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Functional foods, cardiovascular disease and diabetes

Edited by
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1

The potential and limits of functional foods in preventing cardiovascular disease

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1.1 Introduction: diet and cardiovascular disease

Cardiovascular disease (CVD) is still a major cause of death in Western populations and is becoming an important cause of morbidity and mortality worldwide. Thanks to advanced medical knowledge and treatments, many patients survive an initial event. Because of that, prevention of secondary CVD is a growing task for nutritionists and other health professionals.

Cardiovascular risk can be reduced by lifestyle changes, one of which is diet. There is now substantial evidence from epidemiological and clinical studies that a diet rich in fruits, vegetables, unrefined grains, fish and low-fat dairy products, and low in saturated fats and sodium, can reduce the risk of coronary heart disease and hypertension.[1] People who have adopted such diets have benefited by way of a much lower risk of heart disease (see Table 1.1).[1–4] However, such a prudent diet is not typical of what consumers in Western countries eat.[3–5] It appears that consumers today are less likely to invest in long-term health if taste

Table 1.1 Evidence-based strategies to reduce the risk of coronary heart disease

- Substitute nonhydrogenated unsaturated fats for saturated and *trans* fats
- Increase consumption of omega-3 fatty acids from fish, fish oil supplements, or plant sources
- Consume a diet high in fruits, vegetables, nuts, and whole grains and low in refined grain products

Source: Hu and Willett[2]

and convenience are compromised: in 1998 only 24 per cent of consumers ate 'healthy foods' for long-term prevention of disease, as opposed to 45 per cent in 1990.[6] However, almost all consumers indicated that they sometimes buy foods for health reasons. Food industries are aware of this, and market some of their foods with health claims. Indeed, it has been shown that health claims on foods have a positive influence on consumers' perception of the healthiness of foods.[7] Thus, functional foods in the form of palatable and ready-to-use foods that suggest short- or long-term health benefits have a huge market and health potential.

This chapter will discuss how functional foods can play a role in choosing a healthy diet.

1.2 Functional foods defined

The term 'functional foods' is not a standard nutritional term in nutrition textbooks. Regulatory agencies and professional associations of nutrition scientists and dieticians all use different definitions. ILSI Europe, an industry-sponsored forum in which representatives from industry, academia and government address nutrition issues, proposed a definition which in abbreviated form runs:

> 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, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease.

More concise definitions are 'foods that provide a health benefit beyond basic nutrition' or 'foods that have health benefits beyond the traditional nutrients provided'. Examples are soy, garlic, and green tea. What these definitions do not include is the link with the food industry, which uses health claims to market functional foods. An alternative definition could therefore be 'a food which claims explicitly or implicitly to improve health or well-being'.[8]

Functional foods that are marketed with claims to reduce heart disease focus primarily on the risk factors of blood cholesterol, homocysteine and hypertension. This can be done by a *reduced* content of food components that are known to increase risk, such as saturated fat or sodium. More recently products have been designed that are *enriched* in components that are thought to reduce risk. The most common 'protective' ingredients include fibres, soya, omega-3 fatty acids, phytostanols and phytosterols, and (antioxidant) vitamins. These components have cholesterol or homocysteine-lowering abilities in metabolic studies. The added ingredients may be food components that are often deficient in Western diets, such as calcium and folate. Their recommended intake could, however, be achieved by 'normal' foods. The added ingredients may also be nutrients or phytochemicals that are normally

not ingested in effective amounts because natural food sources of these ingredients are scarce or not part of out diet. Examples are phytosterols or probiotics. Examples of functional foods include soya drinks, eggs enriched with omega-3 fatty acids, margarines with plant sterols or stanols, and vitamin-enriched cereals.

In this chapter a distinction will be made between functional foods that help consumers adhere to dietary guidelines and functional foods that offer novel ingredients with claimed or suggested health effects.

1.3 The use of functional foods to meet dietary guidelines

Many consumers struggle to meet dietary recommendations. The United States Department of Agriculture (USDA) reported in 1998 that the average intake of added fats and sugars was too high and the intake of fruits, vegetables, dairy products, lean meats and foods made from unrefined grains was too low compared with serving recommendations.[9] Comparable findings in The Netherlands[10] and the rest of Europe (supplement 2 to the *British Journal of Nutrition* 1999, vol. 81) have been reported.

Functional foods enriched with vitamins, dietary fibres or specific fatty acids, or foods that are designed to be low in sodium or saturated fat, can therefore make a valuable contribution to our diet, as will be discussed in the following paragraphs. The evidence-based strategies for a reduction in CVD risk have been used as a guide.

1.3.1 Substitute nonhydrogenated unsaturated fats for saturated and *trans* fats

Replacement of saturated or *trans* fat in the diet by carbohydrates or other types of fat reduces the risk of coronary heart disease.[11,12] Margarines were rich sources of *trans* fat until about a decade ago, but food manufacturers have markedly reduced the *trans* fat content since reports on adverse health effects.

1.3.2 Increase consumption of omega-3 fatty acids from fish, fish oil supplements or plant sources

Fish oils are listed as functional food ingredients because of their remarkable effect on preventing sudden cardiac death.[13] The recommended consumption of fish in Western countries is one or two portions per week. The average intake varies highly between countries, with a six- to sevenfold variation in total fish consumption in countries in Europe,[14] but is lower than the recommendation. Instead of increasing the amount of fish in the diet, functional foods enriched with the n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can be used. Several foods can be fortified with fish oil, for example margarines, dairy products, sausages, luncheon meat and french onion dip.[15]

Adding these products to an *ad libitum* diet significantly increased plasma and platelet EPA and DHA.[15]

Chicken eggs enriched with n-3 fatty acids may provide an alternative source of EPA and DHA. In populations where egg consumption is higher than fish consumption, in particular, this could be an effective strategy to increase n-3 intake.[16] Chicken eggs can be enriched with n-3 fatty acids by feeding hens diets rich in flax seed or fish meal.[17,18] It has been shown that consumption of two to four enriched eggs/day significantly increased polyunsaturated fatty acid (PUFA) concentrations in platelet phosplipids.[19–21] Effects on blood lipids were variously shown to be absent to beneficial. Omega-3 enriched eggs can be a succesful source of n-3 fatty acids only if they are accepted by the consumer. In a sensory evaluation with 78 untrained volunteers no difference in taste was found, and storage life was no different for enriched and 'normal' eggs.[21] Although three enriched eggs need to be consumed to provide approximately the same amount of n-3 fatty acids as one meal with fish, they can be a good source of n-3 fatty acids for consumers who do not like fish.

1.3.3 Consume a diet high in fruits, vegetables, nuts and whole grains and low in refined grain products

In the US, vegetable consumption is close to the recommended daily intake but fruit consumption is less than half of the recommended amount.[9] In Europe, fruit and vegetable consumption is also below recommendations (supplement 2 to the *British Journal of Nutrition* 1999, vol. 81). As a consequence many consumers do not meet dietary recommendations for fibre, folate, vitamin C and other vitamins. For example, it has been estimated that approximately 50 per cent of Dutch consumers do not meet dietary recommendations for folate.[22]

Many consumers believe that a healthy meal takes more time to prepare.[23] Ready-to-eat salads, fruits and ready-to-cook vegetables can increase consumption in consumers with limited time to prepare foods.

Foods enriched with fibres and vitamins can be an alternative to fruits and vegetables, but only to a certain point. For example, different dietary fibres have different effects on CVD risk: water-soluble dietary fibres such as pectin and guar gum appear to have stronger effects than insoluble fibres such as wheat bran.[24,25] Thus, a mixture of various dietary fibres such as found naturally in fruits and vegetables appears to be necessary for a protective effect on CVD. Also, adding vitamins to foods to compensate for low fruit and vegetable intakes might not have the expected effects. For example, beta-carotene was widely believed to reduce cancer risk in smokers, because intake of carotene-rich foods was associated with less cancer, as were high levels of carotene in blood. However, it was found that carotene supplements increased risk of lung cancer in smokers.[26,27] Large clinical trials of antioxidants have also had disappointing outcomes.[28] Moreover, several other bioactive components from fruits and vegetables, rather than vitamins, may protect against CVD. Enrichment of foods with known vitamins and minerals might therefore not be enough.

Consumption of functional foods can make an important contribution to nutrient intakes. For example, consumption of micronutrient-enriched cereals was associated with significantly increased intakes of iron, B vitamins, vitamin D and fibre in an adult Irish population.[29]

1.3.4 Choose and prepare foods with less salt

Although not part of the strategies as proposed by Hu and Willett in their 2002 paper,[2] reduced salt intake is another strategy to lower risk of CVD.[30] Current average salt intake in Western populations is 9–12 g/day[31] and this should be reduced to 5–6 g/day according to most public health recommendations aimed at lowering blood pressure.[31–33]

Manufactured foods are the largest sources of salt in our diet, whereas cooking salt and table salt provide only 5–35 per cent.[34–36] Thus, reductions in the amount of salt added by food manufacturers have a much larger impact in salt consumption than the advice to use less salt at home.[35] Functional foods with reduced salt content such as soups and snacks – could therefore have a considerable impact on CVD risk.

Replacing sodium in the diet with potassium has been shown to reduce blood pressure.[37] Mineral salts such as LoSalt, in which a third of the sodium has been replaced by potassium, could therefore be a good alternative to regular salt.

1.3.5 Foods with 'novel' ingredients

Most of the claims for benefits from novel ingredients have come from ecological or cohort studies.[38] However, the effects of some ingredients on risk markers have been well investigated in clinical trials,[39] and show promise of reducing disease risk.

Sterols and stanols

Margarines and yogurts have been enriched with plant stanols or sterols, which lower low-density lipoprotein (LDL) cholesterol by 10 per cent and could thus make an important contribution to prevention of coronary heart disease.[40] Many well-controlled trials have documented the efficacy of sterols and stanols for lowering LDL, and no major adverse effects have been noted. However, long-term safety and clinical efficacy have not been evaluated in large-scale clinical trials of the size and duration customary for new drugs. The Health Council of The Netherlands therefore discourages the use of plant sterols by consumers who would not benefit from a cholesterol-lowering effect, e.g. children and pregnant women, and other regulatory agencies have suggested similar limitations.

Polyphenols

High intakes of tea rich in catechins and other flavonoid polyphenols have been associated with a reduced risk of coronary heart disease.[41] A clinical trial to evaluate these effects would seem justified and feasible.

Isoflavones (phytoestrogens)
A high consumption of soy and soy protein has been associated with a low risk of CVD in ecological studies. Besides soy protein, phytoestrogens such as genistein might be responsible for the effects on CVD risk. Phytoestrogens comprise several groups of non-steroidal oestrogens including isoflavones and lignans. There is limited quantitative data on the absorption and metabolism of dietary phytoestrogens. Although it is now known that dietary phytoestrogens are metabolised by intestinal bacteria, absorbed, conjugated in the liver, circulated in plasma and excreted in urine, further clinical trials should determine the potential health effects of these compounds.[42,43]

Functional foods with isoflavones and other phytoestrogens include breakfast cereals, soft drinks, bakery and dairy products and snack bars.[44]

1.4 Do functional foods reach the populations at risk?

Several surveys have shown that a higher socioeconomic status is associated with a healthier diet, that is, a diet closer to the recommendations.[10,45] Consumers with a low or middle socioeconomic status would therefore benefit most from functional foods. It has been suggested that functional foods would appeal most to healthy, well-educated and rich consumers, but this does not appear to be true: in a Dutch survey among 1183 consumers aged 19–91 years, determinants of functional food use depended on the type of food.[46] Stanol-enriched margarines were consumed most by smokers and consumers with a poorer subjective health. A Finnish study, however, showed a higher consumption of such margarines in consumers with higher socioeconomic status.[47] Differences in marketing strategies can possibly account for these differences.

A survey among 238 Dutch dieticians showed that 'functional foods' are still a topic of debate and confusion: although most of them advised patients about functional foods, they felt uncertain about dosage, safety and efficacy.[48] Educating health-care providers on these aspects of functional foods will be an important task for non-commercial nutritionists. A uniform definition of the term 'functional foods' and clear legislation on health claims would further enhance the acceptance and succes of this new generation of foods.

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2

Assessing health claims for functional foods

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2.1 Introduction

According to the traditional concept of nutrition, the primary role of the diet is to provide adequate quantities of nutrients to meet metabolic requirements and maintain optimal health. However, epidemiological, experimental and clinical studies have shown that certain types of food and specific food components can affect a variety of body functions and provide specific health benefits.[1,2] Based on scientific data it is now accepted that diet can have beneficial physiological and psychological effects, beyond well-known nutritional effects, by modulating specific target functions in the body. Therefore, diet not only helps to achieve optimal development and health, but it may promote better health and play an important role in disease prevention by reducing the risk of certain chronic diseases. To meet the optimal diet composition, promote health and reduce the risk of chronic diseases, the concept of functional foods has been developed.

Functional food has been defined in many ways. According to a simple definition proposed by the American Dietetic Association, functional food is 'any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains'.[3] European experts presented the following definition:

> 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, in a way which is relevant to either improved stage of health and well-being and/or the reduction of the risk of a disease.[4]

A functional effect can be provided by essential or non-essential nutrients or by food components of no nutritive value. However, a functional food must be a food: conventional or everyday, consumed as part of the usual diet. It should be composed of naturally occurring components, but one or more components can be added, removed, and/or modified, and/or the bioavailability of one or more components can be modified. These foods may be functional for all members of the populations or for a defined section of the population.

Different types of food claims can be used to promote the benefits of functional food. However, functional and health claims are of great importance. These claims influence consumer behaviour, which potentially affects consumer health.

2.2 Differing types of claim: nutritional and health claims

European experts suggest the following definition of claim:

> A claim is any direct or indirect statement, symbol, suggestion, implication or any other form of communication (including the brand name) that a good has particular characteristics relating to its origin, properties, effect, nature, method of production, processing, composition or any other quality.[5]

For functional foods different types of claims are defined and classified by international bodies such as Codex Alimentarius or the European Commission, or by national organizations/committee/authorities such as the Food and Drug Administration (FDA) in the US or the Food Standards Agency in the United Kingdom.[5–11]

There are nutrition claims, nutrient content claims, comparative claims, claims related to dietary guidelines and healthy eating pattern, nutrient function or 'structure/function' claims, 'enhanced-function' claims, health claims and reduction of disease-risk claims. Nutrient content claims, comparative claims, nutrient function claims, enhanced-function claims and healthy eating claims can be classified as subsections of nutrition claims, while reduction of disease-risk claims can be seen as health claims.

Nutrition claims have been defined as

> any representation which states, suggests or implies that a food has particular nutritional properties including but not limited to the energy value and the content of protein, fat and carbohydrates, as well as the content of vitamins and minerals.[6]

Nutrient content claims refer to the level of a nutrient (nutrients) in a food/food product. These claims can be expressed as 'low fat', 'low in saturated fat', 'high in fibre', 'reduced cholesterol', 'rich in calcium', or 'source of calcium'.

Codex Alimentarius also defines comparative claims, which compare the levels of the nutrient in different food products (two or more) by using such

words as 'more than', 'less than', 'reduced', 'increased'; for example: 'contains 50% more calcium than regular milk'.

Healthy eating pattern claims relate to the dietary guidelines and recommendations of national or international authorities on healthy diets. Examples of such claims are: 'Diet low in saturated fat is recommended by …', '… recommends a daily intake of 800 mg of calcium', '… recommends an enhanced fibre intake.'

Nutrient function claims (or structure/function claims) describe the physiological role of a nutrient and its relation to normal functions of the body, but must not refer to abnormal (pathological) conditions. Examples include 'calcium is necessary for bone structure', 'calcium helps to develop strong bones and teeth', 'vitamin E protects fat from oxidation'. The Codex Alimentarius Committee has stated that nutrient function claims should be related to essential nutrients for which reference values have been established and to nutrients which are mentioned in official dietary guidelines or recommendations.[7,10,12] The food products for which nutrient function claims are made should be an important source of the nutrient in the usual diet.

All these types of nutrition claim are based on established knowledge of nutrients and their physiological functions, which must be widely accepted by the scientific community. So-called 'enhanced function' (functional) claims and reduction of disease-risk or health claims are particularly important for functional food. These types of claims relate to specific beneficial effects of foods or food components, whether nutrients and non-nutrients. Enhanced function or functional claims describe the beneficial effects of food components on physiological or psychological functions, metabolic activities, cellular and biochemical process, beyond the established role of these foods in normal functions of the body. These claims do not refer directly to any healthy benefits or disease risk reduction. Examples of claims of enhanced function are: 'calcium improves bone density', 'antioxidants reduce the risk of oxidative stress', 'folate helps reduce plasma homocysteine levels', 'non-digestible oligosacharides improve the growth of bacterial flora in the gut'.

Claims that suggest that foods or food components have an impact on health – in improving good health or a condition related to a disease – have been called health claims.[5,10] An important subgroup of claims are disease-risk reduction claims. They state that a food or its component may help to reduce the risk of a disease. Examples of such claims include: 'Adequate intake of calcium may help to reduce the risk of osteoporosis', 'Adequate intake of folate by women may reduce the risk of having children with neural tube defect', 'Food low in fat and cholesterol can help to reduce the risk of coronary heart disease'.

Claims have to follow specific criteria to ensure consumers are not misled or confused. Nutritional and health claims must be based on documented scientific information, validated, supported by evidence, complete, objective and verifiable. Claims must be clear and understandable by consumers.[5,12] It is essential that claims for functional foods fulfil these criteria and special attention must be paid to objective scientific validation of functional and health claims.[10,11,13,14]

2.3 Criteria for demonstrating functional effects

Evidence of the effects of functional foods has to be based on objective criteria developed and accepted by the scientific community. Functional foods have to beneficially modulate target functions of the body, which are relevant to improved state of health or well-being, or for reduction of disease risk. As a result, based on current knowledge, the target function to be modulated has to be clearly defined. A set of markers can be used to define the function, demonstrate its modulation and demonstrate the effects of the modulation (Fig. 2.1). Different types of markers may be chosen: biochemical, physiological or behavioural.[4] Markers may directly represent either an event of interest or correlated events. Markers must be specific, sensitive, reproducible, validated and biologically significant. They should be related to: the target functions, function improvement or biological response improvement, or to appropriate intermediate end-points of improved health or reduced disease risk.[4,11,13] Markers should register short- and long-term impact of the foods. It should also

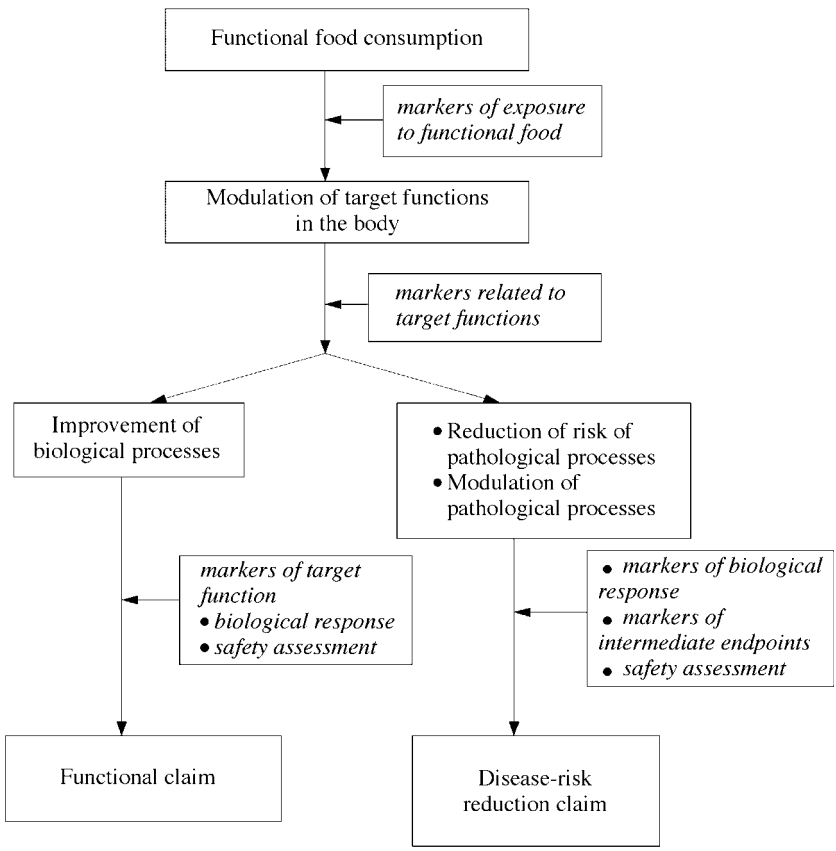

Fig. 2.1 Basis for functional claims and disease-risk reduction claims for functional foods.

be possible to use these markers in safety assessment of functional foods. Markers of exposure to the foods (food components) are also needed. All markers must fulfil standard quality control criteria.

