap Between Epidemiology and Intervention Studies 

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## Abbreviations

| AD | Alzheimer's disease |
| :--- | :--- |
| ADAS-cog | Alzheimer's disease assessment scale-cognitive subscale |
| ADL | Activities of daily living |
| ApoE4 | $\varepsilon 4$ Allele of the apolipoprotein E gene |
| ARCD | Age-related cognitive decline |
| A $\beta$ | $\beta$-Amyloid peptide |
| DASH | Dietary approaches to stop hypertension |
| DHA | Docosahexaenoic acid |
| EPA | Eicosapentaenoic acid |
| LC n-3 PUFA | Long-chain omega-3 polyunsaturated fatty acid |

[^20]MCI Mild cognitive impairment
MMSE
Mini-mental state examination
MRI
Magnetic resonance imaging
PET Positron emission tomography
RCT Randomized clinical trial

## 1 Background

Dementia is one of the most devastating chronic disorders in older persons. Indeed, dementia is characterized by severe cognitive impairment that impacts social and leisure activities, leading progressively to total dependency from others to perform activities of daily living (ADL) [1]. After age 75, about one person in five is affected by dementia [2]. Dementia generates a huge social and economic burden, with an estimated average yearly per-person cost of US\$ 33,329 for care purchased in the market in the US [3]. Given the lack of curative treatment, the main component of the costs is for institutional and home-based long-term care [3]. Indeed, the two main causes of dementia in older persons are Alzheimer's disease (AD), accounting for about $70 \%$ of cases [4], and vascular dementia, including many mixed forms [5]. There is no etiological treatment for these irreversible disorders. However, the dementia stage is preceded by several decades of silent accumulation of neurodegenerative and vascular lesions in the brain before the onset of the first cognitive symptoms [6]. This extremely long time frame allows various environmental factors to accelerate or slow down disease progression and subsequent cognitive decline. Indeed, late-life dementia results from a complex interplay between non-modifiable risk factors such as age and genetics, and potentially modifiable environmental factors including vascular risk factors, metabolic disorders, and lifestyle $[7,8]$. As discussed in another chapter of this book, nutrition is a major component of lifestyle that might modulate neurodegeneration and vascular pathology, preserve or impair cognitive reserve and plasticity, and eventually impact the rate of cognitive decline [9]. Indeed, excessive energy intake may lead to obesity, metabolic syndrome, and diabetes, which have been linked to an increased risk of AD and vascular dementia [10-12]. Conversely, basic research and epidemiological studies suggest a protective effect of several classes of nutrients including long-chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA) [13], antioxidant vitamins C and E [14], carotenoids [15], polyphenols [16], B vitamins [17], and vitamin D [18] against brain aging. Underlying mechanisms include beneficial vascular and metabolic effects, decreased inflammation and oxidative stress, and improved insulin sensitivity, in addition to more specific effects on brain structure and functioning [9].

However, randomized clinical trials (RCT) that have administered various nutritional supplements for the prevention or treatment of cognitive decline have yielded extremely disappointing results so far. Recent meta-analyses or systematic reviews of RCTs with LC n-3 PUFA [13, 19, 20] or B vitamins [21] have concluded
there was a lack of benefit of the supplementation on cognitive function in older people. Regarding antioxidants, the few available large placebo-controlled RCTs did not demonstrate any beneficial impact on cognition [22-28], with the exception of an older RCT in AD patients receiving high-dose vitamin E [29]. The most recent RCT even showed a deleterious effect of an association of high dose antioxidants including vitamins E and C and alpha-lipoic acid on cognitive function [30].

In a recent critical review, Dangour et al. concluded "whether the lack of agreement in findings from mechanistic and observational data and from intervention studies reflects a real absence of benefit on cognitive function from LC n-3 PUFA supplementation, or whether it reflects intrinsic limitations in the design of published studies remains open to question" [31]. Given the discrepancies between the results of epidemiological studies and RCTs, this statement also probably applies to the effect of other nutrients on cognitive function in older people. Methodological limitations of epidemiological studies in the field of nutrition are well known, including measurement error and residual confounding, especially by lifestyle [32]. However, RCTs are not devoid of methodological limitations that may hamper their conclusions. The aim of this chapter was to examine the challenges and potential pitfalls of RCTs involving nutritional interventions for the prevention or treatment of cognitive decline in older people. These methodological considerations could open the door to new, well-designed RCTs in the field of nutrition and cognition that would reconcile the results of basic and epidemiological research with high-grade evidence coming from RCTs.

## 2 Time and Duration of a Nutritional Intervention

The optimal window of opportunity for a nutritional intervention during the long process leading to dementia is not known: should we target healthy individuals for primary prevention, those with mild cognitive symptoms for secondary prevention, or demented patients for treatment (Fig. 20.1)?

Even if there is a continuum of neurodegeneration and clinical symptoms with disease progression, the natural history of AD can be subdivided into three successive stages according to the model proposed by Jack et al. [6]: a long presymptomatic phase without any detectable cognitive impairment with currently available instruments, followed by a phase of incipient cognitive decline named mild cognitive impairment (MCI), that may progressively worsen to reach the final phase of irreversible dementia. During the presymptomatic phase, there is a progressive silent accumulation of the neuropathological hallmarks of AD in the brain: $\beta$-amyloid peptide $(A \beta)$ in senile plaques and hyperphosphorylated tau in neurofibrillary tangles [33]. Neurodegeneration is accompanied by impaired brain glucose metabolism, exacerbated oxidative stress, inflammation with microglia activation, and eventually neuronal death and brain atrophy, especially in the hippocampus [33].


Fig. 20.1 Natural history of Alzheimer's disease and room for prevention

Primary prevention of AD by nutrition, i.e., before neuropathology develops, seems out of reach unless nutritional factors acting in early life can be identified [34]. Indeed, healthy diets providing LC n-3 PUFA along with other essential nutrients are necessary for brain development in infancy [35] and could contribute towards a lifelong sustainable brain reserve. However, evidence from epidemiological studies is weak $[36,37]$, and it is impossible to show the impact of diet in early life on risk of AD decades later by the means of RCTs. Nevertheless, the PREDIMED RCT showed that it was possible to decrease the incidence of cardiovascular disease with a Mediterranean diet enriched with virgin olive oil or nuts in asymptomatic adults [38]. Given the important vascular component of dementia, such a diet could also contribute to the prevention of cognitive decline. Moreover, a recent RCT conducted in New Zealand showed that 6 months supplementation with 1.16 g docosahexaenoic acid (DHA)/d plus 0.17 g eisocosapentaenoic acid (EPA)/d significantly improved episodic memory in women and reaction time of working memory in men aged 18-45 years habitually consuming few fish [39]. Although it is impossible to ensure that these apparently healthy individuals did not have any neurodegenerative lesion in their brains yet, this positive RCT suggests that prevention with nutritional supplements is possible and should probably target younger individuals than those currently included in RCTs.

Secondary prevention of AD would encompass two groups of individuals: those who already have neuropathological changes in their brains but remain still cognitively asymptomatic (preclinical AD) and those with MCI. Co-occurrence of $\mathrm{A} \beta$ and tau pathology seems necessary to produce cognitive deficits [33]. PET imaging shows that $\mathrm{A} \beta$ accumulation generally follows a sigmoidal curve, with a steeper slope during 15 years on average before reaching a plateau [40]. MCI would manifest only once this plateau is reached. This 15 -year interval represents a large potential therapeutic window for secondary prevention with nutritional interventions. However, since individuals do not have any cognitive symptom at this
stage yet, it is very difficult to target those who could actually benefit from such a nutritional supplementation. In addition to biomarkers of AD pathology or impaired brain glucose metabolism [41], dietary or genetic inclusion criteria might be useful, as discussed in Sects. 4 and 5 of this chapter. Moreover, RCTs evaluating the impact of nutritional interventions at this presymptomatic stage would require to follow-up individuals for many years before a significant impact can be evidenced on their rate of cognitive decline or incidence of dementia. Nevertheless, the concept of age-related cognitive decline (ARCD) might prove useful to target individuals for secondary prevention. In the MIDAS trial, ARCD in adults aged 55 years and over was defined as having a subjective memory complaint and a score $\geq 1$ standard deviation below the mean of younger adults aged 25-35 years on the immediate or delayed recall of the Logical Memory sub-test of the Wechsler Memory scale [42]. Thus, these individuals did not reach the criteria for MCI but subjectively, they felt that they were declining, probably indicating a significant neurodegenerative burden. Unfortunately, brain imaging was not available in this RCT [42]. A meta-analysis of RCTs with LC n-3 PUFA for the prevention of cognitive decline showed that in cognitively "healthy" participants defined as having a Mini-Mental State Examination (MMSE) $>25$, supplementation with EPA and/or DHA could have a significant impact on tests of verbal memory but not on other neuropsychological tests [19]. Interestingly, there was no relationship between length of the supplementation (up to 24 months) and its impact on cognitive functions. Similarly, various combinations of antioxidants have been inefficient in RCTs for the prevention of cognitive decline in apparently cognitively healthy elderly [22, 23, 25] except an RCT of supplementation with 50 mg betacarotene on alternate days during 18 years on average, suggesting that supplementation over an extremely long period of time would be necessary to exert beneficial effects [26]. However, this RCT had some methodological limitations including a high selection rate of those participating in the second phase of the study.

At the MCI stage, brain atrophy and synaptic loss are accompanied by objective cognitive deficits. This is an unstable stage: some individuals (about $16 \% / y e a r$ ) will revert to normal cognition at least for a certain period, while others will remain in the MCI state, and about $20 \%$ will progress to irreversible dementia during the same time [43]. Thus, at this stage it would be theoretically easier for a nutritional intervention to have an impact on the rate of cognitive decline or progression to dementia. However, the meta-analysis of Mazereuuew et al. showed an impact of supplementation with EPA or DHA on attention and processing speed but not on other cognitive domains in individuals characterized as "cognitive impairment no dementia" [19]. In particular, there was no impact on episodic memory, which is impaired early in the course of AD [44].

At the dementia stage, all RCTs with LC n-3 PUFA failed to show any impact on cognition [13, 45], except a few exploratory trials with methodological weaknesses conducted in very small highly selected samples [46, 47]. Regarding antioxidants, a single trial showed a benefit of supplementation with $2,000 \mathrm{IU}$ alpha-tocopherol (vitamin E )/d in patients with moderate AD on a primary outcome combining death, institutionalization, loss of the ability to perform basic ADL, or severe dementia,
but only after adjustment for baseline cognition [29]. This beneficial effect of vitamin E was not reproduced in MCI patients with the same dosage [24]. Moreover, at this extremely high dosage vitamin E may have adverse effects including higher risk of mortality [48]. The most recent RCT compared the impact of a combination of several antioxidants (vitamins E and C, lipoic acid), coenzyme Q and placebo for 16 weeks in patients with mild or moderate AD [30]. Unexpectedly, patients receiving the combination of antioxidants experienced faster cognitive decline on the MMSE despite reduction of oxidative stress in the brain as shown by decreased isoprostanes in cerebrospinal fluid.

## 3 Cognitive Outcomes

The choice of the primary outcome in RCTs of nutritional interventions for the prevention or treatment of cognitive decline is closely linked to the disease stage. Two kinds of clinical outcomes are available: time to onset of dementia and rate of cognitive decline. Additional outcomes include biomarkers of disease progression and less specific outcomes such as functional decline in ADLs, institutionalization, and mortality.

The incidence of dementia is too low in individuals without major cognitive symptoms to be used as an outcome in RCTs whose duration ranges from several months to a few years at most. This explains why a recent Cochrane review about the effect of $n-3$ PUFA concluded that direct evidence on the effect of omega-3 PUFA on incident dementia is lacking [20]. Indeed, the sample size required to achieve enough power to show an impact on incidence of dementia is extremely large, as many as 44,000 healthy participants at baseline, depending on hypotheses on dementia incidence and expected risk reduction [49]. Several studies have tried to overcome this difficulty by nesting ancillary cognitive studies in larger RCTs, such as the prevention of AD by vitamin E and selenium (PREADVISE) trial nested in SELECT, a large prostate cancer prevention trial with a $2 \times 2$ factorial design [50]. However, the SELECT trial was discontinued because of an increased risk of prostate cancer in men in the vitamin E only arm [51].

Regarding cognition, global tests such as the MMSE lack sensitivity to change and they are limited by a strong ceiling effect in healthy individuals. No effect of LC n-3 PUFA supplementation on MMSE was observed in the meta-analysis by Mazereeuw et al. whatever the disease stage [19]. Similarly, the three RCTs of LC $\mathrm{n}-3$ PUFA conducted in AD patients did not show any impact on the AD assessment scale-cognitive subscale (ADAS-cog), a global scale of cognitive function [19]. Neuropsychological tests assessing cognitive domains that are affected early in the dementing process are probably the best candidates as primary outcomes in RCTs for the prevention of dementia. However, impairment in specific cognitive domains depends on the etiology of dementia. Impairment in episodic memory is a core feature of early AD [44] while vascular dementia affects more specifically executive functions [52]. At a later stage, multiple domains of cognition are
affected whatever the etiology of cognitive impairment. Most RCTs have used various batteries of neuropsychological tests as main outcomes, giving rise to high heterogeneity between studies. Indeed, some have used single tests to investigate specific areas of cognition (e.g., episodic memory, executive functions, processing speed), while others have combined several tests to encompass each cognitive domain by computing $Z$-scores [39]. In the PAQUID study, we showed that subtle cognitive decline on Isaac's Set Test, a verbal test of semantic memory, could already be evidenced 12 years before dementia onset [53]. Although not specific of early AD, this kind of neuropsychological test could also be used as an outcome in preventive interventions.

Impact on ADLs is one of the mandatory criteria for regulatory agencies when assessing the impact of a drug in AD. This endpoint is less relevant in preventive trials, when participants do not have any limitation on ADLs yet. At the dementia stage, progressive impairment in instrumental and then basic ADLs results from cognitive impairment [54], but also from physical impairments that are often associated in this older population. Hence, little improvement can be expected from nutritional interventions involving single nutrients. By contrast, interventions to prevent or manage undernutrition might have a global impact on functioning in patients with dementia. Unfortunately, this was not the case is the NutriAlz RCT, a nutritional teaching and training intervention targeting home-living patients with dementia and their physicians and caregivers [55].

Biomarkers of disease progression could be useful to evidence the impact of nutrients on specific mechanisms: hippocampal atrophy assessed by magnetic resonance imaging (MRI), amyloid load in the brain assessed by positron emission tomography (PET) with amyloid markers, $\mathrm{A} \beta$ species in cerebrospinal fluid, brain glucose metabolism assessed by FDG PET [44]. However, there is often a lack of correlation between biomarkers and the severity or progression of cognitive impairment. The VITACOG RCT showed that supplementation with folic acid, vitamin B6, and vitamin B12 for 24 months was associated with lower rate of whole brain atrophy, the primary outcome, in elderly individuals with MCI [56]. In the same study, the cognitive benefit assessed as a secondary outcome was less consistent [57]. Indeed, there was no significant overall effect of treatment on MMSE, category fluency, or delayed recall but a significant impact on a test of executive function. Moreover, there was an interaction with baseline plasma homocysteine, the impact of the supplementation on some cognitive tests being more important in individuals with raised homocysteine. This result makes sense knowing that raised homocysteine is a vascular risk factor but also a risk factor of dementia, and hence an expected impact of homocysteine lowering by $B$ vitamins on executive functions. Such prespecified working hypotheses could help to design more powerful studies and allow subgroup analyses if they are mentioned in the original protocol and do not only stem from preliminary statistical analysis.

## 4 The Nutritional Intervention: Diet or Supplement?

RCTs can be classified as explanatory vs. pragmatic according to their aim [58]. In the field of nutrition, explanatory trials aim to evidence the impact of a specific nutrient or combination of nutrients on a select outcome in strictly controlled experimental conditions. The results of explanatory trials can help to derive evidence-based dietary recommendations before assessing their efficiency in the real world by the means of pragmatic trials. An example of explanatory trial is the SUVIMAX RCT whose objective was to demonstrate that a given amount of antioxidant vitamins and minerals close to recommended dietary intake could reduce the risk of major health problems, especially cancer and cardiovascular disease [59]. The final aim was not to recommend a supplementation of the whole population but to base dietary recommendations such as those of the French National Program Nutrition and Health to increase fruit and vegetable intake on the best available evidence.