Using a defined set of markers the modulation of relevant target functions and the improvement of the target biological process must be documented. The functional effects have to be demonstrated in human nutrition studies for all members of a population or for particular group clearly defined by specific markers for example: by age, sex or genetic markers. Based on data on dose-dependent effects, the functional effects of realistic amounts of foods consumed as part of the daily diet has to demonstrated. The assessment of the safety of the amount of foods needed for functional effects has also to be performed, documented and presented according to accepted standards.

2.4 Evidence required to support a health claim

Before functional and health claims are made, scientific evidence for the effects of functional foods is required. Different types of evidence are needed, including data of biological/biochemical observations, epidemiological data and data from human intervention studies. The required data have to describe effects at the molecular, cellular, tissue and organ level, as well as effects on individuals, and also effects at the population level. The evidence must be based on studies in which accepted markers of improved functions or biological response, or markers of intermediate endpoints of disease, are used.

Data of basic studies, such as cellular experiments, data derived from animal studies and different biochemical measurement, have to explain the mechanism of action of functional food components, or give some important insight into the mechanism of action. Epidemiological studies, such as cross-sectional or prospective studies, have to provide data on relationship between food consumption and different outcomes. Meta-analysis of different epidemiological studies can be very useful. Data derived from human intervention studies are very important.

In all studies a set of accepted biomarkers must be used and analysed. The studied population should be similar to the population for which the functional foods are intended. For most food products this will be the general population. However, the general population consists of healthy subjects, high-risk subjects and affected subjects. The efficiency and safety of a functional food product has to be documented for these different population groups. If the functional food products are directed to a subgroup of the general population, for example a high-risk group, the subgroup must be clearly defined and explained in a way that is understandable to consumers.

The data may document the effect of isolated individual food components, but they must also show that the final functional food product is expected to have physiological or health effects. All required data have to be internally consistent, meet appropriate scientific criteria and standards of both biological and statistical significance, developed and accepted by an objective body.

In 2002 the Food and Drug Administration designed a new initiative, called the Consumer Health Information for Better Nutrition Initiative, to

> encourage makers of conventional foods and dietary supplements to make accurate, up-to-date, scientific based claims about the health benefits of their products, and to help eliminate bogus labeling claims by pursuing marketers of human dietary supplements and others who make false or misleading claims about the health benefits or other effects of their products.[15]

In January 2003, the FDA Task Force on Consumer Health Information for Better Nutrition prepared a report and recommendations for evaluating and ranking the scientific evidence for qualified health claims.[15] The quality and strength of the scientific evidence for a proposed health claim are categorized through a grading system consisting of four levels, A, B, C and D, corresponding to high, moderate, low and extremely low levels of scientific support. The highest level 'A' means that the evidence is derived from well-designed studies and therefore there is significant scientific agreement about the health claim. This level is chosen as a point of reference and such a claim is referred to as an 'unqualified health claim'. The second level 'B' applies to claims that are supported by good scientific evidence, but the evidence is not conclusive. The third level 'C' refers to scientific evidence that is limited and inconclusive. The fourth level 'D' should be used when little scientific evidence supports the claim. Claims categorized as B, C or D are called 'qualified health claims' and they require a disclaimer or other qualifying language to ensure that they do not mislead consumers. All qualified health claims have to be reviewed by the FDA before they are used on the food label, a process that involves a careful review of all the available scientific data and may include detailed expert assessment. Such a review process and ranking system should protect consumers from unproved and misleading information.

2.5 Future trends

Functional foods have been expected to play an important role in modern nutrition, which is expected to promote health and reduce the risk of chronic diseases. Functional foods must fulfil all standards of food safety assessment. However, for this type of food, the concept of benefits versus risk of long-term intake has to be elaborated, developed and validated. The safety of intake of low or high amounts of nutrients and high amounts of non-nutrients related to long-term consumption of functional food, as well as interactions between food components and biological processes, have to be monitored. Protocols for pre-marketing nutrition studies on functional food and post-marketing monitoring are needed.

One of the most urgent tasks concerns the conditions for health claims for probiotics. The joint report on this matter was announced by FAO/WHO (Food

and Agriculture Organization/World Health Organization) experts following the meeting of the work group in London, Ontario, Canada in 2002.[16] According to this document, if we want to emphasize specific health effects of probiotic bacteria, we have to refer to a defined strain which has been genotyped and phenotyped. Moreover, such a strain has to be transferred to the international culture collection. The manufacturer is obliged to place information concerning the probiotic bacteria contained in the specific product, using the nomenclature consistent with the validation list published in the *International Journal of Systemic and Evolutionary Microbiology*.

The tests concerning the health-promoting properties of probiotic bacteria have to be conducted not only *in vitro*, but also in clinical conditions.

Taking into account the safety aspects, i.e. the possibility of infection increase, adverse metabolic effects, excessive stimulation of immunological systems and potential gene transfer, the manufacturer of probiotics is obliged to present clinical documentation showing the absence of the above effects.[17] In particular, probiotic bacteria must not significantly increase the risk of transfer of antibiotic resistance and cannot possess haemolytic activity.

The requirements of probiotic food safety indicated by FAO/WHO impose on the manufacturers the obligation to conduct placebo-controlled clinical studies and to evaluate their results in four phases, i.e. Phase 1 (safety), Phase 2 (efficiency), Phase 3 (effectiveness) and Phase 4 (surveillance).

If the manufacturer obtains positive results from one research centre, they should also obtain the confirmation of such results from other researchers and the results have to be published in a peer-reviewed scientific or medical journal.

It seems that similar requirements will have to be enforced in the future with respect to all functional food. It should be borne in mind that during clinical studies we often discover new unforeseen so-called pleiotropic actions of functional food, as in the case of medicines.

In view of the above, it is necessary to consider the possibility of disclosing these facts in the form of additional health claims. An example may be our studies on *Lactobacillus plantarum* 299v, during which we have discovered that leptin and insulin levels are lowered without body mass index (BMI) reduction.[18] Another issue is the effect of certain genetic polymorphism highly prevalent within the population on the effectiveness of action of functional food. An example may be polymorphism of apolipoprotein E and its effect on absorption of cholesterol from food and response to changes in fat intake.[19] To sum up, we should say that there are many factors affecting health claims. Therefore, such claims have to be continuously monitored and changed in correlation with results of clinical and population studies.

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Part I

Diet, cardiovascular disease and diabetes

3

Diet and the prevention of coronary heart disease

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3.1 Introduction

Active prevention of coronary heart disease (CHD) is usually started immediately after its first clinical manifestation. Secondary prevention focuses on risk reduction in people with established CHD who are at high risk of recurrent cardiac events and death. It is important to remember that the two main causes of death in these patients are sudden cardiac death (SCD) and heart failure (HF), often resulting from myocardial ischaemia and subsequent necrosis. The main mechanism underlying recurrent cardiac events is myocardial ischaemia resulting from atherosclerotic plaque rupture or ulceration. Plaque rupture is usually the consequence of intraplaque inflammation combined with a high lipid content of the lesion, high concentration of leucocytes and lipid peroxidation products. Thus, in patients with established CHD, the main aims of the preventive strategy are to prevent malignant ventricular arrhythmia and the development of severe ventricular dysfunction (and heart failure) and to minimise the risk of plaque inflammation and ulceration. This means that the priority of secondary prevention is somewhat different from that of primary prevention. In the context of primary prevention, intervention focuses on traditional risk factors (e.g. blood cholesterol or blood pressure) and surrogate endpoints rather than on specific clinical complications such as SCD. This does not mean that traditional risk factors of CHD should not be measured and, if necessary, corrected in secondary prevention, because they also play a role in the occurrence of CHD complications. It simply means that because complications such as SCD and associated syndromes are often unpredictable, occur out of hospital and far from any potential therapeutic resources in the majority of cases, and account for about 50 per cent of cardiac mortality in secondary prevention, they should be the priority of any secondary

prevention programme. For that reason, in the present text, we will focus our dietary recommendations and comments specifically on clinical efficacy and not on surrogate efficacy.

Whatever the specific clinical aims of the programme, nutritional evaluation and counselling of each individual with CHD must be a key point of the preventive intervention. Nutrition is, however, only one component of such a programme. Exercise training, behavioural interventions (particularly to help the patient abstain from smoking) and drug therapy have equally important roles. The control of risk factors has been seen traditionally in the perspective of prevention. The dietary prevention programme is commonly initiated during hospitalisation for a first CHD event. With the shortening of stay in the coronary care unit (CCU), dietary intervention is initiated during the following days at hospital, then continued in secondary prevention centres and included in cardiac rehabilitation programmes. An individual dietary prevention programme should be developed under the guidance of a specialised dietician and in close collaboration with the patient's cardiologist and primary care physician, so that there is no discontinuity or discrepancy in dietary counselling between the hospitalisation and post-hospitalisation phases of the rehabilitation programme.

3.2 Dietary prevention of sudden cardiac death (SCD): the role of dietary fatty acids, alcohol and antioxidants

SCD is usually defined as death from a cardiac cause occurring within one hour from the onset of symptoms.[1] In many studies, however, investigators used quite different definitions, with a time frame of 3 or even 24 hours in the old World Health Organization definition. The magnitude of the problem is considerable since SCD is a very common, and often the first, manifestation of CHD, and it accounts for about 50 per cent of cardiovascular mortality in developed countries.[1] In most cases, SCD occurs without prodromal symptoms and out of hospital. As a matter of fact, this mode of death is a major public health issue. Since up to 80 per cent of SCD patients had CHD,[2] the epidemiology and potential preventive approaches of SCD should, in theory, parallel those of CHD. In other words, any treatment aimed at reducing CHD should reduce the incidence of SCD.

We now examine whether diet (and more precisely, certain dietary factors) may prevent (or help prevent) SCD in patients with established CHD. We focus our analyses on the effects of the different families of fatty acids, antioxidants and alcohol.[2]

3.2.1 Fish, n-3 fatty acids and SCD

The hypothesis that eating fish may protect against SCD is derived from the results of a secondary prevention trial, the Diet And Reinfarction Trial (DART), which showed a significant reduction in total and cardiovascular mortality (both

by about 30 per cent) in patients who had at least two servings of fatty fish per week.[3] The authors suggested that the protective effect of fish might be explained by a preventive action on ventricular fibrillation (VF), since no benefit was observed on the incidence of nonfatal acute myocardial infarction (AMI). This hypothesis was consistent with experimental evidence suggesting that n-3 polyunsaturated fatty acids (PUFA), the dominant fatty acids in fish oil and fatty fish, have an important effect on the occurrence of VF in the setting of myocardial ischaemia and reperfusion in various animal models, both *in vivo* and *in vitro*.[4–5] In the same studies, it was also apparent that saturated fatty acids are proarrhythmic compared with unsaturated fatty acids. Using an elegant *in vivo* model of SCD in dogs, Billman and colleagues recently demonstrated a striking reduction of VF after intravenous administration of pure n-3 PUFA, including both the long-chain fatty acids present in fish oil and alpha-linolenic acid, their parent n-3 PUFA occurring in some vegetable oils.[6] These authors found that the mechanism of this protection results from the electrophysiological effects of free n-3 PUFA when these are simply partitioned into the phospholipids of the sarcolemma without covalently bonding to any constituents of the cell membrane. After dietary intake, these fatty acids are preferentially incorporated into membrane phospholipids.[7]

Nair and colleagues have also shown that a very important pool of free (non-esterified) fatty acids exists in the normal myocardium and that the amount of n-3 PUFA in this pool is increased by supplementing the diet in n-3 PUFA.[7] This illustrates the potential of diet to modify the structure and biochemical composition of cardiac cells. In the case of ischaemia, phospholipases and lipases quickly release new fatty acids from phospholipids, including n-3 fatty acids in higher amounts than the other fatty acids,[7] thus further increasing the pool of free n-3 fatty acids that can exert an antiarrhythmic effect.

It is important to remember that the lipoprotein lipase is particularly active following the consumption of n-3 PUFA.[8] One hypothesis is that the presence of the free form of n-3 PUFA in the membrane of cardiac muscle cells renders the myocardium more resistant to arrhythmias, probably by modulating the conduction of several membrane ion channels.[9] So far, it seems that the very potent inhibitory effects of n-3 PUFA on the fast sodium current, INa,[10,11] and the L-type calcium current, ICaL,[12] are the major contributors to the anti-arrhythmic actions of these fatty acids in ischaemia. Briefly, n-3 PUFA act by shifting the steady-state inactivation potential to more negative values, as was also observed in other excitable tissues such as neurons.

Another important aspect of the implication of n-3 PUFA in SCD is their role in the metabolisation of eicosanoids. In competition with n-6 PUFA, they are the precursors to a broad array of structurally diverse and potent bioactive lipids (including eicosanoids, prostaglandins and thromboxanes), which are thought to play a role in the occurrence of VF during myocardial ischaemia and reperfusion.[13,14]

Other clinical data show suppression (by more than 70 per cent) of ventricular premature complexes in middle-aged patients with frequent

ventricular extrasystoles randomly assigned to take either fish oil or placebo.[15] Also, survivors of AMI[16] and healthy men[17] receiving fish oil were shown to improve their measurements of heart rate variability, suggesting other mechanisms by which n-3 PUFA may be antiarrhythmic.

Support for the hypothesis of a clinically significant antiarrhythmic effect of n-3 PUFA in the secondary prevention of CHD, as put forward in DART,[3] came from two randomised trials testing the effect of ethnic dietary patterns (instead of that of a single food or nutrient), i.e. a Mediterranean type of diet and an Asian vegetarian diet, in the secondary prevention of CHD.[18,19] The two experimental diets included a high intake of essential alpha-linolenic acid, the main vegetable n-3 PUFA. Whereas the incidence of SCD was markedly reduced in both trials, the number of cases was very small and the antiarrhythmic effect cannot be entirely attributed to alpha-linolenic acid as these experimental diets were also high in other nutrients with potential antiarrhythmic properties, including various antioxidants. These findings were extended by the population-based case-control study conducted by Siscovick and colleagues on the intake of n-3 PUFA among patients with primary cardiac arrest, compared with that of age- and sex-matched controls.[20] Their data indicated that the intake of about 5–6 grams of n-3 PUFA per month (an amount provided by consuming fatty fish once or twice a week) was associated with a 50 per cent reduction in the risk of cardiac arrest. In that study, the use of a biomarker, the red blood cell membrane level of n-3 PUFA, considerably enhanced the validity of the findings, which also were consistent with the results of many (but not all) cohort studies suggesting that consumption of one to two servings of fish per week is associated with a marked reduction in CHD mortality compared with no fish intake.[21,22] In most studies, however, the SCD endpoint is not reported.

In a large prospective study (more than 20 000 participants with a follow-up of 11 years), Albert *et al*. examined the specific point that fish has antiarrhythmic properties and may prevent SCD.[23] They found that the risk of SCD was 50 per cent lower for men who consumed fish at least once a week than for those who had fish less than once a month. Interestingly, the consumption of fish was not related to non-sudden cardiac death suggesting that the main protective effect of fish (or n-3 PUFA) is related to an effect on arrhythmia. These results are consistent with those of DART[3] but differ from those of the Chicago Western Electric Study, in which there was a significant inverse association between fish consumption and non-sudden cardiac death, but not with SCD.[24] Several methodological factors may explain the discrepancy between the two studies, especially the way of classifying deaths in the Western Electric Study.[24] This again illustrates the limitations of observational studies and the obvious fact that only randomised trials can definitely provide a clear demonstration of causal relationships.

The GISSI-Prevenzione trial was aimed at helping in addressing the question of the health benefits of foods rich in n-3 PUFA (and also in vitamin E) and their pharmacological substitutes.[25] Patients (n = 11 324) surviving a recent AMI (<3

Table 3.1 Clinical efficacy of (n-3) PUFA in the GISSI-Prevenzione Trial. See text for comments

	Relative risk (95% confidence interval)
Death, nonfatal AMI and stroke	0.85 (0.70–0.99)
Overall mortality	0.80 (0.67–0.94)
Cardiovascular mortality	0.70 (0.56–0.87)
Sudden cardiac death	0.55 (0.40–0.76)
Nonfatal cardiovascular events	0.96 (0.76–1.21)
Fatal and nonfatal stroke	1.30 (0.87–1.96)

Source: modified from GISSI-Prevenzione investigators.[25]

months) and having received the prior advice to come back to a **Mediterranean type of diet** were randomly assigned supplements of n-3 PUFA (0.8 g daily), vitamin E (300 mg daily), both or none (control) for 3.5 years. The primary efficacy endpoint was the combination of death and nonfatal AMI and stroke. Secondary analyses included overall mortality, cardiovascular (CV) mortality and SCD. The exact definition of SCD was not given in the paper. However, the clinical events were validated by an *ad hoc* committee of expert cardiologists,[25] who presumably used the current definition of SCD. Treatment with n-3 PUFA significantly lowered the risk of the primary endpoint (the relative risk decreased by 15 per cent). Secondary analyses provided a clearer profile of the clinical effects of n-3 PUFA (Table 3.1). Overall mortality was reduced by 20 per cent and CV mortality by 30 per cent. However, it was the effect on SCD (45 per cent lower) that accounted for most of the benefits seen in the primary combined endpoint and both overall and CV mortality. There was no difference across the treatment groups for nonfatal CV events, a result comparable to that of DART.[3] Thus, the results obtained in this randomised trial are consistent with previous controlled trials,[3,18,19] large-scale observational studies[21–24] and experimental studies,[4-7] which together strongly support an effect of n-3 PUFA in relation with SCD.

An important point is that the protective effect of n-3 PUFA on SCD was greater in the groups of patients who complied more strictly with *the Mediterranean diet*. This suggests a positive interaction between n-3 PUFA and some components of the Mediterranean diet which is, by definition, not high in n-6 PUFA and low in saturated fats, but rich in oleic acid, various antioxidants and fibre, and associated with a moderate consumption of alcohol (see below for further comments).

3.2.2 Saturated fatty acids, oleic acid, *trans* fatty acids and n-6 fatty acids

Regarding the other dietary fatty acids, animal experiments have clearly indicated that a diet rich in saturated fatty acids is associated with a high incidence of ischaemia- and reperfusion-induced ventricular arrhythmia,

whereas PUFA of either the n-6 or n-3 family reduce that risk.[4-6] Many (but not all) epidemiological studies have shown consistent associations between the intake of saturated fatty acids and CHD mortality.[26] However, the SCD endpoint is usually not analysed in these studies. In addition, a clear demonstration of a causal relationship between dietary saturated fatty acids and SCD would require the organisation of a randomised trial, which is not ethically acceptable. Thus, besides the effect of saturated fatty acids on blood cholesterol levels, the exact mechanism(s) by which saturated fats increase CHD mortality remain unclear. If animal data, demonstrating a proarrhythmic effect of saturated fatty acids, are confirmed in humans, the first thing to do in order to prevent SCD in humans would be to drastically reduce the intake of saturated fats. In fact, this has been done in randomised dietary trials and, as expected, the rate of SCD decreased in the experimental groups.[18,19] However, as written above about the same trials,[22,23] the beneficial effect cannot be entirely attributed to the reduction of saturated fats, because other potentially antiarrhythmic dietary factors, including n-3 PUFA, were also modified in these trials.

In contrast to n-3 PUFA, few data have been published so far regarding the effect of n-6 PUFA on the risk of SCD. Roberts *et al*. have reported that the percentage content of linoleic acid (the dominant n-6 PUFA in the diet) in adipose tissue (an indicator of long-term dietary intake) was inversely related to the risk of SCD, which was defined in that study as instantaneous death or death within 24 hours of the onset of symptoms.[27] This is in line with most animal data and may suggest that people at risk of SCD may benefit from increasing their dietary intake of n-6 PUFA, in particular linoleic acid, in the same way as for n-3 PUFA. However, n-3 PUFA were more effective on SCD than n-6 PUFA in most animal experiments.[4-6]

In addition, diets high in n-6 PUFA increase the linoleic acid acid content of lipoproteins and render them more susceptible to oxidation,[28] which would be an argument against such diets because lipoprotein oxidation is a major step in the inflammatory process that renders atherosclerotic lesions unstable and prone to rupture.[29–31]

Erosion and rupture of atherosclerotic lesions were shown to trigger CHD complications (see below the section on plaque inflammation and rupture) and myocardial ischaemia and to considerably enhance the risk of SCD.[32-35] As a matter of fact, in the secondary prevention of CHD, diets high in n-6 PUFA failed to improve the overall prognosis of the patients.[36] Also, in the Dayton study, a mixed primary and secondary prevention trial, in which the chief characteristic of the experimental diet was the substitution of n-6 PUFA for saturated fat, the number of SCD was apparently lower in the experimental group than in the control group (18 *vs.* 27) but the number of deaths from other causes, in particular cancers, was higher in the experimental group (85 *vs.* 71), thus offsetting the potential protective effect of n-6 PUFA on SCD and having

no effect at all on mortality.[37] Such negative effects were not reported with n-3 PUFA. Thus, despite the beneficial effect of n-6 PUFA on lipoprotein levels, which could, in theory, reduce SCD in the long term by reducing the development of atherosclerosis, it seems preferable not to increase the consumption of n-6 PUFA beyond the amounts required to prevent deficiencies in the essential n-6 fatty acid, linoleic acid (approximately 4–6 per cent of the total energy intake), which are found in the current average Western diet. As a substitute for saturated fat, the best choice is obviously to increase the intake of vegetable monounsaturated fat (oleic acid) in accordance with the Mediterranean diet pattern. If oleic acid has apparently no effect on the risk of SCD (at least by comparison with n-3 and n-6 PUFA), its effects on blood lipoprotein levels are similar to those of n-6 PUFA and it has the great advantage of protecting lipoproteins against oxidation.[38]

Thus, the best fatty acid combination to prevent SCD (and other complications of CHD) and, in other words, the cumulative antiarrhythmic, antioxidant and hypolipidaemic effects, would result from the adoption of a diet close to the Mediterranean diet pattern.[38,39]

Finally, Roberts *et al*. reported no significant relationship between *trans* isomers of oleic and linoleic acids in adipose tissue and the risk of SCD[40] whereas Lemaitre *et al*. found that cell membrane *trans* isomers of linoleic acid (but not of oleic acid) are associated with a large increase in the risk of primary cardiac arrest.[41] As for the role of *trans* fatty acids on ventricular arrhythmias, it has not been investigated in experimental models.

Thus, although specific human data on the effect of saturated fatty acids on SCD are lacking, results of several trials suggest that it is important to reduce their intake in the secondary prevention of CHD. Despite a possible beneficial effect on the risk of SCD, increasing consumption of n-6 PUFA should not be recommended in clinical practice for patients with established CHD. Diets including low intakes of saturated fatty acid (as well as *trans* isomers of linoleic acid) and n-6 PUFA (but enough to provide the essential linoleic acid) and high intakes of n-3 PUFA and oleic acid (Mediterranean diet pattern) appear to be the best option to prevent both SCD and nonfatal AMI recurrence.[19,38]

3.2.3 Alcohol and SCD

The question of the effect of alcohol on heart and vessel diseases has been the subject of intense controversy in recent years. The consensus is now that moderate alcohol drinking is associated with reduced cardiovascular mortality, although the exact mechanism(s) by which alcohol is protective are still unclear. In contrast, chronic heavy drinking has been incriminated in the occurrence of atrial as well as ventricular arrhythmias in humans, an effect called ‘the holiday heart’ because it is often associated with binge drinking by healthy people,

specifically during the weekend. Studies in animals have shown varying and apparently contradictory effects of alcohol on cardiac rhythm and conduction, depending on the animal species, the experimental model and the dose of alcohol. If given acutely to non-alcoholic animals, ethanol may even have antiarrhythmic properties.

In humans, few studies have specifically investigated the effect of alcohol on SCD. The hyperadrenergic state resulting from binge drinking, as well as from withdrawal in alcoholics, seems to be the main mechanism by which alcohol induces arrhythmias in humans. In the British Regional Heart Study, the relative risk of SCD in heavy drinkers (more than six drinks per day) was twice as high as in occasional or light drinkers.[42] However, the effect of binge drinking on SCD was more evident in men with no pre-existing CHD than in those with established CHD. In contrast, in the Honolulu Heart Program,[43] the risk of SCD among healthy middle-aged men was positively related to blood pressure, serum cholesterol, smoking and left ventricular hypertrophy but inversely related to alcohol intake. In fact, the effect of moderate 'social' drinking on the risk of SCD in non-alcoholic subjects has been addressed so far in only one study.

Investigators of the Physicians' Health Study assessed whether light-to-moderate alcohol drinkers apparently free of CHD at baseline have a decreased risk of SCD.[44] After controlling for multiple confounders, men who consumed two to four drinks per week or five to six drinks per week at baseline had a significantly reduced risk of SCD (by 60–80 per cent) as compared with those who rarely or never consumed alcohol. Analyses were repeated after excluding deaths occurring during the first 4 years of follow-up (in order to exclude the possibility that some men who refrained from drinking at baseline did so because of early symptoms of heart diseases), and also using the updated measure of alcohol intake ascertained at year 7 to address potential misclassification in the baseline evaluation of alcohol drinking.[44] These secondary analyses basically provided the same results and confirmed the potential protective effect of moderate drinking on the risk of SCD. Despite limitations (the selected nature of the cohort, an exclusively male study group, no information on beverage type and drinking pattern), this study suggests that a significant part of the cardioprotective effect of moderate drinking is related to the prevention of SCD. Further research should be directed at understanding the mechanism(s) by which moderate alcohol drinking may prevent ventricular arrhythmias and SCD.

In practice, the current state of our knowledge suggests that in CHD patients at risk of SCD, there is no reason not to allow moderate alcoholic drinking. From a practical point of view, we advise drinking no more than one or two drinks per day, preferably wine, preferably during the evening meal, and never before driving a car or undertaking dangerous work.

3.2.4 Antioxidants and SCD

The issue about the effect of dietary antioxidants on the risk of CHD in general and on SCD in particular is more controversial. Regarding vitamin E, for instance, the most widely studied dietary antioxidant, discrepant findings between the expected benefits based on epidemiological observations[45,46] and the results of clinical trials[47,48] were published. In a recent controlled trial, a significant decrease in nonfatal AMI and a non-significant increase in cardiovascular mortality (in particular in the rate of SCD) were reported with a daily regimen of 400–800 mg of vitamin E in patients with established CHD.[49] Because of certain methodological shortcomings (which we will not discuss here), this trial was said to confuse rather than clarify the question of the usefulness of vitamin E supplementation in CHD, and provided no indication about possible links between vitamin E and the prevention of SCD.

The GISSI-Prevenzione trial brings new information in this regard. Unlike those of n-3 PUFA, the results of vitamin E supplementation do not support a significant effect on the primary endpoint, namely a combination of death and nonfatal AMI and stroke.[25] However, the secondary analysis provides a clearer view of the clinical effect of vitamin E in CHD patients, which cannot be easily dismissed (see Table 3.2). In fact, among the 193 and 155 cardiac deaths that occurred in the control and vitamin E group, respectively, during the trial (a difference of 38, $P < 0.05$), there were 99 and 65 SCDs (a difference of 34, $P < 0.05$), which indicated that the significant decrease in cardiovascular mortality (by 20 per cent) in the vitamin E group was almost entirely due to a decrease in the incidence of SCD (by 35 per cent). In contrast, nonfatal cardiac events and non-sudden cardiac deaths were not influenced.[25] These data suggest that vitamin E may be useful for the primary prevention of SCD in patients with established CHD.

The vitamin E data of the GISSI trial do not stand in isolation. In an *in vivo* dog model of myocardial ischaemia,[50] we also reported a protective effect of vitamin E on the incidence of VF (the main mechanism of SCD) with a 16 per cent rate in the vitamin E group and 44 per cent in the placebo group ($P < 0.05$). Also in line with the GISSI results, infarct size, which is the main determinant of acute heart failure and non-sudden cardiac death, was larger in the supplemented

Table 3.2 Clinical efficacy of vitamin E in the GISSI-Prevenzione Trial. See text for comments

	Relative risk (95% confidence interval)
Death, nonfatal AMI and stroke	0.89 (0.77–1.03)
Overall mortality	0.86 (0.72–1.02)
Cardiovascular mortality	0.80 (0.65–0.99)
Sudden cardiac death	0.65 (0.48–0.89)
Nonfatal cardiovascular events	1.02 (0.81–1.28)
Fatal and nonfatal	0.95 (0.61–1.47)

Source: modified from GISSI-Prevenzione investigators.[25]

group (58.5 per cent of the ischaemic area) than in the placebo group (41.9 per cent, $P < 0.05$). Such ambivalent effects of vitamin E may at least partly explain why its effects were neutral or non-significant in many studies, with the negative effects hiding the beneficial ones. Nevertheless, the GISSI trial showed that cardiovascular mortality and SCD were significantly reduced by vitamin E, and the effect on overall mortality showed a favourable trend ($P = 0.07$). Finally, the recently published HOPE trial, testing the effect of 400 IU of vitamin E daily in patients at high risk of CHD (therefore in primary prevention) and reporting an apparent lack of effect of vitamin E, does not help us to solve the issue of whether or not vitamin E is protective against SCD.[51] In that trial, it is not clear whether the patients actually took the capsules during meals (a prerequisite for intestinal absorption of vitamin E), whether the patients were more or less deficient in vitamin E (no blood measurement), whether some of them were taking vitamin supplements (a common practice nowadays among certain populations), and SCD was apparently not among the predefined endpoints. In addition, patients with left ventricular dysfunction, a major determinant of the risk of SCD, were not eligible.

3.3 Dietary prevention of chronic heart failure (CHF): the role of micronutrients, dietary fatty acids and reduced sodium intake

The incidence of chronic heart failure (CHF), the common end-result of most cardiac diseases, is increasing steadily in many countries despite (and probably because of) considerable improvements in the acute and chronic treatment of CHD, which is nowadays the main cause of CHF in most countries.[52] In recent years, most research effort about CHF has focused on drug treatment, and little attention has been paid to nonpharmacological management. Some unidentified factors may indeed contribute to the rise in the prevalence of CHF and should be recognised and corrected if possible. For instance, CHF is now seen also as a metabolic problem, with endocrine and immunological disturbances potentially contributing to the progression of the disease.[53,54] In particular, the role of the tumour necrosis factor (TNF) is discussed below. Recently it has also been recognised that increased oxidative stress may contribute to the pathogenesis of CHF.[55] The intimate link between diet and oxidative stress is obvious, since the major antioxidant defences of our body are derived from essential nutrients.[56] See below the section about the antioxidant nutrients.

While it is generally considered that a high sodium diet is detrimental (and may result in acute decompensation of heart failure through a volume overload mechanism), little is known about other aspects of diet in CHF in terms of both general nutrition and micronutrients such as vitamins and minerals. In these patients, it is important not only to take care of the diagnosis and treatment of the CHF syndrome itself, and for the identification and aggressive management of traditional risk factors of CHD such as high blood pressure and cholesterol

(because they can aggravate the syndrome), but also for the recognition and correction of malnutrition and of deficiencies in specific micronutrients.

The vital importance of micronutrients for health and the fact that several micronutrients have antioxidant properties are now fully recognised. These may be as direct antioxidants, such as vitamins C and E, or as components of antioxidant enzymes: superoxide dismutase or glutathione peroxidase.[56] It is now widely believed (but still not causally demonstrated) that diet-derived antioxidants may play a role in the development (and thus in the prevention) of CHF. For instance, clinical and experimental studies have suggested that CHF may be associated with increased free radical formation[57] and reduced antioxidant defences[58] and that vitamin C may improve endothelial function in patients with CHF.[59] In the secondary prevention of CHD, in dietary trials in which the tested diet included high intakes of natural antioxidants, the incidence of new episodes of CHF was reduced in the experimental groups.[18,60] Taken altogether, these data suggest (but do not demonstrate) that antioxidant nutrients may help prevent CHF in post-infarction patients.

Other nutrients, however, may be also involved in certain cases of CHF. While deficiency in certain micronutrients, whatever the reason, can cause CHF and should be corrected (see below), it is important to understand that patients suffering from CHF also have symptoms that can affect their food intake and result in deficiencies, for instance tiredness when strained, breathing difficulties and gastrointestinal symptoms such as nausea, loss of appetite and early feeling of satiety. Drug therapy can lead to loss of appetite and excess urinary losses in case of diuretic use. All of these are mainly consequences, not causative factors, of CHF. Thus the basic treatment of CHF should, in theory, improve these nutritional anomalies. However, since they can contribute to the development and severity of CHF, they should be recognised and corrected as early as possible.

Finally, it has been shown that up to 50 per cent of patients suffering from CHF are malnourished to some degree,[61] and CHF is often associated with weight loss. There may be multiple aetiologies to the weight loss,[62] in particular lack of activity resulting in loss of muscle bulk and increased resting metabolic rate. There is also a shift towards catabolism with insulin resistance and increased catabolic relative to anabolic steroids.[63] TNF, sometimes called cachectin (see above), is higher in many patients with CHF,[53,63] which may explain weight loss in these patients. Interestingly, there is a positive correlation between TNF and markers of oxidative stress in the failing heart[64] suggesting a link between TNF and antioxidant defences in CHF (the potential importance of TNF in CHF is discussed below in the section on dietary fatty acids and CHF). Finally, cardiac cachexia is a well-recognised complication of CHF, its prevalence increases as symptoms worsen[65] and it is an independent predictor of mortality in CHF patients. However, the pathophysiological alteration leading to cachexia remains unclear and, at present, there is no specific treatment apart from the treatment of the basic illness and correction of the associated biological abnormalities.

3.3.1 Deficiency in specific micronutrients

As mentioned above, an important practical point is that deficiencies in specific micronutrients can both aggravate and cause CHF. The prevalence of these deficiencies among patients with CHF (and post-infarction patients) is unknown. Whether we should systematically search for them also remains unclear. In particular, we do not know whether the association of several borderline deficiencies that do not individually result in CHF may result in CHF, especially in the elderly. For certain authors, however, there is sufficient evidence to support a large-scale trial of dietary micronutrient supplementation in CHF.[66]

There is not room here to fully explore the present knowledge in this field. Nevertheless, if we restrict our comments to human data, the situation can be summarised as follows. Cases of hypocalcaemia-induced cardiomyopathy (usually in children with a congenital cause for hypocalcaemia) that can respond dramatically to calcium supplementation have been reported.[67] Hypomagnesaemia is often associated with a poor prognosis in CHF,[68] and correction of the magnesium levels (in anorexia nervosa for instance) leads to an improvement in cardiac function. Low serum and high urinary zinc levels are found in CHF,[69] possibly as a result of diuretic use, but there are no data regarding the clinical effect of zinc supplementation in that context. In a recent study, plasma copper was slightly higher and zinc slightly lower in CHF subjects than in healthy controls.[58] As expected, dietary intakes were in the normal range and no significant relationship was found between dietary intakes and blood levels in the two groups. It is not possible to say whether these copper and zinc abnormalities may contribute to the development of CHF or are simple markers for the chronic inflammation known to be associated with CHF.[53,63] Further studies are needed to address the point, since the implications for prevention are substantial.

Selenium deficiency has been identified as a major factor in the aetiology of certain nonischaemic CHF syndromes, especially in low-selenium soil areas such as eastern China and Western Africa.[70] In Western countries, cases of congestive cardiomyopathy associated with low antioxidant nutrients (vitamins and trace elements) have been reported in malnourished HIV-infected patients and in subjects on chronic parenteral nutrition.[71] Selenium deficiency is also a risk factor for peripartum cardiomyopathy.

In China, an endemic cardiomyopathy called Keshan disease seems to be a direct consequence of selenium deficiency. Whereas the question of the mechanism by which selenium deficiency results in CHF remains open, recent data suggest that selenium may be involved in skeletal (and cardiac) muscle deconditioning (and in CHF symptoms such as fatigue and low exercise tolerance) rather than in left ventricular dysfunction.[58] Actually, in the Keshan area, the selenium status coincides with the clinical severity rather than with the degree of left ventricular dysfunction as assessed by echocardiographic studies. When the selenium levels of residents were raised to the typical levels in the non-endemic areas, the mortality rate declined significantly but clinically latent cases were still found and the echocardiographic prevalence of the disease

remained high.[70] What we learn from Keshan disease and other studies conducted elsewhere[58] is therefore that in patients with a known cause of CHF, even a mild deficiency in selenium may influence the clinical severity of the disease (tolerance to exercise).

These data should serve as a strong incentive for the initiation of studies testing the effects of natural antioxidants on the clinical severity of CHF. In the meantime, however, physicians would be well advised to measure selenium in patients with an exercise inability disproportionate to their cardiac dysfunction. Finally, low whole blood thiamine (vitamin B1) levels have been documented in patients with CHF on loop diuretics and hospitalised elderly patients, and thiamine supplementation induced a significant improvement in cardiac function and symptoms.[72]

3.3.2 Dietary fatty acids and sodium intake, cytokines, LVH and CHF

Beyond the well-known effect of high sodium intake in the clinical course of CHF (and the occurrence of acute episodes of decompensation), another important issue is the role of diet in the development of left ventricular hypertrophy (LVH), a major risk factor for CHF (and also SCD), as well as for cardiovascular and all-cause mortality and morbidity.[73,74]

The cause of LVH is largely unknown. Whereas male gender, obesity, heredity and insulin resistance may explain some of the variance in LVH, hypertension (High blood pressure, HBP) is generally regarded as the primary culprit.[75] Thus, the risks associated with LVH and HBP are intimately linked. Recent data have suggested that low dietary intake of polyunsaturated fatty acids and high intake of saturated fatty acids, as well as HBP and obesity, at age 50 predicted the prevalence of LVH 20 years later.[76] Although the source of saturated fatty acids is usually animal fat, the source of unsaturated fatty acids in that specific Scandinavian population and at that time was less clear and there was no adjustment for other potential dietary confounders, such as magnesium, potassium, calcium and sodium. Thus this study did not provide conclusive data on the dietary lipid determinants of LVH.[76] However, it does suggest that dietary fatty acids may be involved in the development of LVH and that this 'diet–heart connection' may partly explain the harmful effect of animal saturated fatty acids on the heart.