### 4.1 RCTs with Nutritional Supplements: More Is Not Better

In opposition to therapeutic trials with drugs whose molecule is not present in the body before treatment, RCTs with nutritional supplements are undertaken in individuals who already have a baseline level of these nutrients, with a great interindividual and intra-individual (day to day) variability, depending on their dietary intake. This variability is particularly important for nutrients such as EPA and DHA coming from fish and seafood which are rarely consumed on a daily basis. Many foods are also fortified with various nutrients, e.g., vitamin D in milk or B vitamins in flour, depending on the country. This baseline variability tends to underestimate the strength of the association between nutrients and outcomes. Optimal quantities and proportions of nutrients that should be ingested daily to prevent or slow down cognitive decline are not known. Epidemiological studies can help to estimate the amount of nutrients that are needed to have a protective effect on cognition, and by difference between needs and actual intake, the amount that should be given in a supplement. Dietary inclusion criteria in RCTs should also be taken into account. Indeed, little benefit and even potentially harmful effects can be expected from a nutritional supplementation in individuals whose dietary intake already meets the recommendations. The Alzheimer's Disease Cooperative Study was a placebocontrolled RCT which included 402 individuals with mild or moderate AD who consumed less than 200 mg DHA/d. There was no impact on cognition or any other outcome of a supplementation with 2 g DHA/d for 18 months [45]. Although participants had a low DHA intake at baseline, such a supplemental dose may lead to an excessive daily amount. By contrast, a supplement with 1.33 g EPA + DHA/d significantly improved reaction time for episodic memory and working memory in adults who infrequently consumed fish (providing less than 200 mg

EPA + DHA/week) [39]. Although these studies are not directly comparable and differ by many other aspects, their discordant results suggest that habitual dietary intake of the nutrient in question should be considered as an inclusion criterion. Moreover, an increasing number of individuals already take various nutritional supplements and should be excluded from RCTs. Identification of individuals who could possibly benefit from a nutritional intervention because of their low dietary intake requires reliable dietary surveys. However, estimation of actual intake of some nutrients may be difficult and time-consuming especially for nutrients which are not consumed on a daily basis such as EPA and DHA, or vitamin D also provided in part by fatty fish. Simple dietary screening tools should be developed and validated for this purpose. These tools could also help to target beneficiaries of the nutritional supplementation in the general population. Biomarkers may be an alternative for example for vitamin D status, but they are expensive and less easy to generalize.

Most RCTs with nutritional supplements have used doses far above the recommended allowances whatever the kind of nutrient. However, higher doses of nutrients do not necessarily mean better effects on cognition or other outcomes. Very variable amounts of EPA and DHA have been used in RCTs for prevention or treatment of cognitive decline. Among the six RCTs conducted in cognitively "healthy" (MMSE $>25$ ) participants reported by Mazereeuw et al. quantities of EPA varied from 0 to $1,093 \mathrm{mg} / \mathrm{d}$ and those of DHA from 59 to $1,700 \mathrm{mg} / \mathrm{d}$, without any clear relationship between dosage and effect [19]. These RCTs did not determine an optimal EPA-DHA ratio either. Because of their multiple double bonds, LC n-3 PUFA are potentially prone to lipid peroxidation that will produce toxic compounds. Intake of $1,600 \mathrm{mg} \mathrm{DHA} / \mathrm{d}$ for 2 weeks is associated with increased urinary isoprostanes, a marker of oxidative stress [60]. Concomitant supplementation with high dose vitamin E at $900 \mathrm{IU} / \mathrm{d}$ does not prevent lipid peroxidation [61]. Indeed, vitamin E may become harmful at high dosages [51]. RCTs with 2,000 IU vitamin E/d such as those previously published [24, 29] in the field of cognition can no longer be undertaken. As for drugs, phase-1 and 2 clinical trials should precede phase-3 RCTs in order to ensure safety and estimate optimal doses of nutrients that should be given in a supplement (Fig. 20.2).

### 4.2 Limits of a Single-Nutrient Approach: A Nutrient May Hide Another One

A major reason for the lack of efficacy of nutritional supplements to prevent cognitive decline may lie in their inability to reproduce the complexity of a healthy diet. Indeed, except as supplements, nutrients are never consumed in isolation but combined with others in food. Observational studies cannot disentangle the impact of a given nutrient from that of others to which it is tightly associated in the diet, such as LC n-3 PUFA and vitamin D provided by fatty fish. Thus, subsequent


Fig. 20.2 Relationship between nutrient intake and health status
intervention studies may have targeted the wrong nutrient, e.g., EPA and DHA instead of vitamin D. Moreover, nutrients may act in synergy. For example, dietary antioxidants might contribute to protect LC n-3 PUFA against peroxidation. Accordingly, we observed that regular intake of fruits and vegetables was necessary to observe a cognitive benefit of fish consumption [62].

The Souvenaid ${ }^{\ominus}$ medical food was derived to improve synapse formation and function [63]. Souvenaid ${ }^{\text {© }}$ includes EPA, DHA, uridine, choline, B vitamins, and antioxidants (vitamins C and E, selenium). The Souvenir II RCT was conducted in 259 drug naïve patients with mild AD consuming $<3$ servings fatty fish/week, randomized to receive either Souvenaid ${ }^{\odot}$ or an isocaloric ( $125 \mathrm{kcal} / \mathrm{d}$ ) placebo for 24 weeks [64]. At 24 weeks, patients receiving Souvenaid ${ }^{\odot}$ significantly improved on the primary outcome, the memory domain Z-score of the Neuropsychological Test Battery. Although this RCT did not show a significant impact of the intervention on executive functions or functional ability, it provides a proof-of-concept that a nutritional intervention can improve cognitive functions in patients with AD.

Other RCTs of multivitamin and multimineral supplementation [65] or other combinations of nutrients (e.g., B vitamins and LC n-3 PUFA [66]) have failed to show any impact on cognitive function in healthy older adults. These discordant findings suggest that the optimal combination of nutrients and their right target need further research.

The brain has a high energy requirement and the aging brain is now known to have regions of lower glucose uptake than in younger adults [67]. Furthermore, asymptomatic individuals at genetic or familial risk of AD have lower regional brain glucose uptake decades before the onset of cognitive decline; with the onset of cognitive decline, the deterioration in brain glucose uptake becomes exacerbated [67, 68]. The declining capacity of the aging brain to acquire sufficient fuel may therefore be a factor limiting the efficacy of nutrient supplements aiming to correct or delay the onset of progression of cognitive deficit. Insulin resistance associated with type 2 diabetes is associated with higher risk of AD and may contribute to deteriotrating brain glucose uptake in the elderly [69]. We therefore consider it to
be of paramount importance that nutritional interventions for the prevention of AD be coupled with interventions aiming to improve brain fuel uptake.

### 4.3 Dietary Interventions

In the field of cognition in older persons, most published RCTs of nutritional interventions have been explanatory so far. However, general dietary recommendations providing several classes of nutrients along with optimal dietary energy intake could be the most efficient nutritional strategy to slow down cognitive decline and postpone the onset of dementia. Indeed, observational epidemiological studies have shown that healthy dietary patterns were associated with better cognitive outcomes [70, 71]. In particular, higher adherence to a Mediterranean type diet rich in fruits, vegetables, legumes, cereals, fish, and olive oil has been associated with a lower risk of AD and cognitive decline [72]. This dietary pattern provides antioxidant vitamins C and E , vitamin B6, and folate which could exert a protective effect against cognitive decline [73]. Higher adherence to a Mediterranean diet is also associated with higher plasma EPA and DHA concentrations [74]. However, unlike in cardiovascular disease [38, 75], no RCT has shown the impact of shifting to a Mediterranean type diet on cognitive function in older persons. Nevertheless, this positive impact of the Mediterranean diet on cardiovascular outcomes suggests a potential effect on the vascular component of cognitive decline.

Very few published RCTs have investigated the impact of a dietary modification on cognitive outcomes. Forty-nine older adults (20 healthy and 29 with amnestic MCI) were randomized to follow a HIGH or LOW diet for 4 weeks [76]. The HIGH diet was high in fat, especially saturated fat, and low in carbohydrates in proportion of total energy intake but with a high glycemic index. Conversely, the LOW diet was low in fat, especially saturated fat, and had a low glycemic index. The proportion of protein was similar in both diets (15-20 \%). Caloric needs were estimated to maintain pre-intervention weight and meals were delivered at home, and hence a strictly controlled condition. As expected, insulin sensitivity improved with the LOW diet. The LOW diet was associated with improved verbal memory but had no impact on other cognitive domains. In another RCT, 124 overweight adults with high blood pressure were randomized to receive either the Dietary Approaches to Stop Hypertension (DASH) diet (rich in fruits, vegetables, low-fat dairy, and low in saturated fat and cholesterol) alone, or the DASH diet combined with a weight management program, or a usual control diet [77]. The DASH diet alone or associated with weight management was associated with better psychomotor speed whereas only combination of the DASH diet with weight management was associated with greater improvements in several tests of executive functions and memory. These findings suggest that the impact of healthy diets could be augmented by simultaneously taking into account other modifiable risk factors of cognitive decline such as overweight or hypertension. Accordingly, several
ongoing RCTs include dietary modifications along with other lifestyle recommendations (e.g., increase physical activity) and management of cardiovascular risk factors to prevent cognitive decline [49].

### 4.4 Definition of an Appropriate Control Arm

Defining an appropriate control arm is a general challenge of RCTs with nutritional interventions. Explanatory trials with nutritional supplements are usually placebo controlled, i.e., the control group receives a similar pill without the presumed efficient nutrient(s). This design allows double blinding of the investigator and the participant. However, the composition of the placebo is not trivial. Indeed, the placebo must not provide a nutrient that could have an opposite effect on cognition (e.g., trial of LC n-3 PUFA vs. placebo containing a high amount of n-6 PUFA such as sunflower oil) and artificially inflate the expected difference between intervention and control arms. Conversely, no beneficial impact on cognition should be expected from the components of the placebo (e.g., trial of LC n-3 PUFA vs. placebo containing olive oil, which provides polyphenols). The placebo must also be isocaloric, as for the Souvenir II trial [64] in order to neutralize the potential effects of higher or lower energy intake on cognition. Finally, the typical taste of fish oils providing EPA and DHA should be neutralized as much as possible or artificially introduced in the placebo so that participants cannot guess their group of randomization. In an RCT of EPA for the treatment of psychological distress and depressive symptoms, $50 \%$ of women in the EPA group stated that the capsules had a fishy taste compared with $6 \%$ in the placebo group [78]. However, there were no differences in the proportions of participants who guessed their allocation group.

RCTs of dietary interventions are complex interventions [79] faced with major challenges. Indeed, it is very difficult to conceive a placebo intervention that would have no impact on cognition without letting participants with the detrimental feeling that they have no benefit to expect from their involvement-a situation that may lead to massive dropout. General dietary recommendations may be used for the control arm. However, they may have a favorable impact on cognition and decrease the apparent efficacy of the specific intervention to evaluate. Blinding is not possible, thus the participants and often field investigators are aware of the group of randomization. Contamination between intervention and control groups is also a concern. Even if participants of the different arms of the study should not have the opportunity to meet at the investigation center, they may exchange about their experience in social occasions, shops, etc. Cluster randomization of districts or practices may limit this risk of contamination but it requires much larger sample sizes. General dietary recommendations, e.g., to increase fish, fruit and vegetables consumption provided by media campaigns may also interact with the intervention and decrease its apparent efficacy. Moreover, many kinds of nutritional supplements are widely available in stores or on the Web. Whatever the intervention, supplement users and those with strong nutritional beliefs should not be included.

Compliance to the intervention must be controlled with dietary surveys and biomarkers in both arms in a blinded fashion, e.g., levels of EPA and DHA in plasma or red blood cell membranes assessed at baseline and at the end of the RCT in order to appreciate their change over time.

## 5 Interactions Between Nutrients and Genetic Polymorphisms: Towards a Personalized Nutrition?

Most epidemiological studies have adjusted their statistical analyses for the $\varepsilon 4$ allele of the apolipoprotein E gene (ApoE4), the main genetic risk factor for AD. However, many studies did not mention whether there was an interaction between nutrients and ApoE4 on cognitive function. When a significant interaction is observed, stratified analyses should be run. The few studies that have presented separate analyses according to ApoE genotype have yielded inconsistent results.

Regarding antioxidants, a few studies have reported significant interactions between ApoE polymorphism and nutrients on cognitive functions. Higher betacarotene levels in serum were associated with a lower risk of cognitive decline over 7 years in ApoE4 carriers but not in ApoE4 negative healthy older adults [80]. Conversely, the protective association of dietary vitamin E against the risk of incident AD was observed only among older persons who were ApoE4 negative [81]. Another study did not find any significant interaction between ApoE4 genotype and dietary intake of several antioxidants (beta-carotene, flavonoids, vitamins C and E) on the risk of AD [82]. ApoE4 carriers had significantly lower selenium (Se) levels measured in nail samples than noncarriers after controlling for estimated dietary Se intake in an elderly Chinese cohort [83]. AD patients with at least one ApoE4 allele have lower serum level of total antioxidant status and lower activity of oxidative stress enzymes catalase and glutathione peroxidase compared to healthy individuals but also to AD patients without the ApoE4 [84]. Another study suggested a functional vitamin E deficiency, and hence increased oxidative stress, in AD patients with the ApoE4 genotype [85]. Taken altogether, these findings suggest that ApoE4 exacerbates the role of oxidative stress in the pathogenesis of AD [86].

Higher oxidative stress or impaired antioxidant defense mechanisms could also explain why ApoE4 carriers seem to be less sensitive to intake of LC n-3 PUFA, which are easily peroxidized. Indeed, several epidemiological studies but also two RCTs [39, 45] have found an interaction between EPA, DHA, or fish intake and ApoE4 in regard to cognitive function (for a review see [87]). In most studies, individuals who are not ApoE4 carriers seem to be more responsive to dietary fat and to its impact on cognitive functions [62, 87-91]. However, controversial findings exist as well, with two studies showing that ApoE4 carriers had a higher impact of increased LC n-3 PUFA on cognition [39, 92]. Underlying mechanisms are still poorly understood. In addition to exacerbated oxidative stress, ApoE4
carriers might have impaired cholesterol or fatty acid transport. Indeed, ApoE4 carriers have an exaggerated postprandial triglycerides elevation [93]. Other potential mechanisms involve impaired metabolism of n-3 PUFA, glucose or ketones, and exacerbated brain inflammation [87]. More generally, the ApoE4 carriers may be more vulnerable to deleterious environmental factors [94].

In addition, other recently discovered genetic polymorphisms associated with the risk of AD could also interact with nutrients. For example, CLU (apolipoprotein $\mathrm{J})$, whose marker rs11136000 is associated with a decreased risk of AD, is one of the most abundantly expressed apolipoproteins in the central nervous system, suggesting a potential role in lipid transport or functionality.

In RCTs with nutritional interventions, stratified analyses according to ApoE4 genotype should be planned in the protocol. Indeed, the impact of antioxidant nutrients might be more important in ApoE4 carriers who have an elevated level of oxidative stress [95] while that of LC n-3 PUFA might be higher in ApoE4 noncarriers. Thus, the resulting effects may be not significant if ApoE4 carriers and noncarriers are mixed. Eventually, when epidemiological studies strongly suggest a modifying effect of a given genotype such as ApoE4, the randomization could be stratified according to genetic polymorphisms to ensure that subgroups are perfectly comparable at baseline of the RCT. This may have important implications in terms of sample size calculation. Indeed, about $20 \%$ of the general population is ApoE4 carrier. Thus, this group should be overrepresented to ensure similar power as in the ApoE4 noncarriers. When antioxidant nutrients and LC n-3 PUFA are provided simultaneously, a $2 \times 2$ factorial design is needed to distinguish their respective or synergistic effects. However, such a design combined with stratification according to ApoE genotype would require extremely large sample sizes. As a result, many RCTs are underpowered. Moreover, participants in RCTs are highly selected individuals whose risk of cognitive decline or incidence of dementia is lower than in comparable participants in observational epidemiological studies, and hence an overestimation of the effect size and underestimation of the requested sample size.