Another 'diet–heart connection' in the context of advanced CHF relates to the recent theory that CHF also is a low-grade chronic inflammatory disease with elevated circulating levels of cytokines and cytokine receptors that are otherwise independent predictors of mortality.[53,63] High-dose angiotensin-converting enzyme (ACE)-inhibition with enalapril, a treatment that reduces mechanical overload and shear stress (two stimuli for cytokine production in patients with CHF), was recently shown to decrease both cytokine bioactivity and left ventricular wall thickness.[77] Finally, various anti-cytokine and immuno-modulating agents were shown to have beneficial effects on heart function and clinical functional class in patients with advanced CHF[78] suggesting a

causal relationship between high cytokine production and CHF. This also suggests that there is a potential for therapies altering cytokine production in CHF. In that regard, it has been shown that dietary supplementation with n-3 fatty acids (either fish oil or vegetable oil rich in n-3 fatty acid) reduces cytokine production at least in healthy volunteers.[79,80] An inverse exponential relationship between leucocyte n-3 fatty acid content and cytokine production by these cells was found, most of the reduction in cytokine production being seen with eicosapentanoic acid in cell membrane lower than 1 per cent, a level obtained with rather moderate n-3 fatty acid supplementation.[80] However, further studies are warranted to test whether (and at which dosage) dietary n-3 fatty acids may influence the clinical course of CHF through an anti-cytokine effect.

Sodium intake is the environmental factor that is currently most suspected of influencing blood pressure and the prevalence of HBP. However, the full damaging potential of high sodium intake for the heart (and also the kidney) seems to be largely independent of the blood pressure effect of sodium. Animal experiments and clinical studies have consistently shown that high sodium intake is a powerful and independent determinant of LVH and that such an effect of salt that is not related to arterial pressure is not confined to the heart.[81,82] Whereas the long-term effect of a reduced sodium intake after a recent AMI is unknown, in particular on LVH, experts claim that even a 50 mmol reduction in the daily sodium intake would reduce the average systolic blood pressure by at least 5 mmHg (in patients aged over 50 years) and CHD mortality by about 16 per cent. Thus, as regards the damaging effect of high sodium intake on the heart, and despite the lack of strong data showing the beneficial effect of reducing sodium intake in that specific group of patients, we believe that cardiologists should extend their dietary counselling about sodium not only to the patients with HBP or CHF but also to all post-infarction patients.

3.4 Dietary strategies to prevent the development of heart disease

For several decades, the prevention of CHD (including the prevention of ischaemic recurrence after a prior AMI) has focused on the reduction of the traditional risk factors: smoking, HBP, hypercholesterolaemia. Priority was given to the prevention (or reversion) of vascular atherosclerotic stenosis. As discussed above, it has become clear in secondary prevention that clinical efficiency needs to primarily prevent the fatal complications of CHD such as SCD. This does not mean, however, that we should not try slowing down the atherosclerotic process, and in particular plaque inflammation and rupture. Indeed, it is critical to prevent the occurrence of new episodes of myocardial ischaemia whose repetition in a recently injured heart can precipitate SCD or CHF. Myocardial ischaemia is usually the consequence of coronary occlusion caused by plaque rupture and subsequent thrombotic obstruction of the artery.

Recent progress in the understanding of the cellular and biochemical pathogenesis of atherosclerosis suggests that, in addition of the traditional risk factors of CHD, there are other very important targets of therapy to prevent plaque inflammation and rupture. In this regard, the most important question is: how and why does plaque rupture occur?

3.4.1 CHD is an inflammatory disease

Most investigators agree that atherosclerosis is a chronic low-grade inflammation disease.[29] Pro-inflammatory factors (free radicals produced by cigarette smoking, hyperhomocysteinaemia, diabetes, peroxidised lipids, hypertension, elevated and modified blood lipids) contribute to injure the vascular endothelium, which results in alterations of its antiatherosclerotic and antithrombotic properties. This is thought to be a major step in the initiation and formation of arterial fibrostenotic lesions.[29] From a clinical point of view, however, an essential distinction should be made between unstable, lipid-rich and leucocyte-rich lesions and stable, acellular fibrotic lesions poor in lipids, as the propensity of these two types of lesion to rupture into the lumen of the artery, whatever the degree of stenosis and lumen obstruction, is totally different.

In 1987, we proposed that inflammation and leucocytes play a role in the onset of acute CHD events.[83] This has recently been confirmed.[32–35] It is now accepted that one of the main mechanisms underlying the sudden onset of acute CHD syndromes, including unstable angina, myocardial infarction and SCD, is the erosion or rupture of an atherosclerotic lesion,[32,33] which triggers thrombotic complications and considerably enhances the risk of malignant ventricular arrhythmias.[34,35] Leucocytes have been also implicated in the occurrence of ventricular arrhythmias in clinical and experimental settings,[84,85] and they contribute to myocardial damage during both ischaemia and reperfusion.[86] Clinical and pathological studies showed the importance of inflammatory cells and immune mediators in the occurrence of acute CHD events,[29,86] and prospective epidemiological studies showed a strong and consistent association between acute CHD and systemic inflammation markers.[88,89] A major question is to know why there are macrophages and activated lymphocytes[29] in atherosclerotic lesions and how they get there. Issues such as local inflammation, plaque rupture and attendant acute CHD complications follow.

3.4.2 The lipid oxidation theory of CHD

Steinberg *et al*. proposed in 1989 that oxidation of lipoproteins causes accelerated atherogenesis.[90] Elevated plasma levels of low-density lipoproteins (LDL) are a major factor of CHD, and reduction of blood LDL levels (for instance by drugs) results in less CHD. However, the mechanism(s) behind the effect of high LDL levels is not fully understood. The concept that LDL oxidation is a key characteristic of unstable lesions is supported by many reports.[29] Two processes have been proposed. First, when LDL particles become trapped in the artery wall,

they undergo progressive oxidation and are internalised by macrophages, leading to the formation of typical atherosclerotic foam cells. Oxidised LDL is chemotactic for other immune and inflammatory cells and up-regulates the expression of monocyte and endothelial cell genes involved in the inflammatory reaction.[29,89] The inflammatory response itself can have a profound effect on LDL,[29] creating a vicious circle of LDL oxidation, inflammation and further LDL oxidation. Second, oxidised LDL circulates in the plasma for a period sufficiently long to enter and accumulate in the arterial intima, suggesting that the entry of oxidised lipoproteins within the intima may be another mechanism of lesion inflammation, in particular in patients without hyperlipidaemia.[30,31,91] Elevated plasma levels of oxidised LDL are associated with CHD, and the plasma level of malondialdehyde-modified LDL is higher in patients with unstable CHD syndromes (usually associated with plaque rupture) than in patients with clinically stable CHD.[30] In the accelerated form of CHD typical of post-transplantation patients, higher levels of lipid peroxidation[92–94] and of oxidised LDL[95] were found as compared to the stable form of CHD in non-transplanted patients. Reactive oxygen metabolites and oxidants influence thrombus formation (see reference 95 for a review), and platelet reactivity is significantly higher in transplanted patients than in non-transplanted CHD patients.[97]

The oxidised LDL theory is not inconsistent with the well-established lipid-lowering treatment of CHD, as there is a positive correlation between plasma levels of LDL and markers of lipid peroxidation[93,98] and a low absolute LDL level results in reduced amounts of LDL available for oxidative modification. LDL levels can be lowered by drugs or by reducing saturated fats in the diet. Reduction of the oxidative susceptibility of LDL was reported when replacing dietary fat with carbohydrates. Pharmacological/quantitative (lowering of cholesterol) and nutritional/qualitative (high antioxidant intake) approaches of the prevention of CHD are not mutually exclusive but additive and complementary. An alternative way to reduce LDL concentrations is to replace saturated fats with polyunsaturated fats in the diet. However, diets high in polyunsaturated fatty acids increase the polyunsaturated fatty acid content of LDL particles and render them more susceptible to oxidation[28] which would argue against use of such diets (see above the section on SCD and n-6 PUFA). In the secondary prevention of CHD, such diets failed to improve the prognosis of the patients (for a review see de Lorgeril *et al*.[36]). In that context, the traditional Mediterranean diet, with low saturated fat and polyunsaturated fat intakes, appears to be the best option. Diets rich in oleic acid increase the resistance of LDL to oxidation independent of the content in antioxidants[99,100] and results in leucocyte inhibition.[101] Thus, oleic acid-rich diets decrease the pro-inflammatory properties of oxidised LDL. Constituents of olive oil other than oleic acid may also inhibit LDL oxidation.[102] Various components of the Mediterranean diet may also affect LDL oxidation. For instance, alpha-tocopherol or vitamin C, or a diet combining reduced fat, low-fat dairy products and a high intake of fruits and vegetables, was shown to favourably affect either LDL oxidation itself or/and the cellular consequences of LDL oxidation[86,103]

Finally, significant correlation was found between certain dietary fatty acids and the fatty acid composition of human atherosclerotic plaques,[104,105] which suggests that dietary fatty acids are rapidly incorporated into the plaques. This implies a direct influence of dietary fatty acids on plaque formation and the process of plaque rupture. It is conceivable that fatty acids that stimulate oxidation of LDL (n-6 fatty acids) induce plaque rupture, whereas those that inhibit LDL oxidation (oleic acid), inhibit leucocyte function (n-3 fatty acids)[106] or prevent 'endothelial activation' and the expression of pro-inflammatory proteins (oleic acid and n-3 fatty acids)[107,108] contribute to pacify and stabilise the dangerous lesions. In that regard, it is noteworthy that moderate alcohol consumption, a well-known cardioprotective factor, was recently shown to be associated with low blood levels of systemic markers of inflammation,[109] suggesting a new protective mechanism to explain the inverse relationship between alcohol and CHD rate. In the same line, the potential of dietary n-3 fatty acids to reduce the production of inflammatory cytokines[79,80] by leucocytes (as discussed in section 3.2 on dietary fatty acids and CHF) should be underlined. As both dietary n-3 fatty acids and moderate alcohol consumption are major characteristics of the Mediterranean diet, it is not surprising to observe that this diet was associated with lower rate of new episodes of CHF in the Lyon Diet Heart Study.[60]

Thus, any dietary pattern combining a high intake of natural antioxidants, a low intake of saturated fatty acids, a high intake of oleic acid, a low intake of omega-6 fatty acids and a high intake of omega-3 fatty acids would logically produce a highly cardioprotective effect. This is consistent with what we know about *the Mediterranean diet* pattern[38,39] and with the results of the Lyon Diet Heart Study,[19,60,110] and was recently confirmed by epidermiological studies.[111,112]

3.5 Dietary prevention of post-angioplasty restenosis

Patients treated with percutaneous transluminal coronary angioplasty (PTCA) have a high (15 to 50 per cent, depending on studies) risk of developing restenosis within the first 6 months after the procedure. At present, with the exception of stents coated with antifibrotic substances[113] and probucol (the later with many unacceptable side effects), there is no drug treatment to prevent that complication. On the other hand, a dietary approach with either n-3 fatty acid or folate supplementation has been proposed.

Several small studies have indeed suggested that supplementation with n-3 fatty acids may inhibit restenosis. However, recent larger trials set up to prove that effect failed to do so.[114–116] In the Coronary Angioplasty Restenosis Trial, in particular, 500 patients were randomly allocated to 5 g per day of a fish oil concentrate or placebo (corn oil) from at least 2 weeks before until 2 months

after PTCA.[116] Compliance was documented by measuring the content of n-3 fatty acids in the blood, and patients receiving n-3 fatty acids had a significant reduction in serum triglyceride levels. However, no effect could be demonstrated on the restenosis rate or coronary atherosclerosis assessed by quantitative coronary angiography after 6 months of treatment.

It is noteworthy that in these negative trials, patients were treated with quite high doses of n-3 fatty acids, up to 8 g per day,[115] and that no previous data did support the use of such doses in the prevention of CHD. In addition, these studies were all performed in patients having had conventional balloon PTCA, and there are no data on patients receiving any type of stent. One major limitation of the dietary approach of the prevention of restenosis is the theoretic requirement to start supplementation at least a few days before PTCA, while many PTCAs are now performed in emergency or during the sub-acute phase of AMI, with no time for a pre-stenting supplementation.

Finally, recent data suggest that high homocysteine levels could be associated with restenosis and accelerated atherosclerosis[117,118] and a combination of folic acid, vitamin B12 and pyridoxine (known to decrease homocysteine levels) was shown to reduce the rate of restenosis in a double-blind randomised trial.[118] In that trial, the need for revascularisation was also significantly lower in patients receiving the treatment (10.8 per cent *vs.* 22.3 per cent). This underlines the potential clinical benefit of the dietary approach of the prevention of restenosis and should be compared with the data of the Lyon Diet Heart Study in which the patients randomised in the experimental group and following a Mediterranean diet, which is typically a folate-rich diet, also had a lower rate of restenosis after PTCA.[60] Further studies are however needed to clarify which component of the vitamin cocktail of the Swiss trial[118] was preventive and which dosage would be the most appropriate. The potential impact of dietary folates in the various clinical manifestations of CHD is also discussed in section 3.6.3 on 'endothelial dysfunction'.

3.6 Dietary control of conventional risk factors: cholesterol, blood pressure, type 2 diabetes and obesity

3.6.1 Diet and blood cholesterol

Cholesterol is a determinant of CHD mortality, and its blood level is at least partly regulated by diet. However, few epidemiological studies have prospectively included analyses of the dietary habits of the studied populations in the evaluation of their risk.[119] In the Seven Countries Study, marked differences in CHD mortality, dietary habits and cholesterol distribution were observed in the different cohorts.[119] Cholesterol levels were high in Northern Europe and in the USA (an average level of 7 mmol/L), and low in rural Japan (an average of 4 mmol/L), and population cholesterol levels were positively associated with CHD mortality. Secondary prevention trials with statins in Northern Europe[120] and Australia[121] confirmed the importance of cholesterol by demonstrating a

reduction by 25–30 per cent of the relative risk of CHD death in patients taking these drugs. Whether the effect of statins was entirely related to their effect on cholesterol remains unknown.

A major (and often underestimated) finding of the Seven Countries Study was the large difference in absolute risk of CHD death at the same level of serum cholesterol in the different cohorts. At a cholesterol level of about 6 mmol/L, CHD mortality was three times as high in Northern Europe as in Mediterranean Europe (18 per cent *vs.* 6 per cent). This suggested that factors other than cholesterol were playing an important role. Because of the similarity of the other traditional risk factors and the large differences in the dietary habits of the cohorts,[122] it was proposed that the difference in CHD mortality between populations was mainly related to their dietary habits, through biological effects independent of cholesterol.[123]

This was the basis of a new 'diet–heart hypothesis' in which cholesterol was not the central issue.[36,123] In fact, the first dietary trials designed for the secondary prevention of CHD were based on the hypothesis that a cardioprotective diet should primarily reduce cholesterol.[36] While the investigators succeeded in reducing cholesterol, they failed to reduce CHD mortality.[41] This was mainly attributed to an insufficient effect of the tested diets on cholesterol, and the conclusion was that cholesterol-lowering drugs should be preferred. However, none of the diets tested in these old trials was patterned from the traditional diets of populations protected from CHD (e.g. vegetarian, Asian or Mediterranean), although these diets are associated with low cholesterol.[119,122] Also, no trial was aimed at testing the cholesterol-lowering effect of a typical Mediterranean diet, probably because this diet was (and often still is) mistakenly regarded as a high-fat diet, allegedly not appropriate to reduce cholesterol. Studies investigated the effect of the main lipid-related features of Mediterranean diets, for instance diets low in saturated and polyunsaturated fat but relatively high in monounsaturated fat.[123–125] Certain aspects of the Mediterranean diet not related to lipids (for instance, the amount of fibre) were not investigated, although they influence lipid metabolism. The consensus now is that a diet low in saturated and poly-unsaturated fat but rich in oleic acid results in a significant reduction of total and LDL cholesterol, and also has an effect on triglycerides and a small positive or no effect on HDL cholesterol.[123–125] It is not certain whether these results can be completely reproduced in patients with established CHD, as none of these studies were conducted in such patients. Finally, as discussed in the different sections of this text, the Mediterranean diet was shown to strongly reduce the risk of CHD complications in secondary prevention[19,60] and should be one of the preferred dietary patterns adopted by post-infarct patients. In the Lyon trial, the lipid-lowering effect of the Mediterranean diet was not different from that of the prudent Western diet followed by the control group, because lipid-lowering drugs were widely used in the two randomised groups. This nonetheless suggested that the Mediterranean diet was cardioprotective through biological effects independent of its effect on cholesterol. In particular, data from the Lyon

trial suggested that the Mediterranean diet might prevent SCD (see above, section 3.2).

3.6.2 Diet and blood pressure

Blood pressure is also related to CHD mortality, and HBP is a common problem in many Western countries. In fact, the relationship between blood pressure and CHD is continuous and there is no abrupt increase in risk at levels of blood pressure regarded as high.[126] This suggests that efforts to prevent blood pressure-related diseases should be focused both on hypertensive and on nonhypertensive persons. In secondary prevention, most patients are taking some blood pressure-lowering drugs (often beta-blockers) as systematic post-infarct treatment. In addition, traditional approaches to control the epidemic of blood pressure-related CHD have largely concentrated on drug therapy in persons with hypertension. However, because of the many side effects, the rate of discontinuation is high with these classes of drugs.[127] Clearly, a non-drug therapy, including lifestyle modifications, may have an important and expanding role as a complement of drug therapy, especially in the long term. Another point is the importance of even small differences in blood pressure in terms of outcome. For instance, a 5mmHg reduction in diastolic blood pressure has been shown to result in a 35–40 per cent lower risk of stroke.[128] Thus, a significant clinical benefit can be expected even from a small decrease in blood pressure resulting from a dietary change.

As regards the influence of diet on blood pressure-related CHD complications, data from the Seven Countries Study again provide major information.[129] CHD mortality varies greatly among populations at each level of systolic or diastolic blood pressure in that study.[129] At a diastolic blood pressure level of 90 mm Hg, CHD mortality was three times as low among Mediterraneans as among the populations from the USA and Northern Europe. On the same reasoning as for cholesterol, it is presumed that the protective factor is the diet of Mediterraneans. The same reasoning probably applies for the Asian (Japanese) diet. Another question is whether dietary factors influence blood pressure. High sodium intake and binge alcohol drinking certainly increase blood pressure.[130] Whether the Mediterranean diet pattern may specifically influence (decrease?) blood pressure is unknown, although this dietary pattern has been reported to protect the arterial endothelium that is responsible for the production and release of several major vasodilators, including nitric oxide.[131] In the (Mediterranean diet) Lyon trial, the extensive use of blood pressure-lowering drugs in both groups prevented any effect on blood pressure from becoming apparent. However, recent research has emphasised the powerful role of total diet in hypertension.[130] An adequate intake of minerals (sodium, potassium, magnesium and calcium), rather than the sole restriction of sodium, was proposed as the focus of dietary recommendations.[132] In this approach, however, the direct role of high sodium intake on the myocardium is not fully taken into account (see above the section about CHF and LVH). Other studies

suggested that dietary n-3 fatty acids may lower blood pressure in subjects with hypertension.[133] The responses were proportional to the changes in phospholipid n-3 fatty acids whereas n-6 fatty acids had no effect, which suggests that the effect did result specifically from the n-3 family. These data implied that in addition to their benefits through mechanisms such as the prevention of ventricular arrhythmias (see above, section 3.2), n-3 fatty acids may be helpful in modulating (endothelial) factors regulating blood pressure.

Another trial, the Dietary Approaches to Stop Hypertension (DASH), tested the effect on blood pressure of either a diet rich in fruits and vegetables or a 'combination' diet rich in fruits, vegetables and low-fat dairy products, and with reduced saturated and total fat.[134] Although this 'combination' diet was not a typical Mediterranean diet, its main characteristics can be included among those recommended in a Mediterranean diet trial. In the first DASH trial, sodium intake was kept constant and the 'combination' diet decreased systolic blood pressure by 5–6 mm Hg in subjects with normal blood pressure; in those with mild hypertension, the blood pressure reduction was twice as great – about 12 mm Hg. Reductions of this magnitude are similar to those observed with antihypertensive medications, but they are obtained at a very much lower cost, particularly in terms of side effects. In fact, the first DASH trial confirmed the meta-analyses as well as earlier indications from observational studies, suggesting that dietary factors other than sodium markedly affect blood pressure.[132] In a second trial, the DASH investigators studied the effects of different levels of dietary sodium in conjunction with the DASH diet.[135] As before, the DASH diet substantially lowered systolic and diastolic blood pressure. In addition, at any level of sodium intake, blood pressure was lower among patients following the DASH diet than among those following the control diet.[135] Thus, combining a reduction of sodium intake to levels below 100 mmol per day and the DASH diet lowers blood pressure to a greater extent than either of the two separately. Whether these dietary changes may reduce the risk of CHD remains to be demonstrated.

3.6.3 Diet and 'endothelial dysfunction'

One mechanism that may contribute to the association between high blood pressure and CHD is called 'endothelial dysfunction'. The endothelium, the innermost layer of all blood vessels, is critical in determining the contractile state of the underlying smooth muscle.[136] Through the release of a number of substances, the endothelium modulates several other functions, including platelet aggregation, leucocyte adhesion and migration, smooth muscle cell proliferation and lipid oxidation, all of which participate in the atherosclerotic process. The term 'endothelial dysfunction' has been used to describe a constellation of abnormalities in these regulatory actions of the endothelium, and 'endothelial dysfunction' has been reported in conditions such as hypercholesterolaemia, HBP, diabetes and hyperhomocysteinaemia. For instance, in patients with HBP, there is an imbalance in the bioactivity of

endothelial factors with proatherosclerotic (endothelin-1) and antiatherosclerotic (nitric oxide) actions[137] that may explain why HBP is a risk factor for CHD, regardless of whether endothelial dysfunction is a cause or a consequence of HBP. Coronary endothelial dysfunction by itself was indeed shown to be of prognostic significance in patients with CHD.[138] Endothelium-derived nitric oxide (NO) plays an important role in the regulation of tissue perfusion, and evidence is accumulating that NO-dependent vasodilatation and NO availability are impaired in the coronary arteries of patients with CHD or with CHD risk factors such as high blood cholesterol or homocysteine levels.

Interestingly, folic acid therapy, either as chronic oral supplementation or as acute intra-arterial administration of the active form of folic acid (5-methyltetrahydrofolate), restores the impaired endothelial function even in patients with CHD risk factors but normal serum folic acid and homocysteine levels.[139,140] Also, a deficient NO-synthase cofactor, tetrahydrobiopterin (BH4), may be involved in blunted endothelium-dependent vasodilatation in humans.[141] In case of BH4 deficiency, uncoupling of the L-arginine-NO pathway is observed, resulting in increased formation of oxygen radicals. Intra-arterial or intra-coronary infusion of BH4 was shown to improve endothelial dysfunction in patients with various clinical manifestations.[141,142] The key point here is that the active form of folic acid is involved in the endogenous regeneration of BH4, suggesting a major interaction between the arginine-NO-synthase pathway and folic acid. Thus an adequate amount of folates in the diet appears to be crucial to protect the endothelium (and for the prevention of 'endothelial dysfunction') and, in general, for the prevention and treatment of CHD far beyond a simple effect on homocysteine (see above, section 3.5).

Another cause of endothelial dysfunction in the context of traditional risk factors of CHD (including HBP, dyslipidaemias and insulin resistance) is an elevated level of ADMA (asymmetric dimethyl arginine), which also inhibits the production of NO.[143] Whereas blood ADMA levels seem to be influenced by dietary fats, further studies are required to clarify the relations between the dietary factors (folates, antioxidants) involved in the regulation of NO-synthase and ADMA metabolism.

A diet rich in vegetables and fruits and the traditional Mediterranean diet provide large amounts of folates. Consumption of legumes (drier beans and peas, fresh peas, peanuts, peanut butter, lentils), which is another typical Mediterranean habit, is a major source of folates and has been shown to be associated with a reduced risk of CHD.[144] Also, tree nuts, such as walnut and hazelnut (common ingredients of vegetarian and Mediterranean diets), can help supply folates. Most nuts are also rich in arginine, an amino acid serving as a substrate for the synthesis of NO.

Because of the importance of NO in cardiovascular diseases, there has been growing interest over the past 10 years in using arginine to prevent and treat cardiovascular diseases.[145] Compelling evidence shows that enteral or parenteral administration of arginine reverses endothelial dysfunction associated with major CHD risk factors in a way very similar to that observed with folic acid and

BH4. Endothelial arginine is derived from the plasma, via intracellular synthesis with citrulline as a precursor, and from the net degradation of intracellular proteins. However, food is the ultimate source of arginine for the body. Dietary arginine intake (the main source being animal products for the Western population) by an adult has been estimated to be around 5 g/day.[145] Because of arginase activity in the intestine and of the limited digestibility of protein-bound arginine, it is assumed that only 50 per cent of dietary arginine enters systemic circulation. The adequate daily arginine requirement is difficult to assess and probably varies in accordance with the presence or absence of CHD risk factors, which are often associated with the presence of endogenous NO-synthase inhibitors. It probably also varies with the amount of folates in the diet, since the active form of folic acid is essential for the regeneration of BH4.

The amount of arginine found in the typical Western diet appears at best barely sufficient to cover the daily requirements of a healthy individual. Furthermore, in the presence of CHD risk factors or established CHD, the intake of arginine would tend to decrease as patients turn away from animal foods which, though protein-rich, are considered 'unhealthy for the heart' in other respects. The exact requirements of these patients have yet to be determined but previous studies suggested that in order to reverse endothelial dysfunction, an amount in the order of 6–9 g above dietary supplies might be necessary. Other factors, such as impaired intestinal absorption, competition with other amino acids (in particular lysine) for cell transport, and the amount of folates in the diet, were not taken into account in these calculations. Whatever the clinical condition, nuts are a convenient natural source of arginine, not only because of the high concentration of arginine in most nuts but also because it is associated with high levels of folates and vitamin B6, the major cofactors involved in the catabolism or recycling of homocysteine. In a certain sense, we can say that nuts are 'natural' functional foods.

3.6.4 Diet and type 2 diabetes

People with type 1 diabetes are rather rare among the patients concerned by the secondary prevention of CHD. Apart from the dietary prevention recommended for non-diabetic patients, there are no specific dietary issues beyond the usual diabetic diet to control blood glucose once the insulin treatment is correctly administered. In contrast, the number of people with type 2 diabetes or insulin resistance is increasing rapidly and cardiologists have to manage that specific problem in more and more post-infarct patients.

Type 2 diabetes mellitus is associated with a three- to fourfold increase in the incidence of CHD,[146] and the risk of CHD death is as high in people with diabetes without CHD as in those without diabetes and with established CHD.[147] The decline in CHD mortality in most Western populations has been mainly attributed to reduction of risk factors, owing to dietary changes in particular. The smaller decline in CHD mortality among people with diabetes, particularly women, may be due to less effective changes in risk factors for those people.

Apart from calorie restriction, the composition of the diet of people with type 2 diabetes remains controversial. The emphasis currently is on a diet low in saturated fatty acids. A reduction of the total fat intake is also suggested when weight loss is a primary issue. Thus, as most type 2 diabetics should lose weight, a low-fat diet is commonly prescribed. However, many physicians are still reluctant to recommend such a diet because they think that a diet high in monounsaturated fats improves metabolic control in these patients better than a low-fat, high carbohydrate diet, and should be preferred.[148] On the basis of a meta-analysis, it is clear that diets high in monounsaturated fat improve the lipoprotein and blood glucose profiles and also lower blood pressure.[148] This type of diet may also reduce the susceptibility of LDL particles to oxidation and thereby reduce their atherogenic potential; in addition, it does not induce weight gain, provided that energy intake is controlled.

Thus, in theory, diets low in saturated fatty acids but rich in monounsaturated fats (two of the main characteristics of *the Mediterranean diet* are advantageous for the prevention of CHD in people with diabetes. Curiously, no diabetic diet has ever been tested in this way for the prevention of CHD in diabetic patients.

An important message from the UK Prospective Diabetes Study and other recent trials is that in the prevention of CHD in people with type 2 diabetes, it is unwise to focus on single risk factors.[149] All known risk factors should be tackled simultaneously, including hyperlipaemia and hypertension. Also, because of a high risk of SCD in people with diabetes, specific recommendations aimed at preventing SCD should be given (see above). Classical risk factors fail to explain the excess CHD rate in Indians as compared with Europeans, although the high prevalence of diabetes in India may play a part.[150] When exploring the contribution of dietary fatty acids in Indian people with diabetes, large differences in phospholipids fatty acids were noted, with lower concentrations of n-3 fatty acids[151] suggesting an explanation for their high CHD mortality.

Considering all of these observations, it seems that the *optimal diabetic diet may be a low-calorie Mediterranean diet*. Not only does this diet protect the heart, improve lipid profiles and reduce blood pressure, but certain components (n-3 fatty acids in association with vegetables and legumes) have also been shown to improve glucose tolerance and prevent the apparition of overt diabetes.[152] These human data confirm animal research that showed the importance of n-3 fatty acids in the action of insulin in various experimental models.[153] Thus, although further studies are required, in particular about the physical structure of foods to modulate glucose metabolism and insulin resistance,[154] it is clear that people with diabetes should be instructed in the basic principles of the Mediterranean diet.

3.6.5 Diet, overweight and obesity

Another question is why has the incidence of type 2 diabetes increased so rapidly? Considerable epidemiological evidence points to excess caloric intake and physical inactivity as the major reasons. There is no room here to discuss the problem of obesity and overweight in people with established CHD. Obesity and overweight are obviously associated with a clustering of CHD risk factors and weight reduction results in favourable changes in the risk factor profile of most individuals. However, it is also obvious that weight reduction efforts have met with limited success in the general population and that the treatment of obesity is complex and difficult. There is no reason (and no published data) to believe that the situation is different among patients with CHD. In addition, there are controversies regarding the efficacy, benefits and consequences of high-carbohydrate or low-fat or very low-fat or high-protein diets, all of which have been proposed as the best way to reduce weight. Thus, despite the fact that having (or maintaining, or reaching) a healthy body weight has been claimed a major goal of the dietary prevention of CHD in primary prevention,[155] we do not believe that this is necessarily true in secondary prevention as well. As discussed in the previous sections of this text (and summarised in the last section), there are other dietary priorities for which we have scientific evidence showing that people with CHD get immediate and major clinical benefits by adhering to them. Of course, if someone with CHD is obese and/or asks spontaneously for a slimming diet, that issue should be appropriately addressed. In line with the principle of 'clinical rather than surrogate efficacy', there is little scientific data on the topic 'slimming diet in CHD patients'. Indeed, no dietary (or pharmacological) trials have been conducted so far to test whether weight loss is associated with improved prognosis in patients with CHD. Finally, whether a high-protein or high-carbohydrate diet or any other diet, in addition to a low energy diet, is preferable is an open question.

3.7 Conclusion: using the 'Mediterranean diet' to prevent coronary heart disease

Despite the increased evidence that dietary prevention is critical in the post-AMI patient, many physicians (and their patients) remain rather poorly informed about the potential of diet to reduce cardiac mortality, the risk of new CHD complications and the need for recurrent hospitalisation and investigation. There are many reasons for that, the main one probably being an insufficient knowledge of nutrition.[156] For that reason (and knowing the resistance of many physicians to accept the idea that diet is important in CHD), we propose **a minimum dietary programme** that every CHD patient, whatever his or her medical and familial environment, should know and follow. This minimum 'Mediterranean' dietary programme has been recently described,[157] and should include the following:

- Reduced consumption of animal saturated fat (for instance, by totally excluding butter and cream from the daily diet and drastic reduction of fatty meat) and increased consumption of n-3 fatty acids through increased intakes of fatty fish (about 200 g, twice a week). For patients who cannot eat fish (for any reason), taking capsules of n-3 fatty acids (for instance, a mix of alpha-linolenic acid and long-chain n-3 fatty acids) is the best alternative. Very importantly, the patients (and their physicians) should be aware that n-3 fatty acid supplementation will be even more cardioprotective if associated with adequate dietary modifications discussed in the text above.[158]
- Increased intake of anti-inflammatory fatty acids (oleic acid and n-3 fatty acids) and decreased intake of pro-inflammatory fatty acids (n-6 fatty acids). The best way is to exclusively use olive oil and canola oil for cooking and salad dressing and canola oil-based margarine instead of butter and polyunsaturated oils and margarines.[159–161] Patients should also systematically reject convenience food prepared with fats rich in saturated, polyunsaturated and *trans* fatty acids.
- Increased intake of natural antioxidants (vitamins and trace-elements) and folates through increased consumption of fresh fruits and vegetables and tree nuts.[162]
- Moderate intake of alcoholic beverages (one or two drinks per day), preferably wine, preferably during the evening meal, and never before driving or making a dangerous technical manipulation.
- Reduction of sodium intake (below 100 mmol per day if possible). This is a very difficult task at the present time because of the high sodium content of many natural (including typical Mediterranean foods such as olives and cheeses) and convenience foods.

However, patients and (physicians) should keep in mind that an optimal (and individual) dietary prevention programme should be managed under the guidance of a professional dietician aware of the most recent scientific advances in the field.

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4

The role of fat-soluble nutrients and antioxidants in preventing heart disease

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4.1 Introduction: oxidative stress and cardiovascular disease

This chapter will focus on the potential roles of fat-soluble nutrients and fat-soluble antioxidants in preventing cardiovascular disease (CVD). Two fat-soluble vitamins will be discussed in detail, i.e. vitamin E and vitamin D. Vitamin E (tocopherols and tocotrienols) is generally considered an antioxidant nutrient, although it may have important functions unrelated to its antioxidant functions (as discussed below). Antioxidant nutrients function by preventing damage to biological systems caused by reactive oxygen species (ROS) and/or reactive nitrogen oxide species (RNOS). Vitamin D (calciferols) is not a true vitamin since it is not required in our diet, can be produced in skin tissue, and is generally not present in plants. Vitamin D is, perhaps, best described as a steroid hormone precursor. Although vitamin D may function as a membrane antioxidant under *in vitro* conditions (Wiseman, 1993), its primary biological role is to maintain plasma calcium and phosphorus homeostasis.

The additional fat-soluble antioxidant nutrient reviewed here will be coenzyme Q10 (ubiquinone or CoQ10), which has strong antioxidant properties. Vitamin E and CoQ10 can protect lipid–protein complexes, such as biological membranes and lipoproteins, from lipid peroxidation. During lipid peroxidation, highly reactive lipid hydroperoxides, peroxyl radicals and reactive aldehydes, such as malondialdehyde (MDA) and 4-hydroxynonenol (4-HNE), are generated. The peroxyl radicals support chain reactions that can rapidly damage oils, biological membranes or lipoproteins containing polyunsaturated fatty acids (PUFA).

The literature reviewed below strongly suggests that oxidative stress plays a key role in the etiology of cardiovascular disease. Oxidative stress is a physiological condition in which pro-oxidant factors outweigh antioxidant defences. Accordingly, the role of oxidative stress in promoting cardiovascular disease and the roles of fat-soluble antioxidant nutrients in potentially protecting from this disease process will be discussed in some detail. Oxidative stress is likely to occur during inflammatory processes, during exercise and from cigarette smoking. The evidence presented below also suggests that vitamin D plays an important and significant role in preventing cardiovascular disease but it is very unlikely that this effect is related to its potential role as an antioxidant.

4.1.1 The impact of cardiovascular disease

Owing to the enormous worldwide impact of cardiovascular disease it must be emphasized that even very modest reductions in risk factors, brought about by the appropriate design and use of functional foods, can have very important health related and economic significance. Statistics from the American Heart Association (see http://www.americanheart.org /statistics/03cardio.html) indicate the enormous impact of CVD. Over 61 million Americans have one or more types of CVD. CVD causes more mortality each year than the next seven leading causes of death combined and the estimated cost of cardiovascular diseases and stroke in the United States in 2003 was $352 billion. In developed countries, childhood obesity has reached epidemic proportions and this will certainly translate into a dramatic increase in type 2 diabetes which is characterized by elevated levels of triglycerides, LDL-C (low-density lipoprotein–cholesterol) and decreased levels of HDL-C (high-density lipoprotein–cholesterol), i.e. a shift towards a highly atherogenic lipid profile. Moreover, the World Health Organization (see http://www.who.int/ncd/cvd) makes a very convincing argument that CVD impact is not just limited to Westernized countries but will reach epidemic proportions in developing countries as well because of demographic and lifestyle changes. It has been estimated that by the year 2020, CVD will be the number one cause of deaths in the world.

4.1.2 Antioxidants, cardiovascular disease, and oxidative modifications of low-density lipoprotein

Both lipid-soluble and water-soluble antioxidants present in blood may be important in preventing cardiovascular disease owing to their ability to prevent the oxidation of lipid–protein complexes called lipoproteins. Lipoproteins are extremely important in cardiovascular disease since we know with certainty that high levels of LDL-C cause atherosclerosis, which is the underlying cause of most cardiovascular disease. In contrast, high levels of HDL-C are a negative risk factor for CVD. Atherosclerosis is the gradual build-up of ‘plaque’ in the arterial wall. LDL-C is the major source of the lipids occurring in these plaques.

There is now considerable evidence that LDL lipids (primarily cholesteryl esters) make their way into plaques by cells in the arterial wall called macrophages. These macrophages take up so much LDL that they become 'foamy' in appearance and are, therefore, called 'foam cells.' This is the very first step (called fatty streak formation) in atherosclerosis and this process begins in childhood. It is surprising, however, that LDL incubated with macrophages does not transform into foam cells. After LDL is oxidized (oxLDL) it will, however, cause macrophages to transform into foam cells. Macrophages have receptors for native LDL but the expression of these receptors is down-regulated by the accumulation of intracellular cholesterol. Unlike native LDL, chemically modified forms of LDL can be taken up by scavenger receptors whose expression is not down-regulated by the accumulation of intracellular cholesterol.

LDL is the primary plasma carrier for both vitamin E and CoQ10, both of which act as antioxidants in LDL by inhibiting lipid peroxidation of lipids containing polyunsaturated fatty acid moieties. Work by Jessup *et al*. (1990) indicates that most of the endogenous vitamin E in LDL must be oxidized before it is converted into a 'high uptake' form of oxLDL capable of transforming macrophages into foam cells. Since antioxidants, such as vitamin E, prevent the oxidation of LDL (Jessup *et al*., 1990) it is logical to suggest that antioxidants could prevent foam cell formation and thereby retard the process of atherosclerosis. This suggestion is called the 'oxidative modification hypothesis.' Although most *in vitro* experiments support this view, not all evidence is supportive (Asmis and Jelk, 2000).

Whether or not oxLDL formation occurs *in vivo* and what the mechanism (s) might be for this oxidation are still open issues (Chisolm and Steinberg, 2000). Despite intensive efforts, there is little evidence for the existence of oxLDL in fresh human plasma. This has led to the hypothesis that LDL could be oxidized in the subendothelial space of arteries rather than in plasma. It is significant, therefore, that LDL isolated from human aortic atherosclerotic intima has extremely high levels of 3-nitrotyrosine (Leeuwenburgh *et al*., 1997). Although the origin of this 3-nitrotyrosine is not clear, it is probably due to the reaction of peroxynitrite ($ONOO^-$) with tyrosine residues in apoB100 (the primary protein component of LDL). The addition of $ONOO^-$ to LDL or bovine serum albumin *in vitro* certainly gives rise to 3-nitrotyrosine. Furthermore, LDL-treated $ONOO^-$ undergoes lipid peroxidation accompanied by the oxidation of alpha-tocopherol to alpha-tocopheryl quinone and is converted to a form recognized by macrophage scavenger receptors (Graham *et al*., 1993; Hogg *et al*., 1993).