## 6 Conclusion

Epidemiological observational studies and RCTs have yielded conflicting results regarding the impact of diet and nutrients on cognitive decline in older adults. More research is needed to better identify the potential beneficiaries of a nutritional intervention for the prevention of cognitive decline, function of their cognitive status, dietary habits and genetic characteristics. Estimation of optimal quantities and proportions of nutrients for the prevention of cognitive decline should be refined, according to actual needs of the target population. Interactions between genetic polymorphisms and nutrients must be identified in order to focus on the most susceptible individuals, but also to better understand the pathophysiological mechanisms linking nutrition and cognitive functions. Epidemiological studies are
still necessary to investigate these new areas in the link between nutrition and cognition and to provide relevant data that will be used to implement more convincing RCTs.

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# Chapter 21 <br> Nutraceutical Regulation of the Neuroimmunoendocrine Super-system 

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## 1 Introduction

There is a strong relationship between nutrition, metabolism and immunity. It is clear that nutrition affects a range of biological processes that are critical to the immune response including cell proliferation and survival, signal transduction and gene expression. On this basis there is an increased interest in the use of dietary strategies to control a range of inflammatory and immune disorders including inflammatory bowel disease, rheumatoid arthritis and allergy [13]. Immunonutrition has also been proposed in early life, to support the development of a healthy immune system [4] and in the elderly to protect against immunosenescence [5, 6]. Two particularly promising nutritional approaches to immunomodulation are the use of probiotics and n3 polyunsaturated fatty acid (n3 PUFAs), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Indeed, both n3 PUFAs and probiotic bacteria are already used to supplement infant formula [7, 8], and there is evidence that they may have synergistic benefits [9, 10].

In recent years a convergence of the fields of immunology, microbiology and nutrition has provided a "diet-microbiota" model that may underlie the increased incidence of a range of immune disorders. The "microbiota hypothesis" proposes that dietary changes and increased antibiotic use in "industrialised" countries lead to changes in the composition of commensal bacteria in the gut, disruption of the normal microbiota-mediated mechanisms of immunological tolerance in the mucosa and consequently to increased susceptibility to immunological disorders [11]. Attempts to exploit potential therapeutic benefits of modulating the gut microbiota in inflammatory and other immune disorders have lead to extensive research efforts in the field of probiotics. Defined as non-pathogenic bacteria that

[^21]promote beneficial health effects when ingested [12]; these "beneficial microbes" are most frequently Lactobacillus or Bifidobacterium species; however, a number of lactic acid bacteria and non-pathogenic E.coli have also been identified as probiotics [13]. There is now good evidence of that bacteria delivered orally can regulate immune responses in the GI tract and beyond. In particular, the ability of specific bacteria to protect against infection and attenuate allergic inflammation has been well documented in animal models and clinical studies (For review see [14, 15]). Based on experimental evidence, a number of mechanisms underlying the protective immune effects of bacteria have been proposed. These include enhanced NK cell activity, altered antigen presentation by dendritic cells and subsequent decrease in IgE responses [16], a skewing of T cell polarisation towards Th1 responses [17, 18], the induction of regulatory T cells [19-21] and inhibition of mast cell responses to antigen [22-25].

Dietary lipids have also been reported to have immunomodulatory effects. Polyunsaturated fatty acids (PUFAs) consist of two main groups of essential fatty acids: omega- 3 ( $n-3$ ) and omega- 6 ( $n-6$ ). The n-6 PUFAs are generally regarded as proinflammatory; diets rich in n-6 PUFAs result in predominance of arachidonic acid in tissues, which in turn gives rise to eicosanoids such as prostaglandin $\mathrm{E}_{2}$ [26]. These eicosanoids subsequently enhance the synthesis of proinflammatory Th2 cytokines and IgE antibodies. Conversely, n-3 PUFAs suppress immune responses. Dietary n-3 PUFAs can be incorporated into cell membranes, displacing arachidonic acid and modulating lipid-protein interactions [27, 28]. Such membrane incorporation can lead to changes in receptor expression, reduction of prostaglandin $\mathrm{E}_{2}$ synthesis and inhibition of the production of pro-inflammatory cytokines (TNF- $\alpha$, IL-1, IL-6) by several cell types [29-33]. n3 PUFAs can also downregulate MHC II expression and function of antigen-presenting cells [34]. Competitive inhibition of the cyclooxygenase (COX) inflammation pathway is another way through which EPA suppresses inflammation. The COX pathway converts the primary n-6 PUFA, arachidonic acid, to pro-inflammatory prostaglandins and prostacyclins. However, high levels of EPA blocks production of arachidonic acid derivatives by using the COX enzyme to form EPA derivatives instead [26].

Within the body, the maintenance of homeostasis and defence against external threats is not the sole preserve of the immune system but is undertaken through the co-ordinated action of the nervous, endocrine and immune systems. These major adaptive systems are in constant bidirectional communication forming effectively a regulatory super system. Crosstalk between systems is facilitated by the expression of common mediators and receptors. For example, many immune cells respond to neurotransmitters, through an array of receptors including adrenergic, cholinergic, neurokinin and NMDA receptors [35]. At the same time immune cells are capable of producing nerve growth factors and a range of neuroactives including catecholamines, histamine, acetylcholine and GABA, as well as endocrine factors such as corticotropin-releasing factor (CRF) [35]. Conversely, neurons can be activated by cytokines allowing them to respond to the immune environment [36, 37].

Disruption of any component of the regulatory super-system can result in loss of homeostatic control, diminished defences and, subsequently, disease. Thus, immune disorders may have origins in disrupted neuroendocrine control and neurological symptoms may result from dysregulated immunity. With this in mind, modulation of neural and endocrine responses may represent a potential therapeutic approach to immune disorders.

The complex relationship between the nervous, endocrine and immune systems is particularly apparent in the gut. The gastrointestinal (GI) tract sees a coming together of the greatest concentration of immune cells in the body with a network of 500 million neurons in what is the body's largest endocrine organ. In addition, the GI tract is the point of interface between the body and the approximately 100 trillion bacteria [38] that constitute the human gut microbiota. Indeed, it is the coordinated action of immune, nervous and endocrine systems that allows the gut to maintain the balance between supporting the advantageous relationship between host and commensal organisms while at the same time maintaining protection from potential pathogens. Also critical to maintaining homeostasis is the constant dialogue between the gut and the brain. This brain-gut axis consists of clear "hard-wired" anatomical connections, involving vagal and spinal nerves, together with humoral components provided by the endocrine and immune systems. It is suggested that defects in gut-brain axis communication are an underlying cause of functional bowel disorders including irritable bowel syndrome (IBS) [39] and potentially inflammatory bowel disease [40]. There is now also strong evidence that through the gut-brain axis changes in gut function and/or the gut microbiota can result in modulation of stress responses, central nervous system (CNS) function and consequently mood and/or behaviour.

The realisation that the gut and gut-microbiota are deeply integrated into many aspects of host physiology, influencing neural and endocrine responses that regulate immune homeostasis, opens the possibility that changes in neuroendocrine environment may have an important role in mediating effects of nutritional interventions on systemic immunity. The following review focuses on the actions of microorganisms and n3 PUFAs on neural and endocrine functions that may contribute to the therapeutic effects of nutritional interventions in immune disorders (Fig. 21.1).

## 2 The Enteric Nervous System

The enteric nervous system (ENS) consists of an estimated $10^{8}$ neurons forming ganglionated plexuses within the intestinal wall. Comprised of parasympathetic and sympathetic systems the ENS and can operate independently of the central nervous system and is essential for life.

The ENS innervates the intestinal mucosa, including the gut-associated lymphoid tissue, which instigates innate and acquired immune responses against


Fig. 21.1 Modulation of the immune system by n3 PUFAs and probiotics: In addition to acting directly on immune cells both n3 PUFAs and probiotic bacteria have effects that include altering activity of the enteric nervous system (ENS) activating the vagal anti-inflammatory pathway and modulating the HPA response, all of which are involved in maintaining immune homeostasis. Ach Acetylcholine, ACTH Adrenocorticotropic hormone, CCK Cholecystokinin, CCKl Cholecystokinin Receptor 1, CORT Corticosterone; CRF Corticotropin-releasing factor
luminal pathogens while at the same time maintaining tolerance to food antigens and commensal bacteria.

ENS neurons secrete acetylcholine (ACh) and large number of other neurotransmitters and neuropeptides including norepinephrine, nitric oxide, vasoactive intestinal peptide (VIP), Calcitonin gene related peptide, neuropeptide Y and Substance P (SP). The immune-modulatory activities of these neurotransmitters have been well described, and they can influence lymphocyte proliferation and cytokine production. For example VIP inhibits the migration of T lymphocytes into Peyer's patches [41, 42] and alters B cell-mediated immunoglobulin synthesis [43, 44]. On the other hand SP stimulates immunoglobulin synthesis in Peyer's patch B cells [43-45]. The ENS may also regulate antigen presentation, being well placed to interact with dendritic cells and macrophages, found throughout the intestine, including the lamina propria of the small and large intestine, the Peyer's patches, intestinal lymphoid follicles and mesenteric lymph nodes. Indeed, both VIP and SP have been demonstrated to confer tolerogenic or regulatory functions on dendritic cells [46, 47] Thus, modulation of the ENS function can influence, not only welldefined roles in regulating intestinal motility and transepithelial ion transport but also intestinal immune functions related to mucosal protection and defence against infection.

By far the richest innervation of intestinal mucosal epithelium derives from the myenteric plexus, which provides more than $90 \%$ of sensory neuropeptide containing fibres to the mucosal layer [48, 49]. Each of these enteric intrinsic primary afferent neurons (IPANs) innervates 80-120 villi [50]. Thus, IPANs are well placed to respond to luminal content and are plausible targets through which nutrients and microbes could influence gastrointestinal physiology and neuroendocrine responses.

Certainly, IPANs have been demonstrated to be cellular targets of putative probiotic bacteria. Using whole cell patch clamp recording, myenteric IPANs in rats fed a Lactobacillus rhamnosus strain were demonstrated to be more excitable than those from controls. This increase in excitability was accompanied by a reduction in the post-action potential slow after-hyperpolarization, which is partially responsible for the neuronal refractory period; the time period following an action potential during which a neuron cannot initiate a subsequent action potential. [51]. Further experiments identified that the molecular mechanism underlying increased IPAN excitability involved a reduction in current of an intermediate conductance calcium-dependent potassium channel $\left(\mathrm{IK}_{\mathrm{Ca}}\right)$ and application of the $\mathrm{IK}_{\mathrm{Ca}}$ channel blocker TRAM-34 mimicked the effects of the L. rhamnosus, [51, 52]. With regard to mechanism of action of $L$. rhamnosus on the IPANs, there are few chemical correlates of the functional effects probiotics have on enteric neurons. Ingestion of Saccharomyces boulardii has been shown to decrease the number of pig myenteric AH cells that express the vitamin D-dependent cytosolic calcium binding protein calbindin-D28k [53]. A change in calcium intracellular buffering, as is suggested by this result, might be expected to alter the opening probability of $\mathrm{IK}_{\mathrm{Ca}}$.

Recently, it was demonstrated that Bacteroides fragilis produces similar effects to L. rhamnosus on the ENS. Significantly, the capsular exopolysaccharide, polysaccharide A (PSA), isolated from B. fragilis completely mimics the neuronal effects of the parent organism. Furthermore, experiments with a mutant strain of B. fragilis lacking PSA showed that the mutant had lost the neuromodulatory activity of the parent bacteria. Overall these experiments identified that complex carbohydrates may play an important role in mediating signals between bacteria and the host nervous system.

It should be noted that modulation of the ENS in addition to regulating gut motility and intestinal inflammation, likely contribute to afferent signalling to the brain [51,52,54] and thus influence a range of physiological responses beyond the gut.

## 3 The Vagus Nerve

The vagus nerve projects from the medulla oblongata in the brain stem to the colon and consists largely of afferent nerve fibres. Innervating the pharynx, larynx and visceral organs, the vagus is the main afferent pathway from the abdominal cavity
to the brain. Sensory vagal inputs arrive in the nucleus of the solitary tract, and then transmitted to widespread areas of the CNS, many of which [55], are associated with stress-related behaviour and affective disorders.

Within the intestine there are $30,000-80,000$ vagal nerves, $90 \%$ of which are afferent [56,57]. Vagal afferents innervate the muscular and mucosal layers of the entire gut with the coeliac branch supplying the intestine from the proximal duodenum to the distal descending colon [58].

Intraganglionic laminar vagal afferent endings are located in the connective tissue capsule of myenteric plexus ganglia, between the longitudinal and circular muscle layers. These fibres respond to muscle tension generated by passive stretch and active contraction of the muscle layers [59]. However, of particular relevance to the sensing of luminal contents, there are also vagal afferent fibres with terminals lying in the mucosa [56]. These mucosal vagal fibres express a large variety of mechanosensitive and chemosensitive receptors [57] and represent a hard-wired component of the gut-brain axis that is critical to many physiologic processes, such as satiety and regulation of digestive activity. While mucosal vagal afferent fibres are not in a position to sense luminal nutrients directly, not crossing the basal membrane to innervate the epithelial layer of the gut [58], they are in close anatomical apposition to the basal membrane of enteroendocrine cells [60]. The chemosensitive receptors of mucosal vagal fibres are the targets of gut hormones and regulatory peptides such as ghrelin, cholecystokinin, glucagon-like peptide-1 and peptide YY, that influence the control of food intake and regulation of energy balance [57].

In addition to responding to gut hormones, the vagus nerve can also sense the immune environment of the intestine and can be activated by inflammatory cytokines. Perhaps the best-described consequence of vagal afferent signalling in response to cytokines is sickness behaviour; the drastic changes in behaviour responsible for reorganising perceptions and actions to enable ill individuals to cope better with infection. The characteristic sickness behaviours are mediated by proinflammatory cytokines particularly IL-1 $\beta$ and TNF [61] and include lethargy, depression, anxiety, loss of appetite, sleepiness and hyperalgesia.

More recently, evidence has emerged suggesting that activation of the vagus by electrical, immune, microbial or nutritional stimuli can have consequences beyond changes in behaviour and digestive activity. While the majority of vagal fibres are afferent, it has been identified that vagal efferents play a critical role in a neural circuit that controls inflammatory responses. In this anti-inflammatory reflex the vagus nerve senses inflammation sending afferent signals to the brain that then activates efferent responses, releasing mediators including acetylcholine that, through an interaction with immune cells, attenuates inflammation.

### 3.1 Vagal Anti-inflammatory Response

The vagus innervates tissues with important immune functions such as thymus, lung, liver, and the gastrointestinal tract. Furthermore, trunks or branches of the vagus are often associated with lymph nodes that drain regions in which immune activation occurs.

Tracey and colleagues were the first to highlight the anti-inflammatory role of the vagus, demonstrating that direct electrical stimulation of the peripheral vagus nerve prevented the development of shock in rats through the inhibition of TNF synthesis by macrophages [62]. This inhibition of macrophage function is mediated by Ach released by the vagus acting on nicotinic receptors expressed by the immune cell. Similarly, macrophages have been suggested to be the main target of the anti-inflammatory function of the vagus nerve in a murine model of inflammatory bowel disease (IBD) [63]. However, it is also clear that the vagus nerve acts to regulate T cell function Sub-diaphragmatic vagotomy leads to a dramatic increase in T cell proliferation and production of inflammatory cytokines when compared to cells from sham-operated animals [64]. The effect of vagotomy is not limited to the spleen as lymphocytes isolated from the mesenteric lymph nodes also demonstrated a significant increase in inflammatory cytokine production. It is possible that the actions of the vagus on T cells can also influence the development of IBD. O'Mahony et al. [65] demonstrated that transfer of CD4+ T cells from vagotomised donors into non-vagotomised with DSS induced colitis reduced the number of splenic Foxp3 ${ }^{+}$regulatory T cells in recipient animals, and was associated with aggravated disease symptoms mimicking the effects of vagotomy on colitis. Overall, data suggests that the vagus nerve is a tonic inhibitor of multiple components of the immune system.

## 3.2 n3 PUFAs and the Vagus

Grundy and colleagues [66] demonstrated that long- and short-chain fatty acids both activate rat jejunal vagal afferent nerve fibres but do so by distinct mechanisms. Butyric acid, a short-chain fatty acid, appears to have a direct effect on vagal afferent terminals while the long-chain fatty acids activate vagal afferents via a CCK-mediated mechanism. Subsequently, Luyer et al. [67] demonstrated that the interaction between long-chain fatty acids and the vagus results in activation of the cholinergic anti-inflammatory pathway. They found that administration of high fat nutrition reduced circulating levels of TNF and IL-6 in rats subjected to hemorrhagic shock. In keeping with this, it has also been demonstrated that continuous enteral lipid application in the form of olive oil during sepsis significantly reduces the inflammatory cytokine output of the gastrointestinal tract and associated septic pulmonary dysfunction [68]. When Luyer et al. repeated their experiments in vagotomized animals, the administration of the high-fat diet no longer prevented
the increase in TNF and IL-6 [67]. In addition, nicotine receptor antagonism blocked the ability of dietary fat to suppress the cytokine increase. Similarly, deafferentation abrogates the protective effects of lipid-rich nutrition on systemic inflammation and loss of intestinal integrity following shock [69]. Overall these experiments provide strong evidence of a nutritional anti-inflammatory pathway whereby the intake of dietary fat suppresses cytokine release through activation of peripheral afferent vagus nerves that in turn initiate the cholinergic antiinflammatory response. The mechanism underlying the protective effects of long chain fatty acids include a role for cholecystokinin (CCK), a neuropeptide that is released after consumption of dietary fat and activates the afferent vagus nerve signals that induce satiety. Administration of CCK receptor antagonists and specifically antagonists of the peripheral CCK-1 impaired the fat-induced suppression of the shock response [67].

Clinically, studies indicate that dietary n-3 PUFA levels and n-3 PUFA supplementation are related to improved heart rate variability suggesting increased vagal tone [70, 71]. The relationship between the immunomodulatory actions of n-3 PUFA and their effects vagal tone has yet to be established. However, a number of studies have associated control of inflammation with heart rate variability in humans [72-75], while animal studies indicate that the threshold of vagus nerve activity that initiates the cholinergic anti-inflammatory pathway is significantly lower than that required to activate a change in heart rate variability [62, 76, 77]. It is possible that diet-induced activation of the cholinergic anti-inflammatory pathway contributes to the reduced mortality from sepsis and organ damage following early enteral feeding in trauma and surgery patients, [78-80].

It has also been suggested that this nutrient activated anti-inflammatory vagovagal reflex may contribute to the highly selective intestinal immune response that is required to preserve homeostasis and intestinal barrier function [76]. This neural feedback loop could help maintain unresponsiveness of the GI tract to luminal antigens, allowing the intestine to perform the dual role of sensing and absorbing essential nutrients while protecting against invasion from potentially damaging agents.

### 3.3 Probiotics and the Vagus

Non-pathogenic bacteria also activate vagal signalling from gut to brain. Tanida et al. [81] demonstrated that intraduodenal injection of the bacterial strain Lactobacillus johnsonii La1 enhanced gastric vagal nerve activity that was associated with reduced renal sympathetic nerve activity and blood pressure. Denervation of vagal nerve fibres surrounding the oesophagus eliminated the ability of L. johnsonii Lal to reduce renal sympathetic nerve activity and blood pressure indicating that vagal signalling is required for at least some of the effects of this bacterium on autonomic nerve responses [81].

The anxiolytic and antidepressant effects of chronic L. rhamnosus ingestion in normal adult Balb/c mice were prevented by subdiaphragmatic vagotomy as was the associated alterations in GABAA $\alpha 2$ mRNA expression in the amygdala [82]. Similarly, the ability of B. longum to attenuate DSS colitis induced anxiety was abolished by vagotomy [83]. Thus, it is clear that certain bacteria can alter gutbrain axis communication through modulation of vagal signalling. Indeed, the anxiolytic and antidepressive effects mediated by gut microbe induced activation of the vagus nerve is in keeping with evidence suggesting direct electrical stimulation of the vagus can lead to a reduction in anxiety and depression associated behaviours [84, 85].

As yet, there is no evidence that the vagus nerve contributes to the immunomodulatory effects of gut bacteria and at least one study suggests that the local protective effect of lactobacillus and bifidobacteria strains in models of colitis does not depend on vagal nerves [86]. However, given what is known of the vagal antiinflammatory reflex it seems plausible that gut microbiota induced modulation of vagal mediated "periphery to brain" signalling could translate into changes in efferent neural pathways controlling immune responses.

## 4 The Hypothalamus-Pituitary-Adrenal Axis

The hypothalamus-pituitary-adrenal (HPA) axis is a major component of the neuroendocrine response to stress. The HPA axis is initiated when neurons in the paraventricular nucleus of the hypothalamus, secrete corticotropin-releasing hormone (CRH). CRH, in turn, stimulates the anterior pituitary to secrete adrenocorticotropin hormone (ACTH) into the peripheral circulation from where it acts on the adrenal glands causing synthesis and release of cortisol. It is binding of cortisol to the intracellular glucocorticoid receptor (GR) in a wide variety of tissues that instigates signalling pathways crucial to an adaptive stress response [87, 88].

One of the major physiological roles for the HPA axis is preventing excessive tissue damage due to inflammation and the immunomodulatory influences of the HPA response have been described extensively [87]. While the initial response to stress involves activation of the HPA axis, over time this activity diminishes and cortisol secretion stabilises below normal levels. Indeed, reduced input from this important negative regulator of inflammation may explain some of the detrimental effects of chronic stress on immune disorders [87]. The immunologic effects of the HPA axis are mediated largely through the action of adrenal corticosteroids on intracellular receptors. For example, on T-cells these receptors in regulate expression of IL-4, 5, and 13 following exposure to allergen [89] while mast cells constitutively express glucocorticoid receptors, where they function to inhibit release of histamine and other allergic mediators, as well as reducing the recruitment and activation of eosinophils [90].

Changes in HPA responsiveness has been shown in a variety of animal models as well as human inflammatory and autoimmune diseases such as rheumatoid arthritis
[91] inflammatory bowel disease [92] multiple sclerosis [93] and allergic disorders including asthma and dermatitis [94, 95]. The potentially protective effects of HPA activation have been well demonstrated in relation to allergic disease. CRH deficient mice develop increased airway inflammation following OVA sensitization and challenge compared to wild type mice [96]. In this case the detrimental effect of CRH deficiency is believed to be the result of severely reduced corticosteroid and catecholamine levels because of the absence of the stimulus for their release from the adrenal gland. Short-term restraint stress inhibits antigen mediated cell influx to the lungs of OVA sensitised mice, an effect that can be prevented by the glucocorticoid receptor antagonist RU486. Similarly, scratching behaviour induced by the mast cell degranulating agent, compound 48/80 increases following treatment with RU486 or surgical removal of the adrenal gland in mice [97, 98]. Furthermore, early-life stress in mice leads to a hypo-responsive HPA in the adult animals with an associated increase in allergic airway response [98]. Several clinical reports also suggest that normal function of the HPA axis might be critical for controlling inflammation with evidence of a hypo-responsive HPA axis in patients with chronic inflammatory disorders [99, 100]. It is clear that a normal HPA response is important in maintaining optimal immune function and correspondingly the ability to alter HPA activity has been identified as a potential therapeutic approach to immune disorders.

### 4.1 Probiotics and the HPA Axis

Sudo and colleagues provided some of the earliest evidence that changes in gut bacteria could alter central responses [101] and demonstrated that germ free animals had an enhanced HPA axis response. This hyper-responsiveness was reversed by reconstitution with faeces from animals kept in a pathogen free environment or with a single probiotic strain, Bifidobacterium infantis [101]. In contrast, mono-association with an enteropathogenic E. coli further exaggerated the response to stress. Alterations in the HPA response has since been demonstrated to be a common effect of gut bacteria in many model systems [82, 102, 103] Overall, studies suggest that development in the absence of gut microbiota leads to HPA hyperresponsiveness [101, 103] while certain commensal bacteria and potential probiotics can attenuate HPA responses to stress [82, 102]. There is also evidence that these observations made in animal models can be translated clinically. In a double-blind, randomised parallel group study, healthy volunteers consuming a mixed preparation of L. helveticus R0052 and B. longum R0175 or placebo for 30 days had lower urinary free cortisol levels indicative of a reduced HPA response to daily stressors [104].

The mechanisms underlying the ability of certain probiotic bacteria $t$ to modulate HPA responses is unclear but may be related to changes in gut permeability. Ait-Belgnaoui et al. [105] demonstrated that treatment with L. farciminis for 2-week attenuated the HPA axis response to acute restraint stress in rats and
prevented stress-induced colonic hyper-permeability and uptake of lipopolysaccharides (LPS) in the portal blood. Use of antibiotics to reduce luminal LPS available for uptake, also lead to attenuation of the neuroendocrine response to stress suggesting the effect of L. farciminis-induced on HPA activity may be related to the ability of the bacteria to enhance the intestinal epithelial barrier, thus, reducing circulating LPS.

Regardless of mechanism of action, the clear ability of certain bacteria to modulate HPA activity [82, 101-103] may contribute to improved homeostatic function and thus to some of the observed beneficial effects of probiotics in allergy and other immune disorders.

## 4.2 n3 PUFAs and the HPA Axis

n3 PUFAs specifically DHA and EPA, have been identified as having stressprotective roles that are mediated potentially through an ability to modulate the HPA response [106-108]. DHA deficiency has been associated with high CRF levels in cerebrospinal fluid, indicating a potentially exaggerated stress responses and hyperactive HPA [109]. Conversely, fish oil supplementation has been demonstrated to attenuate mental stress-induced adrenal activation with associated reduction in epinephrine and cortisol levels in healthy subjects [110] and reduced basal cortisol levels and stress perception in recovering alcoholics [111]. A number of studies have indicated that n3 PUFAs may have potential as therapy or adjunctive treatment in depression and that such effects could also be related to an ability to modulate HPA activity. In animal models, feeding DHA to rats significantly decreased immobility time in the forced swim test, a well-validated indication of antidepressant activity. The DHA induced behavioural change was associated with decreased CRF levels in the hypothalamus and pituitary tissues, an indication of changes in HPA activity [112, 113]. In human studies, Jazayeri et al. [114, 115] reported that fluoxetine and EPA were equally effective in controlling depressive symptoms and that a fluoxetine and EPA combination was superior to either treatment alone. The same authors went on to show that EPA and fluoxetine, alone or in combination, decreased serum cortisol after 8 weeks of treatment in depressed patients leading to the suggestion that EPA may exert its therapeutic effects through reduction of HPA hyperactivity [115]. There is also evidence that action of n-3 PUFAs on the HPA axis may influence immune responses to psychological stressors. For example, low serum n-3 or higher n-6:n-3 ratios in medical students prior to exams was associated with high LPS-stimulated TNF and IFN- $\gamma$ production by peripheral blood leukocytes obtained during exams [116].

While it is unclear how dietary DHA modulates the HPA response, there has been extensive research into the beneficial effects of unsaturated fatty acids or high linolenic acid diets on the CNS. DHA can act on NMDA receptors, increasing the probability of channel opening [117]. DHA also modulates GABA responses [118, 119]. Indeed, DHA mediated attenuation of HPA may be explained by the
demonstration that n-3 PUFAs, can act on GABA $_{A}$ receptors to potentiate GABAergic inhibitory drive on CRF-secreting hypothalamic neurons [120]. In this regard, it is interesting to note that the decreased anxiety and HPA response to stress of mice fed with L. rhamnosus is also associated with changes in the central GABAergic system [82]. In another similarity to the action of probiotics, it should also be noted that DHA and EPA have been demonstrated to support intestinal epithelial barrier integrity [121-123]. Given the evidence that changes in intestinal permeability may be critical to probiotic regulation of the HPA response how this relates to protective effect of n3 PUFAs on stress and HPA activity deserves investigation.

## 5 Mast Cells

Mast cells are best known for their contribution to the inflammatory process and particularly in IgE mediated allergic responses. However, mast cells also play an important role in communication between the nervous, endocrine and immune systems, acting as a "universal translator" between components of the adaptive super-system [124].

A range of neurotransmitters and hormones can activate mast cells while reciprocally, mast cell derived cytokines including TNF and growth factors, such as NGF, modulate the threshold for activation of local neurons and promote nerve fibre growth [125-128]. In addition, a variety of molecules, including histamine and serotonin, synthesised and released by mast cells can influence neuronal activity and endocrine function [129, 130].

Mast cells have been described as the immune-gate to the brain [131] and can influence behaviour, with mast cell deficient mice exhibiting a more anxious phenotype [132]. Mast cells can also regulate the HPA axis [133]. Degranulation of dog brain mast cells evokes HPA responses via histamine and CRH release [133]. Conversely action of CRH on masts cells is critical for stress-induced changes in intestinal and blood-brain barrier permeability [134, 135]. Mast cells are found in close apposition to the vagus nerve in the intestine [136, 137], and there is evidence for a bidirectional functional relationship between the two [136, 138]. There is also strong support for mast cells as important participants in visceral hypersensitivity and pain perception, particularly in IBS [139, 140].

Inhibition of mast cell responses appears to be a component of the immunomodulatory effects of certain bacteria and may be a contributing factor to the ability of candidate probiotic organisms to attenuate allergic inflammation [22-25].

Feeding of a $L$. rhamnosus strain (JB-1) to rats resulted in systemic attenuation of mast cell activity that was associated with inhibition of cell membrane $\mathrm{IK}_{\mathrm{Ca}}$ current [22], the same current inhibited by the bacterium in IPANs of the ENS. The $\mathrm{IK}_{\mathrm{Ca}}$ current has been identified as critical to the function of many immune cells [141-144] including [145, 146] having a key role in potentiating mast cell degranulation. Indeed, the degree of attenuation in response to IgE mediated activation of
mast cells from $L$. rhamnosus-fed animals was similar to that observed in $\mathrm{KCa3}$.1deficient mice [146]. The mechanism through which feeding L. rhamnosus leads to systemic mast cell inhibition is currently unknown. However, the activation of a range of $\mathrm{G}_{\mathrm{s}}$-coupled receptors including $\beta_{2}$-adrenoceptors, $\mathrm{A}_{2 \mathrm{~A}}$ adenosine receptors and EP2 prostaglandin receptors can lead to inhibition of the $\mathrm{IK}_{\mathrm{Ca}}$ current [147149] in the mast cell, and thus, there are many possible mediators of the stabilising effect of $L$. rhamnosus on the cell.

The work of de Kivit et al. [150] also provides a mechanism thorough which modulation of gut bacteria may result in a systemic alteration in mast cell function. In this study a diet containing prebiotic galacto- and fructo-oligosaccharides and a strain of Bifidobacterium breve protected against acute allergic symptoms and suppressed mast cell degranulation in whey-sensitised mice. The anti-allergic effects of the synbiotic treatment were correlated with increased galectin- 9 expression by intestinal epithelial cells and increased levels of galectin-9 in serum [150]. Galectin- 9 is soluble-type lectin that recognises $\beta$-galactoside-containing glycans. Crucially, serum derived from whey sensitised synbiotic treated mice was able to suppress IgE mediated mast cell degranulation and the extent of this suppression was correlated with serum galectin-9 [150]. In vitro, galectin-9 has been demonstrated to suppress antigen mediated mast cell degranulation by binding strongly and specifically to IgE and preventing IgE-antigen complex formation [151]. These findings strongly suggest that galectin-9 produced in response to synbiotic treatment is responsible for the systemic suppression of $\operatorname{IgE}$ mediated mast cell activation. Whether the increase in galectin-9 production applies universally to probiotic and prebiotic treatments that stabilise mast cells remains to be determined.

Long chain fatty acids can act directly on mast cells and have differential effects on the cell function. Generally, results suggest that arachidonic acid and other n-6 PUFA increase degranulation and mediator release from stimulated mast cells, while n-3 PUFA appear to suppress cell activation [152, 153]. Gamma-linolenic acid was observed to increase tryptase activity but decrease histamine release following mastoparan stimulation of a canine mastocytoma cell line [152]. In the same study, DHA attenuated $\mathrm{PGE}_{2}$ production in stimulated cells [152]. More recently van den Elsen et al. [153] have demonstrated that EPA and DHA dosedependently reduced $\mathrm{PGD}_{2}$ release and significantly suppressed IL-4 and IL-13 secretion from human mast cell lines. Overall these studies suggest that changes in mast cell phenotype following dietary supplementation with EPA and/or DHA may contribute to the susceptibility to develop and sustain allergic disease.