If oxLDL were the source of lipids in atherosclerotic plaques one might expect that these lipids would have a very low content of vitamin E. Paradoxically, Suarna *et al*. (1995) have found that human atherosclerotic plaques contain relatively large amounts of alpha-tocopherol and ascorbate (a water-soluble antioxidant). Foam cells are not likely, therefore, to be formed by the uptake of large amounts of oxLDL with very low levels of endogenous tocopherol. Nevertheless, Suarna *et al*. (1995) also found that plaque contains

large amounts of oxidized lipids and a significant level of alpha-tocopheryl quinone, an oxidation product of alpha-tocopherol. These data support the view that oxidative stress is an important factor in atherosclerosis but indicate that oxidized lipids in atherosclerotic plaques may not be derived from oxLDL.

4.1.3 Alternative mechanisms for foam cell formation

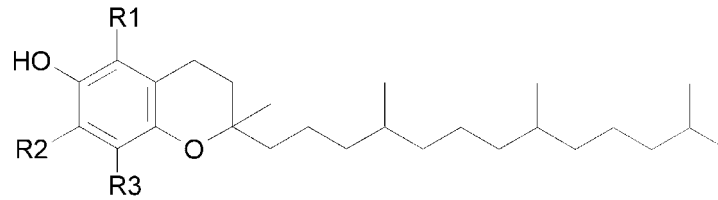
An alternative mechanism for LDL macrophage uptake (and foam cell formation) that does not require prior formation of oxLDL is provided by mast cells. Mast cell degranulation produces neutral proteases, such as chymase, and granules. The released granules bind LDL and this LDL is also degraded and fused by the released proteases. *In vivo* evidence suggests that these non-oxidative modifications of LDL promote its phagocytosis by macrophages leading to foam cell formation in the human arterial intima (Kovanen, 1996). Although antioxidants certainly inhibit oxidative modifications to LDL they may also prevent atherosclerosis by inhibiting mast cell degranulation. It is known that mast cell degranulation and histamine release is stimulated by membrane lipid peroxidation and inhibited by antioxidants such as alpha-tocopherol (Masini *et al*., 1990). Mannaioni and Masini (1988) have provided an excellent review of the evidence linking histamine release with the generation of free radicals. Histamine, in turn, has many potential roles in atherogenesis. For example, histamine has been found to be an activator of human vascular smooth muscle growth by transcriptional stimulation of the *c-fos* proto-oncogene (Satoh *et al*., 1994). Mast cell derived TNF-alpha and TGF-beta 1 may also contribute to the proliferation of smooth muscle cells observed in atherosclerotic plaques.

4.2 The functional properties of vitamin E in preventing heart disease

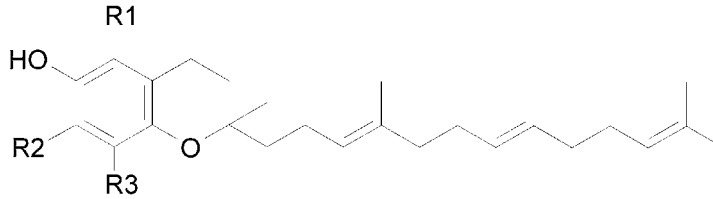
Vitamin E is the main lipid-soluble antioxidant in the body and it must be obtained from the diet since it cannot be synthesized. *In vivo* research has clearly shown that vitamin E prevents lipid peroxidation in humans. Lipid peroxidation is generally viewed as a damaging process that contributes to many chronic diseases as well as to the aging process. When evaluating the scientific literature it is critical to know what form of vitamin E is being used in a study.

4.2.1 Vitamin E is more than one compound

Vitamin E is not a single compound but refers to at least four tocopherols and four tocotrienols (see Fig. 4.1). Dietary vitamin E is primarily gamma-tocopherol (see Fig. 4.2) whereas the vitamin E in supplements is predominantly alpha-tocopherol (natural or synthetic). Alpha-tocopherol has the highest biological activity in protecting from fetal resorption in pregnant rats. Synthetic vitamin E is the most common form found in supplements and the most common



Tocopherol



Tocotrienol

Tocopherols and Tocotrienols

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

Fig. 4.1 Vitamin E is at least eight compounds.

form used in research. Synthetic vitamin E is usually refers to all-racemic alpha-tocopheryl acetate. The 'all-racemic' part of this name refers to the fact that three particular carbons atoms can be in either the *R*- or the *S*- confirmation (these three carbons are shown in Fig. 4.2 by the dotted lines and the solid bars). In natural vitamin E, all three of these carbons are in the *R*-confirmation. The 'alpha-' part of the name refers to the number (and position) of methyl groups on the vitamin E ring structure (these are the 'R' groups in Fig. 4.1). The 'acetate' part of the name refers to the fact that the active part of the vitamin E molecule (which is the phenolic –OH shown in Fig. 4.1) is esterified to an acetic acid molecule. This is done to prevent the vitamin E from oxidizing during storage. Vitamin E will, however, remain inactive until this acetate group is removed in the intestine.

4.2.2 The role of vitamin E in preventing cardiovascular disease: animal models

The role of vitamin E in preventing CVD is not yet fully defined (Meydani, 2000). Although many clinical trials do not support a protective role for vitamin E (as detailed below), these trials often have major design flaws (as discussed

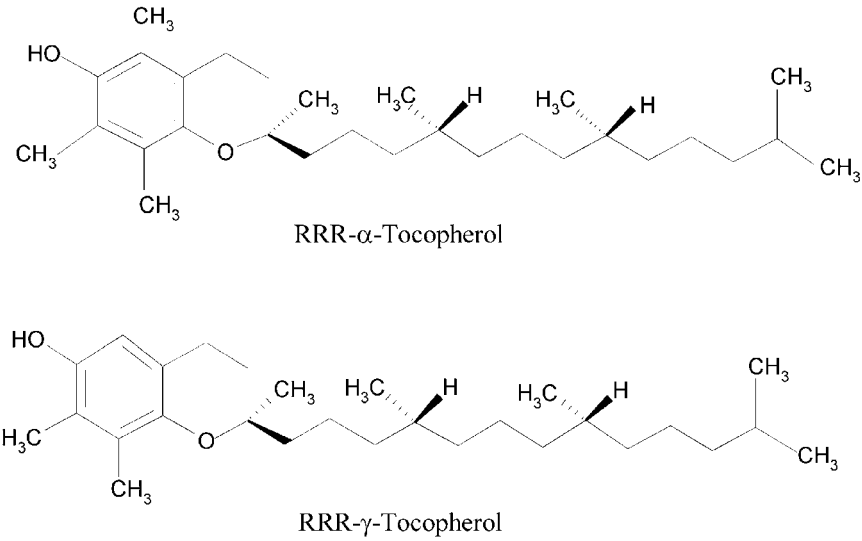


Fig. 4.2 Tocopherols.

below). Recent clinical trials have found that vitamin E supplementation reduces levels of C-reactive protein (CRP), which is a very powerful marker and predictor of CVD. Furthermore, new data suggest that CRP may directly promote atherosclerosis by causing the expression of adhesion molecules on the surface of arteries. The expression of these adhesion molecules is known to be involved in the inflammatory process that leads to CVD.

Excellent animal models are now available for studying the role of functional foods, specific nutrients, or pharmaceutical agents on the process of atherosclerosis. Most animal experiments support a role for vitamin E in preventing the oxidation of LDL and in slowing the development or progression of atherosclerosis (Steinberg, 2000; Chisolm and Steinberg, 2000). Very recent animal data show, however, a tendency to not be as supportive as earlier experiments. For example, in rabbit (Tijburg *et al*., 1997; Djahansouzi *et al*., 2001), hamster (El-Swefy *et al*., 2000) and mouse (Paul *et al*., 2001; Munday *et al*., 1998; Shaish *et al*., 1999) models, vitamin E did not prevent atherosclerosis or fatty streak formation (Tijburg *et al*., 1997; Djahansouzi *et al*., 2001).

A very interesting paper by Terasawa *et al*. (Terasawa *et al*., 2000) recently showed that disruption of the alpha-tocopherol transfer protein increased the severity of atherosclerosis in apoE (−/−) mice and also increased levels of isoprostanes, which are markers of lipid peroxidation. The alpha-tocopherol transfer protein is responsible for selectively transferring alpha-tocopherol from the liver to lipoproteins and animals deficient in this gene develop vitamin E deficiency. This paper provides convincing evidence that lipid peroxidation and atherosclerosis are increased in response to alpha-tocopherol deficiency.

Table 4.1 Observational studies of vitamin E and CVD

Study	Design/follow-up	Outcome
Nurses' health	87 000 women, 8 years	Reduced MI or stroke
Health professionals	39 000 men, 4 years	Reduced MI
Iowa women	34 000 women, 7 years	Reduced fatal MI
Finnish cohort	5000 men and women, 14 years	Reduced fatal MI
EPESE	11 000 elderly men and women, 6 years	Reduced fatal MI
Scottish Heart Health	11 000 men and women, 8 years	No effect
Rotterdam	4800 men and women, 4 years	No effect

4.2.3 Vitamin E and atherosclerosis in clinical studies

Observational studies just look at the association between vitamin E status (either in the diet or in blood samples) and clinical measures of heart disease such as myocardial infarction (MI) or stroke. These studies are summarized in Table 4.1 and generally support the idea that vitamin E helps prevent heart disease. Observational studies do not, however, show cause and effect and have many major limitations. For example, people who are health conscious in general may exercise, maintain an ideal body weight, have a low-fat diet, and also take vitamin E supplements. In this case, vitamin E consumption is just a marker for a healthy lifestyle.

The ultimate experimental design for testing the potential efficacy of vitamin E (or the other nutrients reviewed in this chapter) on atherosclerosis and heart disease is the randomized, double-blind, placebo-controlled clinical trial. These trials are very expensive and therefore often limited to participants who already have documented cardiovascular disease (i.e. a secondary prevention trial).

Although some positive results were obtained, most placebo-controlled clinical studies (see Table 4.2) have not been supportive of a protective role for vitamin E in preventing cardiovascular disease. In the Finnish ATBC study, 50 mg of synthetic vitamin E (all-racemic-alpha-tocopheryl acetate) did not show any positive effect on heart disease. This level of vitamin E may not be sufficient to have an effect.

The British study (CHAOS) study did show a 47 per cent reduction (compared with placebo) in the combined rate of CHD death plus nonfatal infarction in subjects provided with 400–800 IU per day of vitamin E (RRR-alpha-tocopherol) and with a documented coronary heart disease. The positive effect observed in the CHAOS trial has, however, been criticized since the subject population was small, there were numerous imbalances in the base-line characteristics, and it was very short in duration (1.5 years).

The Heart Outcomes Prevention Evaluation (HOPE) Study was a large-scale, randomized, double-blind, and secondary prevention trial with men and women 55 years of age or older (Yusuf *et al*., 2000). In this study, half the subjects received 'natural source vitamin E' and half placebo for 4.5 years. Vitamin E had no effect

Table 4.2 Placebo-controlled vitamin E trials

Study	Population	Treatment	Endpoint	Result
ATBC (primary)	29 000 male smokers, 6 years	50 IU (low)	MI or stroke	Negative
Chinese Prevention (primary)	29 000 men/women, 5 years	30 IU + 15 mg beta-carotene (low)	CVD death	Negative
Cambridge Antioxide Study (CHAOS) (secondary)	2000 men/women with CVD, 1.5 years	400—800 IU	CVD death, fatal or nonfatal MI	Positive for nonfatal MI, no effect on fatal MI
HOPE (secondary)	9500 men/women with CVD 4.5 years	400 IU	MI, stroke, or CVD death	Negative
GISSI-Prevenzione (secondary)	11 300 men/women with CVD, 3.5 years	380 IU	MI, stroke, or CVD death	Negative
ASAP	529 men/women with hyperlipidemia	260 IU plus 500 mg vitamin C	Atherosclerosis progression	Positive in men

on myocardial infarction, stroke, or death from heart disease. Unfortunately, this study had many confounding factors. Many of the HOPE subjects took various medications such as aspirin or other anti-platelet agents (75 per cent), lipid-lowering drugs (28 per cent) or beta-blockers (39 per cent). Some subjects took 'non-trial' vitamin E. No plasma vitamin E levels (or index of oxidative stress) were measured in the subjects to ensure compliance. Unfortunately, this trial does not help us understand the role of vitamin E in preventing heart disease.

The Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) trial was a double-blind, 2 × 2 factorial study looking at the effects of RRR-alpha-tocopherol (2 × 136 IU/day), vitamin C (2 × 250 mg/day) or placebo for 3 years in 520 men and women (45–69 years) with hyperlipidemia (in Finland). RRR-alpha-tocopherol is 'natural vitamin E.' In this study a combined supplementation (E plus C) retarded the progression of carotid atherosclerosis (measured by ultrasonography) in men. Without vitamin C, the antiatherogenic influence of vitamin E was muted. In this study subjects were excluded if they had regular intake of aspirin or antioxidant supplements, but 30 per cent were taking some type of CVD drug. Only 2 per cent were taking statins. In contrast to the HOPE study, plasma levels of tocopherol and ascorbate were measured to confirm compliance. In this study, however, the benefit of taking the vitamin E and vitamin C supplement appeared to be limited to men and possibly only to men who smoke or have increased oxidative stress due to low levels of endogenous antioxidants.

It should be noted that human trials are generally conducted with elderly subjects and utilize endpoints such as myocardial infarction or stroke (that do not occur in animal models) and not the underlying process of atherosclerosis. Atherosclerosis starts during childhood and there is no reason to suspect that vitamin E could reverse atherosclerosis that is fully developed in the elderly. If vitamin E acts by preventing atherosclerosis then the optimal experiment would be a very large-scale clinical study in which children (starting at about 10 years of age) are provided with a vitamin E (or placebo) and followed until they develop cardiovascular disease at 60–70 years of age. This study would be a major undertaking and would be very costly.

4.2.4 Vitamin E and measures of cardiovascular function

Despite the mixed results when the outcome measures are myocardial infarction or stroke, there is considerable evidence that vitamin E has a positive effect on other measures of cardiovascular function. For example, a study by Skyrme-Jones *et al*. (2000) found that 1000 IU of vitamin E (all-racemic alpha-tocopherol) for 3 months improved endothelial function and blood flow in patients with type I diabetes and reduced the oxidative susceptibility of LDL. This study had an excellent study design, i.e. double-blind, placebo-controlled, and randomized. The relationship of oxidative stress to diabetes and the potential use of antioxidants is an area of intensive research (Laight *et al*., 2000). Owing to an epidemic of childhood obesity, the incidence of type II diabetes is expected to dramatically increase in the near future.

Paolisso *et al*. (2000) found that vitamin E therapy (8 weeks, chemical form not identified) was effective in improving brachial artery reactivity. Brachial artery reactivity measures the change in brachial artery diameter after release of an occluding cuff and is a measure of endothelial function. It is thought to be a useful marker for atherosclerosis and coronary artery disease.

4.2.5 Vitamin E therapy in type II diabetes

A number of studies have looked at the potential of vitamin E therapy in people with type II diabetes. Devaraj and Jialal (2000b) studied the influence of RRR-alpha-tocopherol therapy (1200 IU/day for 3 months) on controls and people with type II diabetes (with and without microvascular disease). The vitamin E supplement significantly decreased the monocyte release of O_2^-, IL-1-beta, tumor necrosis factor-alpha, and decreased monocyte-endothelium adhesion in all three groups. Increased levels of IL-I-beta, O_2^-, TNF-alpha, and the increased adherence of monocytes to arterial endothelium are all thought to be markers of inflammation and proatherogenic.

4.2.6 Vitamin E and C-reactive protein

C-reactive protein (CRP) is extremely important because it is emerging as a major risk factor for atherosclerosis and cardiovascular disease (Folsom, 1999). Patrick and Uzick (2001) have written an excellent review on the relationship of cardiovascular disease to CRP. The association between atherosclerosis and CRP is strong even in the absence of classical risk factors such as high cholesterol, triglycerides, and blood pressure (Ridker *et al*., 2001).

CRP is an acute phase protein released by the liver in response to acute injury, infection, or other inflammatory stimuli. It is, therefore, a marker for acute inflammation and infection. It is now known that even mild elevations of CRP may indicate an ongoing inflammatory process and that even values in the 'normal' range may have clinical importance. The recent development of a high sensitivity assay for CRP (hs-CRP) has rapidly accelerated research on this marker of systemic inflammation. Obesity is highly correlated with increased levels of CRP and, as mentioned above, it is dramatically increasing in Western countries (Yudkin *et al*., 1999; Ford, 1999; Visser *et al*., 1999; Lemieux *et al*., 2001; Ford *et al*., 2001; Cook *et al*., 2000).

A number of studies have now shown that vitamin E supplementation reduces levels of CRP. Upritchard *et al*. (2000) studied 57 people with type II diabetes who received placebo for 4 weeks and were then randomized to receive tomato juice (500 ml/day), vitamin E (800 IU/day chemical form not specified), vitamin C (500 mg/day), or continued placebo treatment for 4 weeks. Vitamin E supplementation was found to decrease CRP levels.

Devaraj and Jialal (2000a) tested the effect of RRR-alpha-tocopherol supplementation (1200 IU/day) on CRP and interleukin-6 (IL-6) release from activated monocyte in people with type II diabetes with and without

macrovascular complications compared with matched controls. Vitamin E supplementation was found to significantly lower levels of C-reactive protein and monocyte interleukin-6 in all three groups.

4.2.7 C-Reactive protein and the etiology of atherosclerosis

Since vitamin E has been shown to reduce levels of CRP it is reasonable to suggest that vitamin E supplementation could, thereby, reduce the risk of future CVD. This suggestion rests on the assumption that CRP is a causative factor and not just a marker for CVD. The evidence supporting this assumption is not yet conclusive but is certainly intriguing. New research has shown that CRP directly causes the induction of adhesion molecules on the endothelial cells of both human veins and arteries (Pasceri *et al*., 2000). The expression of these adhesion molecules is known to be essential for the development of CVD. Pasceri *et al*. (2000) concluded that CRP 'may play a direct role in promoting the inflammatory component of atherosclerosis and presents a potential target for the treatment of atherosclerosis.'

Additional evidence suggesting that the lowering of CRP could be important in preventing CVD comes from recent research on the use of statin therapy in the primary prevention (i.e. in people who have no obvious heart disease when enrolled in the study) of acute coronary events (Ridker *et al*., 2001). Statins are very popular and effective drugs that reduce plasma-cholesterol and LDL-C levels. It is very interesting, however, that statins also reduce CRP levels. Work by Ridker *et al*. (2001) found that statin therapy could reduce the risk of acute coronary events associated with CRP even in the absence of elevated blood lipids.

4.3 The functional properties of vitamin D in preventing heart disease

Vitamin D is a hormone critically important for the maintenance of a healthy skeleton and for maintaining calcium and phosphorus homeostasis (Holick, 2003). Vitamin D from dietary sources and from endogenous synthesis is stored in adipose tissue and circulates in the blood bound to alpha2-globulin D-binding protein. Vitamin D occurs primarily in two forms. One form is ergocalciferol (vitamin D2), which is found in irradiated milk, yeast and some plants. The second form is cholecalciferol (vitamin D3), which is formed in human skin by the action of ultraviolet radiation from sunlight. Vitamin D3 is rich in fish liver oils and egg yolks but is generally very low in most other foods.