Overall, there are marked parallels between the neuroendocrine effects of mast cell and those described for probiotic bacteria and n-3 PUFA, (altered intestinal permeability and nociception, HPA regulation and changes in anxiety-like behaviour) and future studies designed to test potential causal relationships between peripheral and central neural regulation and the ability of certain nutritional interventions to modulate mast cell function will be of great interest.

## 6 Concluding Comments

The multiple physiological implications arising from modulating mast cells serves as a good example of an integrated neuroimmunoendocrine system. Alteration in the activity of what is ostensibly an immune cell can impact neural and endocrine responses that in turn influence immunity. Indeed, when considering the physiological processes of host defence and maintenance of homeostasis, distinctions between nervous, endocrine or immune responses are largely artificial as in reality none occur in isolation. This review has focused on potential mechanism through which nutritional modulation of neural and endocrine responses may alter immune cell activity. However, attention should be drawn to fact that due to the complexity of the interactions simple cause and effects relationships between adaptive systems are difficult to discern. For example, immune system dysfunction has been associated with mood disorders [154-156] and approximately one-third of people with depression, without co-morbid disease, have higher levels of inflammatory markers compared to the non-depressed population. Furthermore, inflammatory disorders are associated with greater rates of major depression, while patients treated with cytokines for various illnesses are at increased risk of developing major depression. Conversely, successful treatment with an antidepressant decreases levels of pro-inflammatory cytokines such as IL-6 and TNF [157, 158]. In this context, while dietary/bacterial modulation of vagal afferent vagal signalling to the CNS may alter brain function and behaviour directly, the bidirectional nature of communication between nervous, immune and endocrine systems opens the possibility that, under certain circumstances, the concomitant activation of the vagal antiinflammatory efferent responses may contribute to the antidepressive effects of nerve stimulation. Indeed, there is evidence that pro-inflammatory cytokine levels are reduced in epilepsy patients successfully treated with vagal nerve stimulation [159, 160].

Similarly, inflammation is a stressor, and inflammatory cytokines can act at all levels of the HPA axis to activate stress hormone release. This has lead to the proposal that, in addition to direct effects on the CNS, the ability of n3 PUFAs to reduce the HPA response and improve adaption to stress is, in part, mediated by fatty acid induced suppression of cytokine production from immune cells.

We are still in the early stages of understanding how the gut microbiota and nutritional factors integrate with adaptive systems and how this influences health and disease. In addition to probiotics and n3 PUFAs, other nutritional elements including vitamins, such as vitamin D [161-163], and trace elements, such as zinc $[164,165]$ have been shown to have both immunomodulatory and neuroendocrine effects. Moving forward, it is clear that we need further understanding of the extent to which the putative pathways of neuro-endocrine-immune interaction outlined in this review are actually involved in mediating the beneficial effects of specific functional foods. In the body, nutrition, immunology, neurology and endocrinology converge; it follows that research disciplines should do likewise if we are to understand the common mechanisms underlying many immune disorders as well
as develop probiotic and/or dietary strategies for the treatment and prevention of disease.

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# Chapter 22 <br> Targeting (Gut)-Immune-Brain Axis with Pharmaceutical and Nutritional Concepts: Relevance for Mental and Neurological Disorders 

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## 1 Introduction

The immune system also serves a sensory role, a "sixth sense," to detect factors the body cannot otherwise hear, see, smell, taste, or touch [1]. The immune system has evolved to detect foreign entities such as pathogens, tumors, and allergens with great sensitivity and specificity. Consequently, as a sensory organ, it would be a means to signal and mobilize the body to respond to these challenges including the (central) nervous system. Since individual leukocytes are not physically connected to the nervous system, the question arises how such signaling works. Nowadays a lot of scientific evidence exists demonstrating bidirectional pathways between the (central) nervous system and immune system.

Already in 400 B.C., Hippocrates described the importance of the gastrointestinal tract in health and disease, by stating "bad digestion is the root of all evil." Although written in a period long before the major developments of modern medicine, current understanding of the physiology of the GI tract proves him right in many ways. The intestines have a profound effect on the entire body, including the brain [2]. Discovery of the enteric nervous system (ENS) around 1,900 was pivotal in the field of gut-brain interactions. Consisting of a complexity comparable to the central nervous system (CNS), the ENS is often described as the "second brain" [3]. Bidirectional communications between the brain and the gut occur via various pathways, involving the vagus nerve, autonomic nervous system

[^22]and neuro-immune interactions both in the GI tract and in the brain [4]. Over the past few decades, strong correlations have been observed between the occurrence of gastrointestinal problems and psychiatric disorders, which have augmented the interest in the gut-brain connection [5].

In this review the role of the neuro-immune axis and its targetability in relation to neurological disorders, such as depression, neurodegenerative diseases and autism is discussed.

## 2 Neuro-Immune Axis

First, nerves and immune cell are found in close proximity in the periphery as well as in the CNS. Outside the CNS, this close contact between nerve endings and immune cells is even enhanced during inflammatory responses at interfaces with the external environment for example at mucosal sites of the intestinal tract. Association between nerve fibers and immune cells helps to determine whether there is a local threat that requires an (immune) response: the so-called neurogenic inflammatory response [6, 7]. The nerve endings originating from peripheral sensory neurons have two functions: first to conduct (electrical) signals from the periphery to the CNS and secondly the release of neuropeptides and neurotransmitters that can participate in the immune/inflammatory response close by. Though the CNS has long been regarded as an immune-privileged organ, recent research has shown that the CNS is a highly immunological active organ with complex innate immune responses [8]. Microglial cells are the resident macrophages of the CNS, align neuronal synapses, and are important in controlling neuronal proliferation and differentiation [9]. In addition to microglial cells, in the CNS astrocytes are the most abundant cell type. Astrocytes contribute to the mechanical construction of nervous tissue in the brain and are important for the generation and maintenance of the blood-brain barrier (BBB). There is a lot of evidence that astrocytes can sense inflammatory an environment and consequently can respond by changing their cell phenotype to react in an immunological way. In addition, astrocytes can regulate the lymphocyte immune response in the brain via the release of chemokines and cytokines [10]. Under pathological conditions in the brain, lymphocytes are found crossing the BBB resulting in additive immune responses in the neuronal network of the brain.

Secondly, the expression of cytokines, chemokines, and their receptors has been demonstrated on peripheral as well as central nerves. For examples, enhanced neuronal TNF $\alpha$ and its receptors TNF $\alpha$ R1 and R2 have been demonstrated in dorsal root ganglia (DRG) neurons as well as in neurons of the brain strongly associated with inflammation [11, 12]. Research has also demonstrated that neurons express interleukin and chemokine receptors that could play a role in neuronal inflammation, dysfunction via (de)sensitization of nociceptive receptor, pain and CNS-mediated disease symptoms [1, 13]. Besides cytokines and chemokines, also immunoglobulins and their Fc receptors have been detected in neuronal sources.

Ig-free light chains and IgE are able to mediate antigen-specific responses (sensitization and activation) in cultured murine DRG [14, 15]. More recently, in murine as well as human PNS and CNS neurons, IgG protein has been detected but further research is necessary to elucidate the biological function of this neuronal $\operatorname{IgG}$ in the neuro-immune crosstalk [16]. The direct immunoglobulin-neuron link may reveal a novel potential pathway of antigen-specific neuronal activation in sensations such as pain and itch, but also in local inflammation in chronic inflammatory diseases.

Thirdly, nonspecific leukocytes and lymphocytes produce neurotransmitters and neuropeptides. The neurotransmitter serotonin is long known to have nonneuronal cellular sources such as enterochromaffin cells in the gut and mast cells [17]. Acetylcholine and other ligands for nicotinic acetylcholine receptors are synthesized by activated B and T lymphocytes and are thought to regulate local innate immunity [18] or inhibit vagus-induced cytokine production in an autocrine way [19]. In addition, several neuropeptides are released by lymphocytes, macrophages, dendritic cells, eosinophils, and mast cells upon innate activation [20, 21]. Cytokineprimed lymphocytes can locally secrete opoid peptides to induce local analgesic effects via JAK/STA1/3 activation in the cell [1, 22]. Opioid peptides are found in mast cells, granulocytes, lymphocytes, and macrophages. The prevailing peptides are b-endorphin and Met-enkephalin, but dynorphin and endomorphins were also detected. It is suggested that in a stressful (e.g., inflammation) situation, opioids are tonically released in inflamed tissue and activate peripheral opioid receptors to attenuate clinical pain [23]. Another example is the production of neurotrophins such as brain-derived neurotrophic factor (BNDF) and nerve growth factor (NGF) by activated lymphocytes that are suggested to be involved in a neuroprotective effect during autoimmune reactions in the brain [24]. Vice versa, nonspecific leukocytes and lymphocytes were reported to express classical neuronal receptors. Besides opoid receptors, a prominent example is nicotinic cholinergic receptors. Nonneuronal $\alpha 7$-nicotinic cholinergic receptors upon activation exerts antiinflammatory and immunomodulating activities on multiple cell types, including as T cells, B cells, dendritic cells, mononuclear phagocytes, and polymorphonuclear leukocytes [25, 26]. Dendritic cells express various receptors for neurotransmitters and neuropeptides like acetylcholine, norepinephrine, and vasoactive intestinal peptide that alter dendritic cell co-stimulatory molecule expression, cytokine release, and subsequent T -cell activation in an anti-inflammatory fashion [26].

Lastly, cytokines like interleukin $1 \beta$ (IL1 $\beta$ ), IL6, and tumor necrosis factor- $\alpha$ (TNF $\alpha$ ) can directly act on the nervous system to affect behavior. Cytokines are important for development and normal brain function, and have the ability to affect neural activity and neurotransmitter systems that results in behavioral changes. Inflammation (e.g., activation of the innate and/or adaptive immune system) or inflammatory cytokine administration produces adaptive behavioral responses that serve to safeguard energy use to fight infection or recovery from injury (so-called sickness behavior) [27-29]. However, chronic exposure to elevated inflammatory cytokines and long-lasting alterations in CNS neurotransmitter levels may contribute to the development of mental disorders such as autism, schizophrenia, and
depression [28, 30-32]. Mechanisms of cytokine-induced behavioral effects involve activation of inflammatory signaling pathways in the brain that results in changes in monoaminergic, glutamatenergic, and neuropeptidonergic systems, and decreases in growth factors, including BNDF [33, 34].

The hypothalamic-pituitary-adrenal (HPA) axis deserves special attention. Glucocorticosteroids play an important role in regulating homeostasis under basal and (immune) challenged conditions. Glucocorticosteroids protect the host from the consequences of an overactive inflammatory immune response and have been shown to be one of the most potent anti-inflammatory compounds ever. A disturbed HPA axis response has been associated with allergic and autoimmune diseases as well as with psychiatric and neurodevelopmental disorders. The latter disorders are in turn associated with an enhanced inflammatory status. Pro-inflammatory cytokines such as TNF $\alpha$, IL1, and IL6 act at all three levels of the HPA axis: (1) paraventricular nucleus of the hypothalamus resulting in the release of corticotrophin releasing hormone (CRH), (2) the pituitary that secretes adrenocorticotropic hormone (ACTH), and (3) the adrenal cortex. The overall chronic inflammation or stress-induced glucocorticosteroid response will eventually lead to glucocorticosteroid resistance at the level of the glucocorticoid receptor [35].

## 3 Afferent Pathways of the Neuro-Immune Axis

The afferent nerve pathways can be regarded as an immune-sensing pathway. Either innate or adaptive activation of the immune system regulates CNS activity through the release of inflammatory mediators such as cytokines, chemokines, and even immunoglobulins that bind to receptors located peripherally on the vagal or sympathetic nerve endings or centrally within the CNS or at the BBB. Cytokines and chemokines act on afferent parasympathetic, sympathetic, and sensory nerve endings to cause sickness behavior and, in relation to chronic inflammation, will eventually leading to behavioral and cognitive changes that are associated with mental disorders. Lymphocyte-derived neuropeptides and neurotransmitters modulate pain sensation by acting on peripheral sensory nerves and under chronic conditions may lead to hyperalgesia. Inflammation-induced cytokine release can also act on the HPA axis to produce CRH and ACTH, respectively, resulting in a glucocorticosteroid response. Finally, white blood cell-derived neurotransmitters, neuropeptides, and hormones cross the BBB and affect signaling within the CNS [1, 36].

## 4 Efferent Pathways of Neuro-Immune Axis

The psychological or inflammatory stress-triggered CNS communicates to the immune system by activating the sympathetic and parasympathetic neurons or the HPA axis to release the neurotransmitter norepinephrine, acetylcholine, or corticosteroid hormones, respectively. Lymphocytes and nonspecific leukocytes express receptors that bind norepinephrine, epinephrine, acetylcholine, and corticosteroids, providing a mechanism for these ligands to activate intracellular signaling pathways, which regulate the level of immune cell activity. Vagal acetylcholine acts on macrophages or dendritic cells to blunt proinflammatory cytokine synthesis and consequently downregulate the adaptive immune system. Sympathetic outflow also can regulate the function of immune tissues and their cells. Neuroendocrine hormones from the HPA axis modulate lymphocyte function [1, 36].

## 5 The Neuro-Immune Axis in Major Depressive Disorder

Major depressive disorder (MDD) is characterized by persistent depressed mood, loss of interest, and the inability to experience pleasure (anhedonia) that affects day to day life. As described above cytokines have been demonstrated to influence neurocircuitry and neurotransmitter systems in the CNS resulting in behavioral and cognitive changes [1, 33, 37]. Chronic exposure to pro-inflammatory cytokines results in persistent alterations in neurotransmitter function and behavior that in turn may contribute to the development of mental disorders such as MDD [33]. A growing body of evidence shows increases of pro-inflammatory cytokines, such as TNF $\alpha$, IL1 $\beta$, and IL6 in blood and cerebrospinal fluid of patients suffering from MDD [38, 39]. It has been shown that cytokines (for example, IL2 or interferon- $\alpha$ used for anti-tumor therapy) induce depression in humans and laboratory animals [40-42]. In addition, patients suffering from MDD show increased inflammatory responses to stress [43]. Polymorphisms of genes encoding for immune and inflammatory molecules have been identified in association with MDD, further strengthen the role of the neuro-immune axis in depression [44-46]. Nevertheless, the etiology of cytokine-induced MDD is largely unknown.

## 6 Future Directions for Treatment of Major Depressive Disorder Targeting the Neuro-Immune Axis

### 6.1 The Link Between Immune Factors and Monoamine Transporters

The role of serotonin and the serotonin transporter (SERT) have been studied intensively in MDD and an important role for altered serotonergic neurotransmission in depression has been proposed [47]. In addition, selective serotonin reuptake inhibitors (SSRIs), the first-line treatment for MDD, have been demonstrated to decrease proinflammatory cytokines, such as IL1 $\beta$, IL6, IL12, TNF- $\alpha$, and transforming growth factor- $\beta$ as well [48-50]. Furthermore, the pro-inflammatory cytokines TNF- $\alpha$, interferon- $\alpha$, and IL1 $\beta$ increase SERT function [51-54].

Lipopolysaccharide (LPS), a component of the outer membrane of gramnegative bacteria that binds to toll-like receptor 4 (TRL4) leading to the rapid systemic release of pro-inflammatory cytokines, induces anhedonia in rats and mice as shown by increased thresholds in an intracranial self-stimulation (ICSS) paradigm [55-57]. LPS-induced anhedonia was associated with increased extracellular levels of monoamine metabolites of serotonin and dopamine in the nucleus accumbens and prefrontal cortex, suggesting increased SERT and dopamine transporter (DAT) function [58]. Similar results, though less profound, were found after peripheral administration of TNF $\alpha$. Anhedonia induced by LPS was totally abolished in SERT( $-/-$ ) rats and as expected was still present in SERT(+/+) and to a lesser extent in SERT(+/-) rats [57]. Moreover, simultaneous inhibition of the reuptake of dopamine, serotonin, and norepinephrine by a triple reuptake inhibitor (partly) attenuated the LPS-induced increase in monoamine metabolite formation in the brain. This triple reuptake inhibitor induced a long-lasting hedonic effect assessed by the ICSS paradigm in rats [59]. In conclusion, intact SERT function is needed for pro-inflammatory cytokine-induced anhedonia and therefore these cytokines can be regarded as novel targets in MDD.