The major source of vitamin D is normally from skin synthesis but it is only the UV-B component of sunlight light that produces vitamin D, i.e. the component blocked by sunscreen with a sun protection factor of 8 or greater. Vitamin D, either as D3 or D2, does not have significant biological activity and must be metabolized in a two-step process. In the first step, cholecalciferol is

hydroxylated to 25-hydroxycholecalciferol within the liver. In the second step, the 25-hydroxycholecalciferol is further hydroxylated within the kidney to 1,25-dihydroxycholecalciferol, which is the biologically active form of vitamin D.

Vitamin D deficiency can occur due to low dietary intake, by limited exposure to sunlight, by inability of the kidney to convert vitamin D to its active form, or by poor absorption from the gastrointestinal tract. Unfortunately, the only means to test for vitamin D deficiency is by a blood assay. Vitamin D deficiency or insufficiency can be quite common during winter months, particularly for people living at latitudes distant from the equator. 'Even in sunny southern California vitamin D deficiency or insufficiency is prevalent in part due to avoidance of midday sunlight and the use of sunscreens' (http://sunlightandvitamind.com/main.html).

Excessive intake of vitamin D in fortified food, over-the-counter supplements or excessive ingestion of anti-rickets pharmaceuticals can result in vitamin D poisoning. An acute toxic dose has not been established but the chronic toxic dose is more than 50 000 IU/day in adults for 1–4 months and, in children, 400 IU/day is potentially toxic. Acute toxicity effects may include muscle weakness, apathy, headache, anorexia, nausea, vomiting, and bone pain. Chronic toxicity effects include the above symptoms and constipation, anorexia, polydipsia, polyuria, backache, hyperlipidemia, and hypercalcemia. Hypercalcemia may cause permanent damage to the kidney (see http://www.emedicine.com/emerg/topic638.htm). Arterial hypertension and aortic valvular stenosis can also result from hypervitaminosis D.

Recent research has revealed that vitamin D has an importance beyond mineralization of bone tissue since vitamin D receptors have been found in a wide variety of cells. The active form of vitamin D is now known to bind to intracellular receptors that, in turn, function as transcription factors to modulate gene expression.

4.3.1 Vitamin D as an antioxidant

Work by Wiseman (1993) has shown that vitamin D3 (cholecalciferol), its active metabolite 1,25-dihydroxycholecalciferol, Vitamin D2 (ergocalciferol), and 7-dehydrocholesterol (pro-Vitamin D3) are all membrane antioxidants by virtue of their abilities to inhibit iron-dependent liposomal lipid peroxidation. There are very few studies focusing on the potential role of vitamin D as an antioxidant in biological systems. One such study by Sardar *et al*. (1996) found that vitamin D3 may function as an *in vivo* antioxidant in the rat liver with an effectiveness higher than that observed with vitamin E supplementation. It is unlikely that vitamin D in plasma could be an effective antioxidant since its levels are very low, i.e. the levels of 25 (OH)D3 and other vitamin D metabolites in healthy persons are between 60 and 100 nM. In contrast, plasma vitamin E levels are between 15—30 μM, which is at least 250 times higher than the typical plasma levels of vitamin D. At present, there are no data suggesting that vitamin D functions to prevent cardiovascular disease by virtue of its potential role as an antioxidant.

4.3.2 Vitamin D and heart disease

Vitamin D deficiency is a risk factor for the development of cardiovascular disease and diabetes but the molecular mechanisms are not yet fully understood (Timms *et al*., 2002). A variety of possible mechanism will be discussed and critically evaluated below.

Squalene metabolism

Grimes *et al*. (1996) investigated the potential relationship between geography and incidence of coronary heart disease. They suggested that sunlight deficiency could increase blood cholesterol by allowing squalene metabolism to progress to cholesterol synthesis rather than to vitamin D synthesis. They did indeed find higher levels of blood cholesterol during the winter months and suggested this could be due to reduced sunlight exposure. This suggestion is, however, somewhat speculative since it is not clear that vitamin D and its metabolites are a quantitatively significant fraction of squalene metabolism.

Vitamin D, insulin secretion, and diabetes

Vitamin D deficiency appears to have a very plausible relationship to type II diabetes in which defects in insulin secretion or in insulin signaling may be important factors. In animal models it has been found that 1,25-dihydroxyvitamin D3 deficiency inhibits the pancreatic secretion of insulin (Norman *et al*., 1980) and that this effect is only partially dependent upon serum calcium levels (Kadowaki and Norman, 1984, 1985). Subsequent work also showed that vitamin D3 improved impaired glucose-tolerance *in vivo* as well as insulin secretion in rats deficient in vitamin D3 (Cade and Norman, 1986).

Boucher *et al*. (1995) assessed the vitamin D status of Bangladeshi Asians living in East London and found that serum 25-OH vitamin D was reduced in those at risk for developing type II diabetes compared with subjects not at risk. The subjects in this study were also subjected to an oral glucose tolerance test (OGTT), in which a load of 75 g glucose in 300 ml water was consumed after an overnight fast and venous blood samples drawn at 0, 15, 30, 60, and 120 min for blood glucose, serum insulin and serum C-peptide assays. Early phase insulin secretion was assessed by measuring serum insulin 30 min after the oral glucose load. Boucher *et al*. (1995) found a positive correlation between early phase insulin secretion and serum levels of 25-OH vitamin D. Glucose intolerance was also correlated with vitamin D deficiency. Short-term vitamin D replenishment increased insulin secretion in a subset of subjects but did not alter the subjects' glucose intolerance.

The mechanism whereby vitamin D influences insulin secretion is not clear but it is reasonable to suggest that vitamin D receptors could be important in this regard. It is very interesting, therefore, that the insulin-producing beta cells of the pancreas have receptors that are specific for 1,25-dihydroxyl vitamin D3 (Ishida *et al*., 1988). Moreover, Hitman *et al*. (1998) found that vitamin D receptor gene polymorphisms can influence insulin secretion in Bangladeshi Asians. Subsequent work has shown that the expression of the vitamin D receptors is a determinant of insulin secretory capacity in Bangladeshi Asians.

Vitamin D, syndrome 'X', and inflammation

The concepts behind syndrome 'X' are very important because they bring focus to a cluster of related symptoms or disorders that are rapidly becoming a major health concern in Western society. In particular, syndrome X refers to group of health problems that can include type II diabetes, an atherogenic lipoprotein profile, obesity, and high blood pressure. This cluster of disorders is also characterized by increased levels of inflammation, which, in turn, contribute to a variety of chronic diseases such as cardiovascular disease, cancer, Alzheimer's disease, and perhaps premature aging. Boucher (1998) has made a convincing argument that inadequate vitamin D status contributes to syndrome X and that appropriate nutritional and lifestyle changes could help reduce the severity of this syndrome. As detailed above, plasma levels of CRP are very good marker of systemic inflammation. It is significant, therefore, that vitamin D status negatively correlates with plasma CRP levels and that significant reductions in CRP levels occur following vitamin D supplementation (Timms *et al*., 2002).

Vitamin D, calcium, and heart disease

In a preliminary study of 10 000 women over the age of 65, Varosy (2002) reported that women taking a vitamin D supplement (primarily from multivitamins) lowered their risk from heart disease by 31 per cent. It should be noted that this was a descriptive epidemiological study that did not utilize a randomized, double-blind, placebo-controlled experimental design. There is, however, increasing evidence of a link between CVD and osteoporosis (a gradual loss of bone calcium and increases bone fragility) (Burnett and Vasikaran, 2002). Women with osteoporosis have more calcium in their arterial walls than women with normal bones. In general, the degree of coronary artery calcification correlates very well with the degree of atherosclerotic plaque formation (Arad *et al*., 1998; Schmermund *et al*., 1998; Fiorino, 1998). Similarly, Watson *et al*. (1997) found an inverse correlation between serum 1,25-dihydroxy vitamin D levels and the extent of coronary calcification in 173 subjects with high and moderate risk for coronary heart disease.

It is reasonable to suggest, therefore, that loss of calcium from bones is associated with an increased accumulation of calcium in atherosclerotic plaque and that vitamin D or its metabolites could play a role in this process. Accordingly, Arad *et al*. (1998) measured serum concentrations of 1,25-dihydroxy vitamin D and the degree of coronary calcification in 50 subjects undergoing angiography. In contrast to the work cited above, these authors found no correlation between serum concentrations of 1,25-dihydroxy vitamin D and coronary calcification or the ratio of coronary calcification to the extent of coronary stenosis (a measure of atherosclerotic plaque formation).

Vitamin D, inflammation, and atherosclerosis

Atherosclerosis is an inflammatory vascular disease mediated by inflammatory cells, cytokines and chemokines. The T-cell plays an important role in mediating atherosclerosis and the effect of vitamin D on T-cell function may be an

additional pathway by which this vitamin modulates heart disease. It is interesting, therefore, that 1,25-dihydroxyl vitamin D has been found to inhibit Th1 and Th2 cell differentiation in human cord blood cells (Pichler *et al*., 2002) and diminish the production of interleukin-2 (IL-2), interferon gamma (IFN-gamma), IL-1, and tumor necrosis factor-alpha (TNF-alpha) in peripheral blood mononuclear cells. The inhibition of Th1 and Th2 cell differentiation and the decreased production of inflammatory cytokines associated with increased serum 1,25-dihydroxy vitamin D suggests an ameliorating effect on atherogenesis (Smith *et al*., 1999; Krishnaswamy *et al*., 2002). On the other hand, a recent study from India demonstrated elevated levels of serum 25-dihydroxy vitamin D in South Indian patients with ischemic heart disease, suggesting a pathogenic role in atherosclerosis (Rajasree *et al*., 2001). Because of these controversies, the actual role of vitamin D in promoting or preventing atherosclerosis is unclear. It is likely that a dose-dependent effect or an age-related effect may by discovered if well-controlled studies are conducted.

Vitamin D and heart failure

Vitamin D nutriture, in addition to playing a potential role in atherosclerosis, may also be an important factor in the pathogenesis of congestive heart failure (Zittermann, 2003; Zittermann *et al*., 2003). Congestive heart failure (CHF) can have multiple etiologies but is characterized by a reduced amount of blood being pumped from the left ventricle of the heart and, therefore, a reduced amount of blood reaching other organ systems. This disease is often the end stage of cardiac disease and, as more cardiac patients survive their initial problems, the opportunity for developing CHF increases. In aging Western societies, CHF is projected to reach epidemic proportions (http://www.nhlbi.nih.gov/health/public/heart/other/CHF.htm). Zittermann *et al*. (2003) have hypothesized that disturbances in calcium homeostasis could play a role in CHF since it is known that calcium plays a key role in the contractility of cardiac muscle. Observational studies have demonstrated an association of vitamin D deficiency in patients with severe CHF (Shane *et al*., 1997). Zittermann *et al*. (2003) found that patients with CHF have reduced levels of 25-hydroxy vitamin D and 1,25-dihydroxy vitamin D compared with controls. These authors speculate that low circulating levels of vitamin D metabolites could contribute to the etiology of CHF.

4.4 The functional properties of ubiquinone (CoQ10) in preventing heart disease

Ubiquinone or CoQ is a lipid-soluble micronutrient present in animal cells and in many plants. Ubiquinol is the reduced form of CoQ and it functions as an antioxidant as further detailed below (Frei *et al*., 1990). CoQ can be synthesized *in vivo* and is not, therefore, a true vitamin. There are, however, circumstances in which the utilization of CoQ surpasses its rate of synthesis. For example, the use

of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMG-CoA) inhibitors for the treatment of elevated LDL-cholesterol can result in a deficiency of CoQ10. Fortunately, CoQ10 is well absorbed by oral supplementation, resulting in increased levels of serum CoQ. In humans, the primary form of CoQ is CoQ10, in which ten isoprenoid units are present. In contrast, mice and rats have CoQ9 as the dominant form of CoQ.

The most well-characterized function of CoQ10 is in mitochondrial ATP synthesis where it plays a key role in oxidative phosphorylation (Crane, 2001). In particular, CoQ10 is the coenzyme for at least three mitochondrial enzymes essential for the production of ATP. In rats, lifelong supplementation with CoQ10 does not increase lifespan (Lonnrot *et al*., 1995).

4.4.1 Ubiquinol as an antioxidant

The role of ubiquinol as an antioxidant is much more controversial than its role in ATP synthesis (Beyer, 1992). In liposomes, ubiquinol has antioxidant ability similar to that of alpha-tocopherol but, unlike alpha-tocopherol, is not recycled by vitamin C (ascorbate) (Frei *et al*., 1990; Shi *et al*., 1999). There is, however, evidence suggesting that the semiquinone form of CoQ10 may be a pro-oxidant and generate superoxide radicals (Beyer, 1992). *In vitro* data suggest, however, that CoQ10 can conserve vitamin E in rat liver microsomes and mitochondrial membranes and thereby increase the resistance of these membranes to oxidative damage (Hiramatsu *et al*., 1991).

Dietary supplementation with CoQ10 is known to increase the level of ubiquinol in LDL and to increase the resistance of LDL to the initiation of lipid peroxidation (Mohr *et al*., 1992). As detailed above, the ability of dietary antioxidants to prevent the formation of oxLDL may be an important factor in preventing the very early stages of atherosclerosis, i.e. foam cell formation (Giugliano, 2000). In a very well-designed clinical study, Kaikkonen *et al*. (2000) compared the antioxidant effectiveness of CoQ10 and RRR-alpha-tocopherol (the natural form of vitamin E) in mildly hypercholesterolemic subjects using a randomized placebo-controlled experimental design. In this study, only vitamin E supplementation increased the resistance of LDL to oxidation. In subjects taking both vitamin E and CoQ10 supplements there was no enhanced effect of vitamin E to increase the resistance of LDL to oxidation. This result is somewhat surprising since *in vitro* experiments suggest that ubiquinol can regenerate alpha-tocopherol from the alpha-tocopheroxyl radical (an oxidized form of vitamin E) and thereby enhance the antioxidative effectiveness of vitamin E (Cabrini *et al*., 1991).

4.4.2 CoQ10 in heart disease

Compared with vitamin E, there has been only very limited research on the potential cardiovascular benefits of CoQ10. Singh *et al*. (1998) have reviewed the role of CoQ10 in CVD. CoQ10 deficiency has been observed in a wide

variety of cardiovascular disorders, e.g. congestive heart failure, angina pectoris, coronary artery disease, cardiomyopathy, hypertension, mitral value prolapse (Singh *et al*., 1998). In the *apoE* gene knockout mice (an excellent model of human atherosclerosis) supplementation with both vitamin E and CoQ10 was found to inhibit atherosclerosis better than with vitamin E or CoQ10 alone (Thomas *et al*., 2001). It is not known, however, if CoQ10 supplementation in humans can decrease atherosclerosis.

Although ubiquinol may inhibit the formation of oxidized and atherogenic forms of LDL, it is likely that the primary mechanism whereby CoQ10 could prevent heart disease is through its ability to improve ATP synthesis in cells with a high ATP demand such as cardiac myocytes. As an antioxidant, ubiquinol could also inhibit the free radical damage to the myocardium that arises during ischemia-reperfusion injury. Heart failure (due to cardiomyopathy and congestive heart failure), as discussed above, is a major and increasing worldwide health problem. It is logical to suggest that dietary CoQ10 supplementation could increase ATP production and thereby improve myocardial contractility. A meta-analysis of eight randomized controlled studies looking at the effect of dietary CoQ10 supplementation on congestive heart failure indicates an improvement in stoke volume, ejection fraction, cardiac output, cardiac index, and end diastolic volume index (Soja and Mortensen, 1997). These results certainly support a role for dietary CoQ10 supplementation as an adjunctive treatment for congestive heart failure.

4.5 Future trends

Nutritional science has made enormous strides in the past few decades and has particularly benefited from a molecular approach combined with the use of specific animal models with well-defined genetic characteristics. The design of functional foods that go beyond basic nutritional needs and attempt to improve and optimize human health is now a realistic goal. Just as pharmaceuticals of the future will benefit from knowing a subject's genetic background (i.e. pharmacogenetics), it is likely that optimizing an individual's diet must also take into account possible genetic factors (i.e. nutrigenomics). The new science of nutrigenomics will provide a molecular basis for understanding how an individual's diet could be changed, depending upon their specific genetic make-up, to optimize their long-term health status.

In this chapter, we have reviewed the information relating three lipid-soluble nutrients (vitamin E, vitamin D, and coenzyme Q10) to heart disease and type II diabetes, diseases with a clear genetic predisposition. For all three nutrients, there is a clear need for more research in both animal models and in long-term, well-designed clinical studies. Some tentative conclusions can, however, be reached. The three lipid-soluble nutrients reviewed above all have antioxidant properties and antioxidants are, in general, anti-inflammatory. Dietary supplementation with vitamin E or vitamin D is associated with decreased

levels of CRP, which is a marker for inflammation and increased risk of cardiovascular disease as well as in type II diabetes (Rodriguez-Moran and Guerrero-Romero, 1999, 2003; Arnalich *et al*., 2000). Surprisingly, there are no published studies on the potential role of CoQ10 in reducing plasma CRP levels.

In the case of vitamin E, there should be increased consideration for the non-alpha-tocopherol forms, particularly the potential anti-inflammatory properties of gamma-tocopherol. For CoQ10, the available data strongly supports a role for supplementation (along with conventional therapy) for the treatment of congestive heart failure. Vitamin D is remarkably under-researched considering its very promising role as an antiatherogenic factor. Lifestyle modifications, i.e. reasonable exposure to sunlight, may be more important than nutritional considerations in the case of vitamin D.

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5

Vitamin E and other antioxidants in the prevention of cardiovascular disease

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5.1 Introduction

Oxidative stress is believed to play a crucial role in the initiation and progression of atherosclerosis disease. Steinberg and colleagues[1] were among the first to postulate that modified low-density lipoprotein (LDL) could account for the accumulation of lipid within macrophages, a critical early step in the formation of the atherosclerotic plaque. In the early phases, native LDL may amass in the subendothelial arterial space and may be minimally oxidized by resident vascular cells through the activity of such enzymes as 12/15-lipoxygenase. In turn, this minimally modified LDL leads to the production of chemotactic factors and granulocyte and macrophage colony-stimulating factors, which enhance recruitment of circulating monocytes and their differentiation to macrophages in the vessel wall.

Plaque stability is believed to be influenced by levels of inflammatory mediators locally, which may stimulate expression of a number of proteolytic enzymes that lead to plaque fragility and rupture. These inflammatory actions encourage further oxidization of LDL, leading to both structural and functional changes in the vessel.[2] Macrophages avidly accumulate LDL particles modified by oxidation or acetylation through a number of scavenger receptors, including CD36 and scavenger receptors A-I/II, leading to the formation of foam cells and development of the atherosclerotic plaque.[3] At the same time, oxidized LDL species are directly toxic to vascular cells, and lead to endothelial injury and dysfunction, disabling, among other things, the intrinsic antiplatelet effects of this protective barrier, as well as the generation of nitric oxide, with deleterious effects on vascular tone and reactivity.[2]

The importance of oxidized LDL in atherogenesis has been further confirmed by the use of specific antibodies to oxidized LDL, which have been shown to be local to atherosclerotic lesions in the vessel wall.[4] Oxidative stress may contribute to atherogenesis by mechanisms that are not necessarily linked to LDL oxidation. For example, free radical oxygen species such as superoxide anion can rapidly react with and inactivate nitric oxide, enhancing proatherogenic mechanisms (e.g. leucocyte adherence to endothelium, impaired vasorelaxation, platelet aggregation).[5]

Although enzymatic and nonenzymatic oxidation of LDL seems to be involved, its relevance in the evolution of human atherosclerosis is still unclear. An important matter of discussion is the evident discrepancy between experimental and clinical trials with antioxidants, that, in fact, provided divergent results. Most trials with antioxidants in experimental models of atherosclerosis demonstrated that this treatment is able to retard the progression of atherosclerosis while the results of clinical trials are conflicting,[5] in that positive as well as negative effects has been reported. The investigation of antioxidants for prevention of atherosclerosis stems from observational trials that demonstrated the existence of an inverse relationship between the consumption of antioxidant vitamins and the risk of cardiovascular events. However, meta-analysis of the observational studies indicated that among antioxidant vitamins, vitamin E was the only one that exerted a beneficial effect against atherosclerotic complications.[6]

On the basis of these data almost all the trials have been based on the assumption that supplementation with vitamin E would represent a useful approach for preventing cardiovascular disease. However, candidates for antioxidant treatment were not accurately defined: any patient at risk of cardiovascular events has been indiscriminately enrolled in those trials. We argue that antioxidant status represents an important marker of oxidative stress,[7] its determination may be useful for better identifying candidates for antioxidant treatment. In order to substantiate this hypothesis, data inherent to oxidative stress and antioxidant status in patients at risk for cardiovascular disease and in patients included in observational and interventional trials have been reviewed. As antioxidant vitamin E has been the subject of the most important research in this field, our analysis is essentially concentrated on the clinical relevance of this vitamin in patients with cardiovascular disease.