### 6.2 Targeting the Cytokines TNF , IL1ß, and IL6 in Major Depressive Disorder

Nowadays, monoclonal antibodies as well as small molecules targeting cytokines are commonly used for the treatment of chronic inflammatory diseases, such as rheumatoid arthritis or inflammatory bowel diseases. Only limited data are available on the effects of these cytokine blocking approaches in MDD. Anti-depressant and anxiolytic effects of the TNF- $\alpha$ receptor antagonist, etanercept, were demonstrated in rats [60]. Recently in humans, the anti-TNF- $\alpha$-antibody, infliximab, has been shown to improve depressive symptoms in MDD patients that were resistant to
anti-depressive treatments [61]. The potential beneficial antidepressant effect of infliximab depended on the baseline levels of inflammatory biomarkers. Furthermore, IL1 receptor $-/-$ mice and mice that have brain restricted overexpression of IL1R antagonist are resistant to develop chronic mild stress-induced depression [62]. In addition, in elderly people with high plasma levels of IL1R antagonist, which was associated with a low grade of inflammation, have a higher risk of developing depressive symptoms over time [63]. These results suggest that lowering IL1 $\beta$ brain levels might be beneficial for patients suffering from MDD [64].

In addition to IL1 $\beta$, IL6 has been identified as a potential biological target for the treatment of MDD. In a meta-analysis, an association between MDD and IL6 has been demonstrated [38]. In addition, in women suffering from MDD high IL6 levels were associated with low performance in verbal memory [65]. No reports have been published on the effects of blocking IL6 in MDD patients, but in a clinical trial the anti-IL6 receptor antibody, tocilizumab, improved significantly rheumatoid arthritis-associated fatigue in $62 \%$ of the patients [66, 67].

### 6.3 Immunomodulating Drugs

In animal models for depression induced by LPS and chronic stress, cyclooxygenase (COX) inhibitors have been demonstrated effective [68-70]. In patients suffering from MDD, celecoxib, a selective COX2 inhibitor, as well as acetylsalicylic acid, a nonselective COX blocker, improved the antidepressant effect of reboxetine, a norepinephrin reuptake inhibitor [71, 72]. Similar effects were demonstrated for omega- 3 fatty acids that have shown to have potent antiinflammatory effects [73]. In addition, a meta-analysis reported direct effects of omega-3 fatty acids in MDD [74]. In addition, p38 mitogen-activated protein kinase (p38 MAPK) inhibitors may have potential antidepressant effects [33, 54]. P38 MAPK is an inflammatory intracellular signaling molecule that currently is in clinical investigation as target in chronic inflammatory diseases. Nuclear factor (NF)- $\kappa \mathrm{B}$ and nitric oxide, other inflammatory signal transducers, might also be a novel target of interest in treatment of MDD. Inhibition of both signal transduction molecules have been shown to have anti-depressant effects in animal models [33].

### 6.4 Nutritional Concepts

Intestinal problems have been reported neurological and mental diseases, such as in Parkinson's disease (PD) [75], Alzheimer disease [76, 77], depression [78, 79], and anxiety disorders [79, 80]. A causal link between CNS and intestinal symptoms in PD has been demonstrated [75]. However, the relationship between intestinal distress with other neurodegenerative disorders, such as Alzheimer's disease and depression remains unknown. Nevertheless, the involved of the intestinal tract
creates a potential target for treatment using nutritional concepts. Furthermore, the lack of effective pharmaceutical treatments has created an urgent need for novel and more integrative approaches.

Neuronal death occurring in neurodegenerative disorders is multifactorial in origin with a complex set of pathological pathways [81-83]. Therefore, simultaneous manipulation of the various pathways involved may exert higher therapeutic efficacy. Dietary components have emerged as potential prevention and/or treatment for neurodegenerative disorders without the adverse side effects of the current pharmo-therapies [84-86]. Recently, it was demonstrated that a combination diet of food supplements impeded cognitive decline and neurodegeneration in a rodent model for neurodegenerative diseases. The multifunctional experimental diet composed of zinc, melatonin, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), uridine, choline, curcumin, and piperine [87]. These ingredients have been shown to attenuate glutamate excitoxicity, exert potent anti-oxidant/antiinflammatory properties, and improve synaptogenesis; processes that all have been implicated in neurodegenerative diseases. These new results as well as human studies [86] support the hypothesis that simultaneously targeting multiple disease etiologies might be more beneficial than the currently accepted "magic bullet, single target" approach in the treatment of neurodegenerative diseases. The diverse properties of our dietary intervention suggests that this multi-targeted treatment might be a stepping stone into a new direction for the development of therapeutic strategies in delaying/preventing neurodegeneration-related pathologies.

## 7 The Gut-Immune-Brain Axis in Autism Spectrum Disorders

Autism spectrum disorder (ASD) is a heterogeneous cluster of severe neurodevelopmental disorders. It is characterized by impairments in social interaction and communication and the presence of restricted, repetitive, and stereotyped interests and behaviors [88]. Increased immune activation is repeatedly reported in ASD patients. In postmortem brains of ASD patients as well as in various animal models, marked activation of astroglia and microglia is observed, indicative of neuroinflammation [89-93]. In addition, enhanced levels of a wide range of cytokines and chemokines were found in the brain and in the cerebrospinal fluid of autistic children [94, 95]. Peripheral immune abnormalities in autistic individuals have also been reported, including differential monocyte responses to in vitro stimulation, dysfunctional natural killer (NK) cells and altered serum immunoglobulins, cytokine and chemokine levels [24, 96-106].

The intestinal tract has a very important immune function, but also exerts an important neurological function and is called "the second brain," because of its abundant amount of enteric nerves and networks. Via these nerves, but also through
other pathways, the intestinal tract is able to affect the brain and vice versa [2, 107]. Evidence is emerging that intestinal immune disturbances can influence the brain and consequently behavior and cognition. A higher prevalence of ASD was found in pediatric patients with chronic gastrointestinal diseases [108]. In addition, gastrointestinal discomfort, changes in gut microflora, food aversion, and increased intestinal permeability, has been shown to correlate with the severity of disturbed behavior in ASD patients [109-115]. Worthwhile mentioning is the fact that, besides the immunomodulatory role of the microbiome, recent accumulating data now exist showing that intestinal bacteria can communicate with the CNS through neuronal, immune, and endocrine pathways and consequently influence brain function, behavior, and cognition [116]. Although still under debate, (non-)IgEmediated food allergy has been suggested to be involved in ASD [117-120]. In ASD children allergic reactions against milk protein have been suggested to trigger behavioral abnormalities and milk intake was reported to be a predictor of constipation in this population [101, 121]. A gluten and milk protein-free diet improved autistic behaviors and reduced the enhanced intestinal permeability [113, 122, 123].

## 8 Future Directions Targeting the Gut-Immune-Brain Axis by Nutrition in ASD

Since existing evidence indicates involvement of the gut-immune-brain axis in ASD, targeting the intestinal tract using immunomodulating medical food concepts could be of potential therapeutic value [124, 125]. In murine models for food allergy disturbed social interaction, repetitive behavior, anxiety, food aversion, and cognitive deficits, all characteristics of ASD, have been demonstrated to be associated with neuroinflammation and changed neuronal activation and different monoamine levels in brain areas that are related to social, emotional, and cognitive behavior [115, 126-130]. Moreover, recent reports exist that ASD and accompanying gastrointestinal symptoms are characterized by distinct and a less diverse gut microbiome [117, 130-132]. Modulation of gut bacteria with short-term antibiotic treatment has been shown to lead to improvement in behavioral deficits in ASD [131]. Specific beneficial bacteria (so-called probiotics, lactic acid producing bacteria, and bifidobacteria) influence the microbiome composition, intestinal barrier and alter the mucosal immune response and possibly influence the brain [133]. In addition, the underlaying mechanism of non-digestible oligosaccharides (so-called prebiotic fibers) includes improved microbiome via the induction of growth of beneficial bacteria and via direct action on epithelial cells restoration of intestinal immune homeostasis [134]. Thus treatment with specific beneficial bacteria in combination with non-digestible oligosaccharides to induce alterations in the microbiome, restoration of intestinal epithelial barrier, and mucosal immune homeostasis could be a novel approach to ameliorate gastrointestinal problems and even behavioral symptoms in ASD. Several studies have reported that dietary
intervention with specific beneficial bacteria in combination with non-digestible oligosaccharides prevented food allergy in mice and man [134-137]. A recent study of food allergic reaction in mice towards hen's eggs protein demonstrated that besides increased levels of antigen-specific IgE levels, diarrhea and disturbed antigen-specific Th2/regulatory T cell balance in the ileum, impaired behavior, and memory deficits were evident [129]. These aberrations ran in parallel with decreased expression of mRNA of BDNF and a disturbed BBB in the hippocampus. In addition, hippocampal neuroinflammation was found characterized by increased numbers of activated macrophages and Th cells. Dietary intervention with specific beneficial bacteria in combination with non-digestible oligosaccharides (Bifido Breve with short chain galacto-oligosaccharides and long-chain fructo-oligosaccharides, $B b / \mathrm{GF}$ ) normalized OVA-induced aberrant behavior and cognition and cellular and molecular changes in the brain. These data demonstrate that food allergic peripheral inflammation modifies the brain inflammatory status and dampens the behavioral and cognitive abilities, suggesting that food allergy may play a role in the development and/or progression of neurodevelopmental disorders. In addition, targeting the gut-immune-brain axis with dietary intervention may have implications for treatment of patients suffering from ASD. The molecular mechanism by which specific beneficial bacteria in combination with non-digestible oligosaccharides is protective in food allergy involves galectins. Galectins are soluble type lectins that bind galactose/b-galactoside containing glycans [134, 138]. Intestinal epithelium-derived galectin 9 is responsible for the immunomodulatory anti-allergic effects of $B b / \mathrm{GF}[137,142]$. Not much is known about the role of galectins in neuroinflammation and brain development and function. Microglial galectin 3 is involved in brain injury and neuroinflammation [139-141]. Neuronal galectin 4 is required for neuronal differentiation in CNS [143]. Astrocyte-derived galectins-1 plays a protective role in inflammation-induced neurodegeneration and is involved in neurogenesis [144, 145]. As for galectins-9, increased expression is found in IL1 $\beta$-stimulated human astrocytes and in spinal fluid of ALS patients [141, 146]. Dietary and/or pharmacological modulation with small molecules targeting the galectin response in neurodevelopment disorders such as ASD could be a future therapeutic approach.

## 9 Conclusions

In this review the role of disturbed bidirectional pathways between the (central) nervous system and immune system, regarded as the (gut)-immune-brain axis, in neurological and mental disorders has been described.

The management of these multifactorial mental disorders needs a new and integrated therapeutic approach and prospects for novel treatment are as follows:

1. Targeting the neuroinflammatory response in the CNS that disturbs neurotransmitter levels and connectivity, with existing immunomodulatory and antiinflammatory drugs or/and medical food concepts such as omega-3 fatty acids.
2. Targeting the HPA axis and resolve glucocorticosteroid resistance.
3. Targeting the disturbed (intestinal) immune system with existing immunomodulatory drugs such as cytokine-specific therapeutic antibodies.
4. Targeting peripheral enteric, parasympathetic, or sympathetic nerves with antiinflammatory neurotransmitters and neuropeptides.
5. Targeting the disturbed intestinal barrier with immunomodulatory drugs and/or medical food concepts, such as nondigestible oligosaccharides and specific beneficial bacteria.
6. Targeting the disturbed intestinal microbiome with antibiotics, specific beneficial bacteria, and/or nondigestible oligosaccharides.

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# Chapter 23 <br> Nutritional Approaches for Healthy Aging of the Brain and the Prevention of Neurodegenerative Diseases 

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## 1 Brain Aging: An Inevitable Physiological Process

The aging of the brain is characterized by a decline in several physiological abilities, including sensory, motor, and cognitive functions [78, 85, 87]. In mice, brain aging is typically accompanied by substantial cognitive deficits, beginning in late adulthood at around 12 months of age [90, 127]. Impaired function of signaling mechanisms, altered gene expression, and perturbed energy production are signs of aging on the cellular level. On the molecular level, oxidative stress results in the accumulation of damaged proteins, lipids, carbohydrates, and nucleic acids [33, 104]. Physiological changes that occur during normal aging of the brain may be exacerbated in vulnerable populations of neurons, initiating pathological processes that finally lead to neurodegenerative disorders [87].

## 2 Aging: An Important Risk Factor for Neurodegeneration

To understand the onset and progression of neurodegenerative diseases is one of the major challenges of the twenty-first century. The United Nations estimate that the number of people suffering from age-related neurodegeneration, particularly from AD , will exponentially increase from 25.5 million in 2000 to an estimated 114 million in 2050 [143]. Several meta-analyses have consistently estimated the global prevalence of dementia in people aged over 60 to be approximately $4 \%$ [101]. The global annual incidence of dementia is estimated to be about 8 per 1,000 population

[^23][29], with no substantial variations across continents, except Africa [100]. The incidence rate of dementia increases exponentially, doubling approximately every 5-6 years with age and incidence rates of dementia are quite similar across regions [100, 101, 148]. The largest increase in absolute numbers of old persons will occur in developing countries [100]. Thus, the global trend in the phenomenon of population aging has dramatic consequences on public health, health-care financing, and health care delivery systems in the world [100].

## 3 Alzheimer's disease: A Devastating Neurodegenerative Disorder

The clinical symptoms of Alzheimer' disease (AD) include a progressive loss of memory and impairment of cognitive abilities. Severe neurodegenerative alterations occur in AD brains, including loss of synapses and neurons, atrophy, and the selective depletion of neurotransmitter systems (e.g., acetylcholine) in the hippocampus and cerebral cortex-two brain regions involved in learning and memory [6]. Such defects are mainly observed in the later stage of the disease and have also been partially demonstrated using transgenic animal models of AD [71, 118].

AD is considered as a protein aggregation disorder, based on two key neuropathological hallmarks. One hallmark is the hyperphosphorylation of the tau protein, resulting in the formation of neurofibrillary tangles (NFTs), and the second hallmark is the increased formation and accumulation of amyloid-beta peptide (A $\beta$ ) oligomers and fibrils derived from amyloid precursor protein (APP) [42]. Although the exact underlying causes initiating the onset of AD are still unclear, an imbalance in oxidative and nitrosative stress, intimately linked to mitochondrial dysfunction, characterizes early stages of AD pathology [90].

## 4 Mitochondrial Dysfunction: A Common Event in Brain Aging and Alzheimer's Disease

Increasing evidence suggests that mitochondrial dysfunction plays an important role in brain aging and in the pathogenesis of neurodegenerative diseases, including AD [24, 48, 78, 82, 83, 86, 116, 135, 141]. Mitochondria are complex, networkforming organelles, involved in different metabolic pathways, e.g., citric acid cycle (TCA), energy transformation, amino-acid metabolism, and urea cycle [95]. Mitochondria consist of inner and outer membranes composed of phospholipid bilayers and proteins. The inner mitochondrial membrane harbors the proteins of the electron transfer system (ETS), responsible for oxidative phosphorylation. The mitochondrial oxidative phosphorylation (OXPHOS) system is the final biochemical pathway that produces energy in form of ATP by consuming oxygen. Electrons are transferred through the complexes of the mitochondrial respiratory system chain
and simultaneously, an electrochemical proton gradient is built across the inner mitochondrial membrane, generating the proton-motive force that drives the production of ATP [13, 124].

Alterations of mitochondrial efficiency and function are mainly related to alterations in mitochondrial mass, amount of respiratory enzymes, or changes in enzyme activities [11, 34, 65, 98]. A reduction in mitochondrial content or lowered ETS results in a general limitation of cellular energy production. Dysfunction of single complexes of the respiratory system are frequently accompanied by deleterious side effects, such as loss of mitochondrial membrane potential (MMP) and subsequently decreased ATP levels, but also production of reactive oxygen species (ROS) [91].

Apart from ROS enzymatically produced by NADPH oxidases, cytochrome P450-dependent oxygenases, and xanthine dehydrogenases, mitochondria are regarded as the primary site of ROS production within cells. The ETS constantly generates ROS, which are usually kept in balance by various defense mechanisms, i.e., anti-oxidative molecules (e.g., glutathione (GSH) or vitamin E) and antioxidant enzymes (e.g., superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione reductase), as long as ROS levels are in the physiological range. Furthermore, slight uncoupling of the ETS, e.g., by uncoupling proteins, may also reduce ROS production. Low levels of ROS are produced constantly which might have physiological functions as signaling molecules [38]. Functional failure of this system can lead to deleterious effects, which may exaggerate the consequences of mitochondrial dysfunction [46]. Insufficient defense mechanisms and excessive ROS production (e.g., as superoxide anions) can lead to cell damage. The major sources of superoxide anions are redox centers of complex I and III of the ETS, and different mitochondrial flavoproteins. Superoxide is a rather weak radical, but it is the precursor of various, potentially more toxic $\operatorname{ROS}$ [13, 69, 92]. Its transformation into hydrogen peroxide and hydroxyl radicals, as well as its participation in the formation of peroxynitrate, creates strong oxidants [31].

The proteins of the OXPHOS system and lipids are key targets of the deleterious effects of ROS, potentially leading to membrane depolarization and subsequently, impaired mitochondrial function [46, 90]. Thus, mitochondria play an important role in producing energy, but also as major source of ROS. Therefore, efforts to increase mitochondrial function should be accompanied by equal efforts to limit deleterious ROS generation.

Early defects in the expression of several subunits of respiratory system chain complexes [106], decreased mitochondrial respiration (mainly mediated by a decline in complex I and complex IV function), and reduced MMP and ATP levels have been detected in several AD cell culture and animal models [59, 73, 106, 141]. Direct effects of APP and A $\beta$ on mitochondrial function may induce this early dysfunction. Accumulation of APP in mitochondria, which has been found in both transgenic cell lines and animals, correlates with mitochondrial dysfunction. This may provide one causal link explaining the impaired energy metabolism and subsequent rise in ROS/RNS in models of AD [5, 37, 58]. Aside from APP, A $\beta$ itself has also been suggested to affect mitochondrial function (Fig. 23.1). Data


Fig. 23.1 Increasing evidence suggests that mitochondrial dysfunction plays an important role in brain aging and in the pathogenesis of neurodegenerative diseases. Dysfunction of single complexes of the respiratory system are frequently accompanied by deleterious side effects, such as decreased adenosine triphosphate (ATP) levels, but also production of reactive oxygen species (ROS). Direct effects of $A \beta$ peptides on mitochondrial function may induce early mitochondrial dysfunction and explain the impaired energy metabolism in models of AD. Physiological changes that occur during the normal aging of the brain may be exacerbated in vulnerable populations of neurons, initiating pathological processes that finally lead to neurodegenerative disorders. Rice bran, curcumin, anthocyanin-rich fruits, and olive polyphenols represent promising nutraceuticals for modulating mitochondrial function in the brain
show that the presence of one of the key enzymes in $\mathrm{A} \beta$ release, namely, $\gamma$-secretase, pinpoints to a direct production of A $\beta$ in these organelles [45].

Recently, Leuner et al. showed that mitochondria-derived ROS are sufficient to trigger amyloidogenic APP-processing in vivo, and that A $\beta$ itself leads to mitochondrial dysfunction and increased ROS levels (Fig. 23.1) [73]. Finally, increasing evidence suggests that mitochondrial dysfunction in AD originates not only from the deleterious impact of $\operatorname{APP} / \mathrm{A} \beta$ but also from its interplay with hyper-phosphorylated Tau protein on the mitochondrial level [59].

## 5 Brain Aging, Dementia, and the Impact of Nutrition

The survival of any organism crucially depends on its nutrient intake, which provides all molecules for cell formation, maintenance and repair, in the form of either ready-made building blocks or precursors [55]. In the case of humans, the importance of nutrition becomes obvious in the form of distinct patterns of clinical symptoms caused by the inadequate intake of one of the macronutrients, vitamins, or minerals [137]. The increase in life expectancy observed in the twentieth century in many populations throughout the world attests to the impact nutrition (in conjunction with better hygiene and medical practice) exerts on human health [97]. At the same time, however, human aging beyond 50 years of age is typically accompanied by the occurrence of one, often more, chronic, age-related diseases, such as cancer, cardio-vascular dieseases, and neurodegeneration [14, 32]. Due to its physiological characteristics, the brain is particularly prone to damage induced by noxious changes or fluctuations in cellular homeodynamics [103, 111]. Thus, the quest for primary prevention of neurodegeneration is imperative.

As stationary autotrophs, plants have evolved numerous pathways for the synthesis of secondary plant metabolites. These phytochemicals act, for example, as free radical scavengers or as defense against infectious microorganisms, with the aim of increasing a plant's chances for reproduction and survival [60].

In the following sections we discuss rice bran, curcumin, anthocyanin-rich fruits, and olive polyphenols as promising nutraceuticals for modulating mitochondrial function in the brain (Fig. 23.1).

### 5.1 Rice Bran

With an annual worldwide production of over 600 tons in 2006, rice is one of the most important staple foods, especially in Asian countries. The outer layer of the rice grain is called rice bran and is removed during the rice milling process to produce white rice. As a by-product of the rice milling process, rice bran has an annual production rate of $40-70$ million tons per year and usually is used as animal food [28,53]. Since rice bran contains the enzyme lipase which quickly renders the bran rancid and inedible it has to be stabilized before using it as human aliment, for example in the form of oils and extracts as health food products [53].

Key components of the rice bran are tocopherols, tocotrienols (Fig. 23.2), and $\gamma$-oryzanol. Known beneficial health effects of rice bran include anti-inflammatory, cholesterol-lowering, antioxidant, and antidiabetic effects [3, 18, 57, 72]. We recently found that a stabilized rice bran extract (RBE) also improves brain mitochondrial function in guinea pigs by increasing mitochondrial content and resistance against oxidative and nitrosative stress [43]. Therefore, RBE might be a suitable substance for the prevention of mitochondrial dysfunction seen in brain aging and neurodegenerative diseases like AD.
a

b


|  | $\mathrm{R}_{1}$ | $\mathrm{R}_{2}$ |
| :--- | :---: | :---: |
| $\alpha$-derivative | $-\mathrm{CH}_{3}$ | $-\mathrm{CH}_{3}$ |
| $\beta$-derivative | $-\mathrm{CH}_{3}$ | -H |
| -derivative | -H | $-\mathrm{CH}_{3}$ |

Fig. 23.2 Chemical structure of $\alpha$-, $\beta$-, and $\gamma$-tocopherol (a) and tocotrienol (b)

Tocopherols and tocotrienols are ingredients of rice bran and very likely play an important role in mediating the above mentioned health-promoting effects [21, 30]. In micromolar concentrations, tocopherols as well as tocotrienols act as radical scavengers that react with free radicals to produce less reactive radicals, thus for example preventing lipid peroxidation in membranes and lipoproteins [96, 144]. At a concentration of $5 \mu \mathrm{M}$, a tocotrienol-rich fraction from palm oil was, for example, able to inhibit oxidative damage in lipids and proteins from rat brain mitochondria induced by ascorbate- $\mathrm{Fe}^{2+}$, the free radical initiator azobis(2-amidopropane) dihydrochloride (AAPH) and photosensitization [54]. Supplementary vitamin E also prevented mitochondrial dysfunction in rat liver perfused with tertbutylhydroperoxide to induce lipid peroxidation by decreasing oxidative stress [44]. Altogether, tocotrienols seem to be better antioxidants than tocopherols, probably due to their faster recycling and better membrane distribution [113, 117]. Micromolar concentrations of tocopherols and tocotrienols that show antioxidative effects can usually not be reached in plasma and brain tissue by means of oral administration. On the other hand it has been reported that nanomolar


Fig. 23.3 Chemical structure of cycloartenyl ferulate, an exemplary member of the $\gamma$-oryzanol family which is a mix of ferulic acid esters of triterpene alcohol and phytosterols
concentrations of tocotrienols are sufficient to exert neuroprotective effects since they are able to modify several enzymes and signaling pathways in brain cells. Nanomolar concentrations of tocotrienols can be reached in human plasma after tocotrienol supplementation [30]. Among the cellular targets of tocotrienols are prenyl transferases [146], phospholipase A2 [61], 12-lipoxygenase [62], and NF-кB (nuclear factor kappa-light-chain-enhancer of activated B-cells) [35, 129].

Vitamin E has also been reported to directly interact with mitochondria [81], another possible mechanism for the neuroprotective potential of vitamin E. Dietary supplementation of rats with vitamin $\mathrm{E}(2.0$ or $5.0 \mathrm{~g} / \mathrm{kg}$ of food) for 3 months, for example, restored the age-dependent decrease in mitochondrial respiration and prevented an increase in oxidation products [93]. These effects are comparable to the increase in mitochondrial function seen in guinea pigs fed with RBE for 3 weeks [43], indicating that vitamin E at least partly accounts for the mitochondriaprotective effects of RBE.

Another key ingredient of rice bran is $\gamma$-oryzanol, a mix of ferulic acid esters of triterpene alcohol and phytosterols (Fig. 23.3) [72]. Antitumoral as well as antioxidant properties (e.g., inhibition of lipid peroxidation) and the lowering of blood cholesterol levels are the main known biological effects of $\gamma$-oryzanol [3, 64, 99]. Since $\gamma$-oryzanol is not water-soluble, it has a very low bioavailability when orally administered [63]. It is largely de-ferulated in the gut [12], but no enhanced ferulic acid concentrations could be detected in plasma after oral administration of RBE to guinea pigs [43], confirming the low bioavailability.

We found that RBE enhances mitochondrial function by increasing mitochondrial content via activation of the peroxisome proliferator-activated receptor gamma coactivator-1 $\alpha$ (PGC-1 $\alpha$ ) [43]. One activator of PGC-1 $\alpha$ is AMP-activated kinase (AMPK) which is, amongst others, induced by certain polyphenols, including resveratrol [77]. Therefore, it seems likely that RBE contains polyphenols or similar compounds able to activate PGC-1 $\alpha$. Identification of these compounds will have to be the subject of further upcoming studies.

Due to the observed beneficial effects of its main ingredients on neurons and the beneficial effects of RBE on mitochondrial function, rice bran appears to be a very

Fig. 23.4 Chemical structure of curcumin (keto form)

promising substance for the long-term prevention of neurodegeneration and the development of neurodegenerative diseases. Further studies need to be accomplished to examine the effects of rice bran administration in aging and neurodegenerative conditions.

### 5.2 Curcumin

Curcumin, demethoxycurcumin, and bisdemethoxycurcumin are derived from the rhizome of the plant curcuma longa (Fig. 23.4). This plant has long been known as a spice, a dye, and a remedy especially in Asian countries before it became generally and worldwide common as main ingredient of curry powder. Apart from the use as spice, curcumin is also applied as food additive (E100) and a pigment in textile and cosmetic industry [27]. Curcumin has been associated with various beneficial health effects, among them antioxidant, anti-inflammatory, antiviral, antibacterial, antifungal, wound-healing, and anticancer properties [1, 40, 149]. Additionally curcumin has been shown to inhibit $A \beta$ aggregation and reduce amyloid plaque burden in transgenic mouse models of AD [10, 75]. Taking all these effects into account, curcumin is assumed to have potential to act against various chronic diseases like diabetes, allergies, arthritis, and AD [1].

Epidemiologic evidence suggests that regular curry consumption decreases AD risk in elderly people. The Indo-US Study compared AD incidence rates in a rural, population-based cohort in India to those of a reference US population in Pennsylvania and found that AD incidence rates in India, where people consume curry spice on a daily basis, are much lower than those in the USA [16]. Ng and coworkers reported that regular curry consumption is correlated with better cognitive function in non-demented elderly Asians [94].

The antioxidative and anti-inflammatory effects of curcumin as well as its ability to inhibit protein aggregation seem to be the most important properties for the potential of curcumin against neurodegenerative diseases [22]. A lot of preclinical in vitro and in vivo studies have been accomplished to verify the beneficial effects of curcumin in neurotoxicity and AD. Apart from the reduction of amyloid plaque formation, curcumin also decreased oxidative injury, DNA damage, cytokine formation and memory deficits in mouse and rat models of AD [20, 22]. In a
homocysteine-induced rat neurotoxicity model, i.p. curcumin treatment (5 and $50 \mathrm{mg} / \mathrm{kg}$ body weight) for 10 days led to a significant decrease in malondialdehyde and superoxide anion levels, rescued hippocampal cells, and improved learning and memory [8]. In an Alzheimer transgenic APPsw mouse model (Tg2576), 6 month curcumin administration via a pelleted diet ( 160 and $5,000 \mathrm{ppm}$ ) decreased oxidized protein and interleukin- $1 \beta$ content in the brain. Insoluble and soluble $A \beta$ concentrations as well as plaque burden were decreased in mouse brains by low-dose curcumin administration [75].

Curcumin has been shown to have beneficial effects on mitochondrial function, for example by inhibiting lipid peroxidation and protein oxidation in rat liver mitochondria [142]. It further counteracted tert-butyl hydroperoxide (t-BHP)induced oxidative damage in rat cortical neurons by rescuing mitochondrial membrane potential, decreasing cytochrome c release and preventing apoptosis [147]. In the brains of streptozotocin-induced diabetic rats, activities of respiratory complexes I and IV were downregulated, and ATP levels were reduced. Curcumin administration to these rats ( $120 \mathrm{mg} / \mathrm{kg}$ bw p.o. for 4 weeks) rescued respiratory enzyme complex activities and restored ATP levels [102]. We recently showed that 5 month feeding of curcumin ( 500 mg curcumin per kg diet) was able to compensate mitochondrial dysfunction in a mouse model of accelerated aging. Thereby curcumin elevated the mitochondrial membrane potential, ATP levels, restored mitochondrial fusion processes, and elevated protein levels of PGC1 $\alpha$ [25]. Since mitochondrial dysfunction plays a major role in aging as well as in the development of neurodegenerative diseases, this mechanism of action of curcumin might very well contribute to the observed beneficial effects of curcumin on neurodegeneration. Various in vitro and in vivo studies have also reported beneficial effects of curcuminoids in chemically induced cell culture and rodent models of Parkinson's Disease (summarized by [22]).

Molecular mechanisms of action of curcumin mainly comprise its activity as radical scavenger, its antioxidant and anti-inflammatory effects mediated through nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and NF-кB as well as epigenetic modulations. Due to its chemical structure, curcumin is a potent scavenger of free radicals [2, 133]. Additionally, curcumin also exerts antioxidant effects by activating Nrf2, a transcription factor that controls the expression of antioxidant and phase-II enzymes, for example heme oxygenase and glutathione synthesis enzymes [109, 145]. By diminishing ROS production via radical scavenging and upregulation of antioxidative enzymes, curcumin contributes to keeping oxidative stress in the cell low, thus amongst others protecting mitochondrial function [102, 125]. Curcumin inhibits NF- $\kappa$, a transcription factor controlling the expression of pro-inflammatory molecules (e.g., cytokines) [52, 56, 128]. In conditions of neurodegeneration or AD , microglia in the brain become activated and produce pro-inflammatory responses via NF-кB [84]. Curcumin is able to inhibit these inflammatory responses in microglia cells, thus contributing to the prevention of neurodegeneration and AD development [49, 139].

Despite these very promising in vitro and in vivo results, no clinical studies testing curucmin in MCI or AD patients have reported positive outcome so far. Two

24-week intervention studies with patients with possible/probable or mild to moderate AD receiving curcumin doses up to $4 \mathrm{~g} /$ day reported no changes in clinical or biomarker measures between the study groups [9, 107]. Probable reasons might be the choice of subjects or the low bioavailability of curcumin. The best time for prevention in sporadic AD is the preclinical stage when neurodegeneration has already started but no clinical symptoms have yet occurred [136]. Therefore, neurodegeneration might have been too far advanced in the subjects included in these studies to be able to detect positive curcumin effects. Phase I clinical trials have proven that curcumin is well tolerated even in high doses (up to 12 g ), but oral bioavailability is very low (plasma levels often below $1 \mu \mathrm{M}$ ) [27]. Curcumin concentrations in the brains of mice were in the low ng/g tissue range 45 min after oral administration of 120 mg curcumin $/ \mathrm{kg}$ body weight (unpublished data). Probable reasons for the low bioavailability are poor absorption, rapid metabolism, and rapid systemic elimination of curcumin [4, 17].

To increase curcumin bioavailability, several different approaches have been pursued. One is the simultaneous administration of other secondary plant compounds like piperine which inhibit hepatic and intestinal metabolism of curcumin and are able to increase curcumin bioavailability significantly [120]. Other approaches comprise the production of curcumin nanoparticles or liposomeencapsulated curcumin [70, 130]. We recently showed that administration of curcumin micelles (AquaNova, Darmstadt, Germany) for 45 min increased curcumin plasma concentrations 50 -fold in C57BL/6 mice, curcumin brain concentrations were increased sixfold (unpublished data).

Altogether, curcumin appears to be a promising food ingredient to help in the prevention of neurodegeneration seen in aging and, for example, in Alzheimer's Disease. To display its protective effects, data from clinical and epidemiological studies suggest that curcumin might have to be administered over an extended period of time starting before the onset of clinical symptoms of neurodegeneration. This long-term preventive effect of curcumin will have to be proven in upcoming clinical trials.

### 5.3 Anthocyanin-Rich Fruits

In the last decade, colorful fruits have emerged as potential neuroprotective food components. Many animal intervention studies with blueberry, blackberry, strawberry, mulberry, Concord grape, and pomegranate provide evidence of the beneficial effects of colorful fruits on aging (especially on age-related cognitive and motor decline) and neurodegeneration. Anthocyanins, a flavonoid subgroup (Fig. 23.5) with high antioxidant potential, are responsible for the characteristic bright colors in these fruits and may also account at least in part for their neuroprotective activity [110, 140].

In the late nineties, James Joseph and colleagues showed that feeding diets with high antioxidant potential might prevent and even reverse age-related deficits in motor and cognitive behavior in Fischer 344 (F344) rats. However, although based


Fig. 23.5 Basic structure of anthocyanin aglycone and substituents of the 6 main structures found in food. In plant material anthocyanins are usually present as 3 -glycoside and $3^{\prime} 5$-glycoside [19]
on equal antioxidant activity, the supplementations with blueberry and strawberry did not lead to the same improvement in behavioral performance. Blueberry supplementation ameliorated both motor and cognitive performance, whereas strawberry supplementation only led to an improvement in motor performance suggesting that simple antioxidant activity is not the sole explanation for the neuroprotective activity [51]. To date, the theory that flavonoids like anthocyanins exert their effects by direct scavenging of reactive-oxygen-species (ROS) is more and more replaced by the assumption that they act by indirect antioxidant activity and activation of signaling pathways [7, 112]. Supporting evidence for this theory comes from bioavailability studies that often report $<0.1 \%$ recovery of ingested anthocyanins in the urine [88, 110]. Like all polyphenols, anthocyanins are subject to various degradation and biotransformation processes leading to a variety of metabolites in the human body. Moreover, to exert direct effects in the brain anthocyanins also have to cross the blood-brain barrier (BBB) separating the CNS from the body periphery. Feeding studies with rodents and pigs have been shown that anthocyanins are able to cross the BBB. After 15 days of supplementation with blackberry extract [131] and 2 h of administration of the anthocyanidin pelargonidin ( $50 \mathrm{mg} /$ body weight) [26], 0.25 nmol anthocyanins $/ \mathrm{g}$ and 0.16 nmol pelargonidin/g tissue, respectively, were detected in rat brain. Moreover, data from pigs suggest that anthocyanins may accumulate in brain tissue. After a feeding period with $1-4 \%$ blueberries for 4 weeks, fasted pigs showed anthocyanin concentration of about $0.3-0.4 \mathrm{ng} / \mathrm{g}$ tissue in the brain but not in plasma or urine. However, there is a lack of data concerning the presence of anthocyanin metabolites which are suggested to account at least in part for the in vivo effects of anthocyaninrich fruits [110].

Regarding neuroprotection, the most extensively studied anthocyanin-rich diet in rodents is the $2 \%$ blueberry-supplemented diet which led to improvements in cognitive and motor performance of aged rats or models of increased oxidative stress or inflammation [15, 23, 36, 105, 122]. The recent work by Rendeiro and colleagues strengthens the theory that the containing flavonoids represent causal
neuroprotective agents in the blueberry diet. Purified blueberry anthocyanins in equivalent doses to the whole blueberry extract led to the same improvement of spatial working memory performance as whole blueberry-diet [105]. Several other anthocyanin-rich fruits have shown to have a beneficial impact on behavioral performance of rodents. However, there seem to be differences in the activity that might be fruit or flavonoid specific as well as concentration-dependent. For example, Concord grape juice in a concentration of $10 \%$ in drinking water improved cognitive performance in aged F344 rats whereas a concentration of $50 \%$ of the juice ameliorated motor function [121]. $2 \%$ blueberry and $2 \%$ strawberry extract diets protected differently from ${ }^{56} \mathrm{Fe}$ particle irradiation which induces oxidative stress, inflammation and behavioral deficits similar to those seen in aging. Strawberry-supplemented rats had a better ability to retain place information (reduced spatial deficits) linked to hippocampus-mediated behavior, blueberrysupplemented rats, in contrast, showed improved reversal learning which is more dependent on intact striatal function [122]. Nevertheless, a recent study showed that ${ }^{56} \mathrm{Fe}$ particle irradiation causes downregulation of genes involved in protective stress signaling which could be ameliorated by blueberry- or strawberrysupplemented diets to a similar extent [123].

A great deal of research has concentrated on the impact of berry fruits and flavonoids on signaling cascades (reviewed in [89, 126]). In this regard, studies on the mechanism of cognitive effects of blueberry diet in rats revealed an involvement of neurogenesis, neurotrophic factor insulin-like growth factor-1 (IGF-1) and its receptor, as well as mitogen-activated protein (MAP) kinase signal transduction [15]. Grape powder in drinking water of rats ( $15 \mathrm{~g} / \mathrm{L}$ ) prevented the L-buthionine( $\mathrm{S}, \mathrm{R}$ )-sulfoximine induced oxidative stress and cognitive impairment as well as prevented the activation of brain extracellular signal-regulated kinase- $1 / 2$ (ERK-1/2) and decrease of glyoxalase-1 (GLO-1), glutathione reductase-1 (GSR-1), calcium/calmodulin-dependent protein kinase type IV (CAMK-IV), cAMP response element-binding protein (CREB), and brain-derived neurotrophic factor (BDNF) levels [150]. Research also concentrated on the effects of purple sweet potato color (PSPC) which is composed of a mixture of anthocyanins. PSPC ( $100 \mathrm{mg} / \mathrm{kg}$ ) attenuated D-galactose-induced aging related changes in mouse brain after oral administration for 4 weeks. The improvement of behavioral performance was accompanied by an enhanced activity of the antioxidant enzymes copper/zinc superoxide dismutase and catalase, less oxidative brain damage measured as malondialdehyde, and diminished parameters related to neuroinflammation (e.g., nuclear translocation of NF- $\kappa$ B) [118]. Further studies using this model also revealed the ability of PSPC to counteract the onset of neuronal apoptosis by promoting survival mechanisms which involves ERK 1/2, phosphoinositide 3-kinase (PI3K), Akt, and c-Jun NH2-terminal kinase (JNK) [80]. Recently, PSPC ( $200 \mathrm{mg} / \mathrm{kg}$ for 4 weeks) has also been tested in a mouse model of cognitive impairment induced by hippocampal mitochondrial dysfunction in mice that were treated with the neurotoxin domoic acid. The study results suggest that better cognitive performance involved estrogen receptor- $\alpha$-mediated mitochondrial biogenesis signaling, restored mitochondrial dysfunction, decreased ROS and
protein carbonyl levels, and suppressed endoplasmic reticulum stress-induced apoptosis [79]. Further evidence for the amelioration of mitochondrial dysfunction is provided by a study with anthocyanins from grape skin in rats with transient memory impairment and mitochondrial dysfunction induced by scopolamine. The i.p. treatment with $200 \mathrm{mg} / \mathrm{kg}$ grape skin anthocyanins reversed the impairment of memory and restored ATP levels in hippocampus and cerebral cortex [41]. Mitochondrial dysfunction has also been investigated in cell cultures treated with protocatechuic acid, a well-known metabolite of the anthocyanidin cyanidin, which has been detected in the bloodstream of humans [138] and rats [134] after consumption of cyanidin glucoside/cyanidin-glucoside-rich foods. Protocatechuic acid was effective to decrease mitochondrial dysfunction and apoptotic cell death induced by rotenone [39] and 1-methyl-4-phenylpyridinium ion [76] in the neuronal-like cell line PC 12. Moreover, treatment of human neuroblastoma SK-N-MC cells with metabolites obtained from in vitro digestion of wild blackberry extract was effective in diminishing ROS, modulating GSH and maintaining high mMP at levels approaching concentrations that are described for human plasma [132].

Mitochondrial dysfunction, oxidative stress, and inflammation occur not only in aging but also in age-related neurodegenerative changes (Fig. 23.1). Slowing down or even preventing aging processes in the brain by nutritional approaches might therefore as well contribute to the prevention of neurodegenerative diseases like Alzheimer's disease. Anthocyanin-rich fruits have a beneficial in mouse models of AD . In amyloid precursor protein/presenilin 1 (APP/PS1) transgenic mice diet supplementation with $2 \%$ blueberry extract from 4 months of age prevented behavioral deficits assessed at 12 months of age as well as enhanced memoryassociated neuronal signaling. No changes in $\mathrm{A} \beta$ burden were observed [50]. However, less accumulation of soluble A $\beta 42$ and amyloid deposition was observed in the hippocampus of APP transgenic mice after the treatment with pomegranate juice concentrate in drinking water ( $1: 80$ or 1:160 dilution) for 6.5 months [47]. In APP/PS1 transgenic mice drinking water supplemented with pomegranate extract ( $6.25 \mathrm{~mL} / \mathrm{L}$ ) for 3 months led to improved spatial learning and memory, decreased $\mathrm{A} \beta$ plaque load, reduced microgliosis as well as lowered tumor necrosis factor a (TNF- $\alpha$ ) concentrations and nuclear factor of activated T-cell (NFAT) transcriptional activity [108]. Additionally, 0.18 or $0.9 \%$ mulberry extract supplemented diet for 3 months led to a decreased accumulation of $A \beta$ as well as higher antioxidant enzyme activity and less lipid oxidation in the brain of senescenceaccelerated mouse prone 8 (SAMP8) mice [119].

Importantly, preliminary studies in older adults with mild cognitive impairment (MCI) show beneficial effects of Concord grape and blueberry juice [66-68]. MCI is the first clinical appearance of neurodegeneration accompanied by increased risk for dementia. In many individuals MCI progresses to AD. The consumption of wild blueberry juice ( 6 and $9 \mathrm{~mL} / \mathrm{kg}$ ) for 12 weeks improved paired associate learning in the Verbal Paired Associate Learning Test (V-PAL) and word list recall in the California Verbal Learning Test (CVLT) in 9 older adults with MCI [68]. In a similar study 12 older adults with MCI showed improved verbal learning in CVLT
and a trend toward improved performance with respect to delayed verbal recall and spatial memory after the consumption of Concord grape juice ( 6 and $9 \mathrm{~mL} / \mathrm{kg}$ ) for a period of 12 weeks [67]. Recently, Concord juice treatment of MCI individuals for 16 weeks reduced semantic interference on memory tasks and led to a relatively greater activation in anterior and posterior regions of the right hemisphere detected using functional magnetic resonance imaging [66].

### 5.4 Olive Oil Polyphenols

Olive oil is a typical component of Mediterranean diets which have been related to many health beneficial effects including the improvement of cognitive decline. Interestingly, the health benefits of extra virgin olive oil (EVOO) seem to be not only due to its high amount of mono-unsaturated fatty acids but also due to phenolic minor components such as hydroxytyrosol [110]. The phenols present in the native olive fruit differ from those in EVOO. Olives mainly contain the glycosides oleuropein and ligstroside that are degraded to their aglycones and various derivates during ripening. The aglycones and derivates are the most abundant phenols in olive oil. Hydroxytyrosol and tyrosol are the end products of the hydrolysis of those aglycones in olive oil (Fig. 23.6) [154].

Recently, EVOO showed beneficial effects in SAMP8 mice, a model of age-related learning/memory impairment associated with increased amyloid- $\beta$ protein and brain oxidative damage [151]. The oral administration of EVOO $(75 \mu \mathrm{~L} / \mathrm{kg}$ body weight) for 6 weeks improved cognitive function and oxidative brain damage in aged SAMP8 mice. Interestingly, mice that received EVOO with enhanced amount of olive oil polyphenols showed a greater improvement in both cognitive function and oxidative damage than mice that received regular EVOO [151]. Additionally, mice treated with EVOO (10 \% wt/wt dry diet) rich in phenols $(6 \mathrm{mg} / \mathrm{kg}$ polyphenols/day) from middle age to senescence had improved contextual memory in the step-down test and a better performance in motor coordination in the rotarod test [152].

Data from human and animal studies indicate that olive oil phenols are well absorbed and underlie biotransformation processes common for polyphenols in general [154]. As ortho-diphenol, hydroxytyrosol contributes significantly to the oxidation stability of olive oil and is attracting particular attention as antioxidant $[154,110]$. However, the intake of phenols in the amounts provided by dietary olive oil is suggested to be too low for direct antioxidant activity in the human body [154]. Several studies therefore concentrated on hydroxytyrosol-rich extracts. Importantly, conjugated hydroxytyrosol was detected in brain tissue of rats ( $50 \mathrm{nmol} / \mathrm{g}$ ) after a single dose of a phenolic extract of olive cake ( $3 \mathrm{~g} / \mathrm{kg}$ body weight) [153]. An interesting source of hydroxytyrosol is olive mill water waste which is currently discarded. Olive mill water waste is very rich in polyphenols that can be recovered by ad hoc techniques [110]. Hydroxytyrosol-rich extract, prepared from olive mill water waste administrated to mice $(100 \mathrm{mg} / \mathrm{kg})$ for 12 days led to a

Fig. 23.6 Olive oil phenols (a) Oleuropein aglycone (b) Hydroxytyrosol


b

moderate, although statistically significant hyperpolarization of mitochondria in dissociated mouse brain cells [115] which is an effect that has been related to a decreased rate of cell death [74]. Moreover, hydroxytyrosol-rich extract was effective to reduce iron-stimulated lipid peroxidation ex vivo, suggesting a neuroprotective effect of hydroxytyrosol intake [115]. Recent in vitro data mainly confirm our previous observation of promising cytoprotection of brain cells by HT-rich olive mill waste water extract in different stressor paradigms [114]. Furthermore, correlation analyses revealed that the observed cytoprotective effects in PC12 cells are likely due to HT present in the extract.

In summary, aging of the brain is characterized by a decline in several physiological abilities, including sensory, motor, and cognitive functions. Physiological changes that occur during normal aging of the brain may be exacerbated in vulnerable populations of neurons, initiating pathological processes that finally lead to neurodegenerative disorders, especially to $A D$. The incidence rate of $A D$ increases exponentially, doubling approximately every 5-6 years with age. The global trend in the phenomenon of population aging has dramatic consequences on public health, health-care financing, and health care delivery system in the world, especially in developing countries. Increasing evidence suggests that mitochondrial dysfunction plays an important role in brain aging and in the pathogenesis of neurodegenerative diseases. The survival of any organism crucially depends on its nutrient intake, which provides all molecules for cell formation, maintenance and repair, either in the form of ready-made building blocks or precursors. Rice bran, curcumin, anthocyanin-rich fruits, and olive polyphenols are promising nutraceuticals for modulating mitochondrial function in the brain and might contribute to the prevention of AD .

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