5.2 Risk factors for coronary heart disease (CHD): the role of oxidative stress

Endothelial dysfunction and intimal–media thickness are considered the early steps in atherosclerosis. Rassel Ross[8] has modified atherosclerosis pathogenetical theories because numerous pathophysiological observations in humans and animals have led to the formulation of the response-to-injury hypothesis of atherosclerosis. Each characteristic lesion of atherosclerosis represents a

different stage in a chronic inflammatory process in the artery. The lesions of atherosclerosis represent a series of highly specific cellular and molecular responses that can be described as an inflammatory disease. Possible causes of endothelial dysfunction leading to atherosclerosis include hypercholesterolaemia, hypertension, diabetes mellitus, cigarette smoking, elevated plasma homocysteine concentrations, infectious microrganisms and ageing. Framingham's studies have shown how each factor and combination of these factors are associated with atherosclerotic diseases.[9] All these factors can be associated with oxidative stress.[10–15] The beneficial effect of alpha-tocopherol and ascorbic acid is mediated by their antioxidant actions in preventing atherosclerosis. On the other hand, the effect of alpha-tocopherol could also be mediated by its antiplatelet and anti-coagulant actions, which would prevent the thrombotic consequences of atherosclerosis.[16,17]

Cigarette smoking, hypertension, diabetes mellitus, genetic alterations, elevated plasma homocysteine concentrations, infectious microorganisms, such as herpes viruses or *Chlamidia pneumoniae*, have proinflammatory actions, increasing the formation of hydrogen peroxide and free radicals such as superoxide anion and hydroxyl radicals in plasma. These substances reduce the formation of nitric oxide (NO) by endothelium. Nitric oxide is a free radical with an unpaired electron in its highest orbital. This is why it behaves as a potential antioxidant agent by virtue of its ability to reduce other molecules. *In vitro* experiments support this concept inasmuch as NO is able to inhibit lipid peroxidation. However, NO is rapidly inactivated by the peroxide anion (O_2^-) to form peroxynitrite (NO_3^-) which is a potent oxidant. Therefore, in the presence of O_2^-, NO behaves as a potent pro-oxidant. This is the mechanism that accounts for the low-density lipoprotein (LDL) oxidation that occurs when NO and O_2^- are simultaneously present in the medium. As NO and O_2^- are simultaneously released by cells, such as endothelial cells, the balance between these two radicals is crucial in understanding the net effect of NO on lipid peroxidation. Thus an excess of NO will favour lipid peroxidation inhibition, while an excess of O_2^- or equimolar concentrations of NO and O_2^- will induce lipid peroxidation. Modulation of this balance may have important clinical implications, particularly in the atherosclerotic process, in which oxidative stress seems to play a pivotal role in the onset and progression of vascular lesions.

Several studies have strongly suggested that enhanced oxidative stress may represent an important trigger for atherogenesis elicited by angiotensin II (Ag II). Free radical formation mediates some of the effects of hypertension. Angiotensin II concentrations are often elevated in patients with hypertension and it is a potent vasoconstrictor. It also increases smooth-muscle hypertrophy and lipoxygenase activity, which, in turn, can increase inflammation and the oxidation of LDL.

Grienling *et al.*[18] examined the effect of Ag II on superoxide anion (O_2^-) production by smooth muscle cells and demonstrated that 4 to 6 hour exposure of these cells to Ag II elicited enhanced production of O_2^-. This effect was mediated by NADH and NADPH oxidase activation probably via intracellular

mobilization of fatty acids such as arachidonic acid. Experimental studies in animals demonstrated that Ag II infusion enhanced simultaneously blood pressure and vascular production of O_2^-; this last effect was dependent upon NADH/NADPH oxidase, further suggesting the role of this pathway in Ag II-mediated O_2^- production. These findings have important pathophysiological implications owing to the effect of O_2^- on vascular motility. The oxidative stress may have a role in hypertensive patients, in whom a reduced vasodilating response to acetylcholine has been demonstrated. Thus, in patients with hypertension, the administration of the antioxidant vitamin C has been able to restore acetylcholine-induced vasorelaxation, suggesting a role for oxygen free radicals in inducing vascular dysfunction in patients with hypertension. Cigarette smoke contains large amounts of free radicals which may degrade nitric oxide release from the endothelium and also produce highly reactive intermediates resulting in endothelial injury. Antioxidants such as vitamin E can also reduce free-radical formation by modified LDL.[19]

Blood analysis of lipid peroxides or measurement of urinary excretion of isoprostanes has provided evidence that oxidative stress is enhanced in patients with diabetes.[20] The impact of these data in the context of atherosclerosis progression is still unclear, but there is some evidence supporting a role for oxidative stress in contributing to deteriorating vascular disease. For instance, an important finding is the demonstration that endothelium-dependent vasodilation is reduced in patients with diabetes and that vitamin C is able to prevent it, so indicating a role for oxygen free radicals in reducing vasodilatory property of endothelium.[21] Oxidative stress could also contribute to worse metabolic disturbance by interfering with glycaemic control. Thus it has been demonstrated that, in diabetes, oxidative stress impairs insulin activity and antioxidants prevent it.[22] That hyperglycaemia is a risk for enhanced oxidative stress has been further corroborated by a study in patients with type II diabetes, in whom an increased urinary excretion of PGF2m-III, which derives from arachidonic acid and interaction with oxygen free radicals, has been demonstrated.[23] It is of note that a significant reduction of urinary PGF2cx-III was observed when patients underwent a strict glycaemic control, further reinforcing the relationship between hyperglycaemia and oxidative stress.

Hyperglycaemia may enhance oxidative stress and in turn induce vascular damage via several pathways, including the formation of the advanced glycated end products that are proatherogenic and prothrombotic (Fig. 5.1 panel C).[24] Furthermore, glucose may alter the balance between free radicals such as $O_2^{\bullet}$ and NO in endothelial cells; thus NO exerts its vasodilatory and antioxidant effect unless it is converted to ONOO by interaction with $O^{\bullet}$. This deleterious effect occurs in endothelial cells exposed to glucose, which, in fact, favours the formation of $O_2^{\bullet}$ and in turn promotes oxidation.[25]

An interesting mechanism potentially accounting for enhanced production of reactive oxygen species (ROS) by glucose is reported in Fig. 5.1. Hyperglycaemia was shown to enhance endothelial $O_2^{\bullet}$ generation via activation of cyclooxygenase pathway which is known to generate ROS with a mechanism

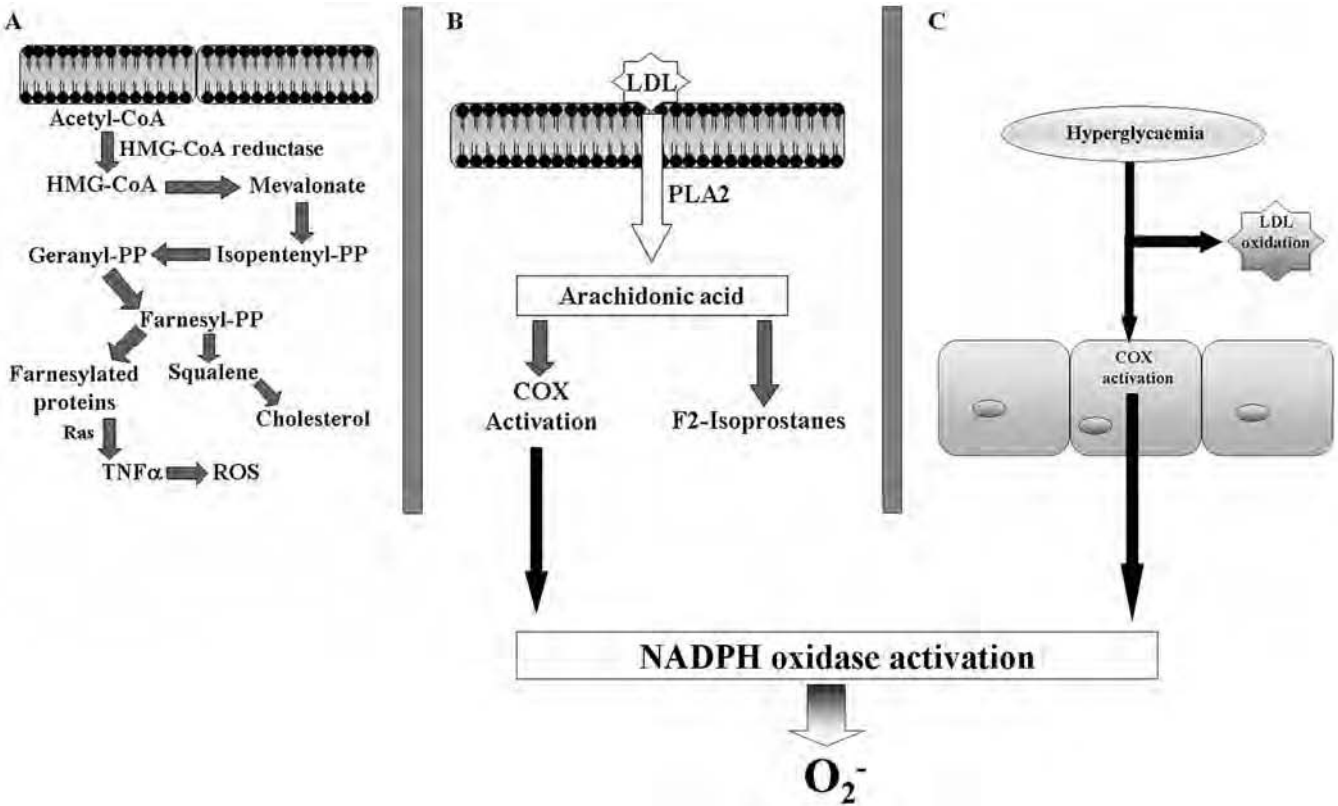
A
Acetyl-CoA
HMG-CoA reductase
HMG-CoA
Mevalonate
Geranyl-PP
Isopentenyl-PP
Farnesyl-PP
Farnesylated proteins
Squalene
Cholesterol
Ras
TNFα
ROS
B
LDL
PLA2
Arachidonic acid
COX Activation
F2-Isoprostanes
C
Hyperglycaemia
LDL oxidation
COX activation
NADPH oxidase activation
O_2^-

Fig. 5.1

involving NAD(P)H oxidase.[26] The potential role of this enzyme in inducing oxidative stress has been recently demonstrated by Guzik *et al*. who studied the expression of NAD(P)H oxidase in the vessel wall of people with and without diabetes.[27] They found that, compared with controls, vascular expression of NAD(P)H oxidase submits, p22 phox and p47 phox, were overexpressed in those with diabetes.

There is experimental and clinical evidence indicating that hypercholesterolaemia is associated with enhanced oxidative stress. Oxygen free radicals, such as $O_2^{\bullet}$, and F_2-isoprostanes, have been found elevated in the artery of hypercholesterolaemic animals and in the urine of patients with high serum cholesterol respectively.[28,29] The relevance of these findings in the context of the pathophysiology of atherosclerosis is unclear, even if there is some evidence that in this setting oxidative stress may have a role in reducing the vasodilation of endothelium.[30] Conversely, these is no evidence yet that the increase of these markers actually represents a marker of progression of atherosclerotic disease.

Two hypotheses can be suggested to explain why hypercholesterolaemia enhances oxidative stress (Fig. 5.1, panel A,B). Cholesterol has been recently shown to activate the metabolism of the arachidonic acid pathway,[31] which in turn seems to be associated with NAD(P)H oxidase activation.[26] This hypothesis has been recently underscored by our group showing that platelet incubation with cholesterol enhanced $O_2^{\bullet}$ production and that inhibition of PLA2 or NADPH oxidase enzymes significantly reduced O_2 formation (Fig. 5.1 panel B).[31]

The cascade of cholesterol biosynthesis may represent another pathway leading to enhanced oxidative stress. Intracellular metabolism of mevalonate leads, in fact, to the formation of protein isoprenylation, which has a key role in the production of proinflammatory and pro-oxidant cytokines such as tumor necrosis factor alpha (TNF; Fig. 5.1, panel A).[32] Accordingly, treatment of hypercholesterolaemic patients with an inhibitor of HMG-CoA-reductase was associated with reduced monocytes formation of TNF, suggesting a relationship between cholesterol and intracellular formation of pro-oxidant cytokines.[33] The association between hypercholesterolaemia and oxidative stress has been further corroborated by an interventional study with statin in people with hypercholesterolaemia in whom simvastatin reduced the urinary excretion of PGF2cx-III, probably by lowering serum cholesterol.[34] However, the existence of a mechanism independent of cholesterol lowering was not investigated. The relationship between hypertriglyceridaemia and oxidative stress has not been fully investigated. We found only one report aimed at analysing whether people with hypertriglyceridaemia had enhanced oxidative stress. Pronai *et al*. measured scavenging property and $O_2^{\bullet}$ formation by peripheral monocytes of hypertriglyceridaemic patients with and without diabetes.[35] They found a significant positive correlation between $O_2^{\bullet}$ generation and plasma triglycerides and a significant negative correlation between superoxide scavenging property and plasma triglycerides.[35]

5.3 Dietary antioxidants and the prevention of CHD: epidemiological evidence

A large number of epidemiological studies have evaluated potential relationships between dietary intake of antioxidants and coronary heart disease (CHD). These are summarised in Table 5.1. Among these, the Nurses' Health study,[36] included over 87 000 female nurses 34 to 59 years of age, who completed dietary questionnaires that assessed their consumption of a wide range of nutrients, including vitamin E. During follow-up of up to 8 years 552 cases of major coronary disease were documented. As compared with women in the lowest fifth of the cohort with respect to vitamin E intake, those in the top fifth had a relative risk of major coronary disease of 0.66 after adjustment for age and smoking. Further adjustment for a variety of other coronary risk factors and nutrients, including other antioxidants, had little effect on the results. Similarly, the Health Professionals' Follow-up study, among almost 40 000 males of 40–75 years, followed up for four years, showed a lower risk of coronary disease among men with higher intakes of vitamin E.[37]

Kushi *et al.* studied over 34 000 postmenopausal women with no cardiovascular disease who in early 1986 completed a questionnaire that assessed, among other factors, their intake of vitamins A, E and C from food sources and supplements.[38] After 7 years of follow-up, results suggested that in postmenopausal women the intake of vitamin E from food was inversely associated with the risk of death from coronary heart disease. This association was particularly striking in the subgroup of 21 809 women who did not consume vitamin supplements (relative risks from lowest to highest quintile of vitamin E intake, 1.0, 0.68, 0.71, 0.42 and 0.42; P for trend = 0.008). After adjustment for possible confounding variables, this inverse association remained (relative risks from lowest to highest quintile, 1.0, 0.70, 0.76, 0.32 and 0.38; P for trend = 0.004). By contrast, the intake of vitamins A and C was not associated with lower risks of dying from coronary disease.[38]

On the other hand, a negative result came from the Rotterdam Study in which 4802 participants aged 55–95 years, who were free of myocardial infarction (MI) at baseline and for whom dietary data assessed by a semiquantitative food frequency questionnaire were available, were followed up for 4 years: an association between vitamin C or vitamin E and MI was not observed.[39]

Other studies have evaluated plasma levels of different antioxidants, such as vitamins E, C and β-carotene in populations affected or not by CHD. The WHO/Monica project has been one of the largest studies that have analysed the intake of these vitamins in populations with different incidence of CHD mortality.[40] In populations with similar values of serum cholesterol and blood pressure, an inverse correlation between CHD mortality and vitamin E plasma levels was observed; conversely, no relation existed between CHD mortality, and other vitamins. In areas with low and medium coronary mortality, plasma levels of vitamin E were 26–28 μM, while at sites with most frequent CHD mortality

Table 5.1 Summary of observational studies

Observational studies	Patients' characteristics	Location of study population	Year	Data analysed	Results
Nurses' Health study[36]	87 245 female nurses 34–59 years of age, followed up for 8 years	USA, multicentre	1993	Cardiovascular events and vitamin E intake	As compared with women in the lowest fifth of the cohort with respect to vitamin E intake, those in the top fifth had a relative risk of major coronary disease of 0.66
Health Professionals' Follow-up study[37]	Male, health professionals 40–75 years, followed up for 4 years	USA, multicentre	1993	Cardiovascular events and vitamin E intake	As compared with the men in the lowest quintile group for vitamin E intake, the men in the highest quintile group had a relative risk of coronary disease of 0.59
Kushi *et al.*[38]	34 000 postmenopausal women, followed up for 7 years	USA, multicentre	1996	Cardiovascular events and vitamin E intake	Vitamin E consumption appeared to be inversely associated with the risk of death from coronary heart disease. Relative risks from lowest to highest quintile, 1.0, 0.70, 0.76, 0.32, and 0.38; *P* for trend = 0.004
Rotterdam Study[39]	4802 participants aged 55–95 years, 4 years follow up	Netherlands, single centre	1999	Myocardial infarction and vitamin E intake	No association with risk of MI was observed for tertiles of vitamin E
WHO/Monica project[40]	More than 100 000 middle-aged men	16 European populations	1991	CHD mortality and vitamin E plasma levels	With low and medium coronary mortality plasma levels of vitamin E were 26–28 pM, while at sites with most frequent CHD mortality plasma levels were 20–21.5 pM

Riemersma *et al.*[43]	110 cases of angina, 394 controls	United Kingdom	1991	Plasma concentrations of vitamins A, C, and E and carotene	Patients with a history of angina had a lower vitamin E/cholesterol ratio than controls (3.66 vs 3.86 pmol/mmol, $P < 0.01$
Singh *et al.*[44]	Cross-sectional survey within a random sample of 595 elderly people (72 with CVD)	India, single centre	1995	Coronary artery disease and vitamin E plasma levels	Adjusted odds ratios for CVD between the lowest and the highest quintiles of vitamin E levels were 2.53
Feki *et al.*[45]	62 angiographically confirmed coronary atherosclerotic patients and 65 age- and sex-matched controls	Tunisia, single centre	2000	Coronary artery disease and vitamin E plasma levels	Vitamin E/cholesterol concentrations were significantly lower in coronary patients than in controls (4.35 ± 1.03 *vs.* 4.82 ± 1.23 mmol/mol)
Mezzetti *et al.*[46]	102 apparently healthy subjects age 80 and older, followed up for 47.4 months	Italy, single centre	2002	Cardiovascular events and vitamin E plasma levels	Subjects with vitamin E levels in the highest quartile had a risk of cardiovascular events one-sixth those with vitamin E levels in the lowest quartile (relative risk = 0.16)
Iannuzzi *et al.*[47]	310 women, examined by B-mode ultrasound to detect early signs of carotid atherosclerosis	Italy, multicentre	2002	Occurrence of atherosclerotic plaques at the carotid bifurcation, vitamin E levels	An inverse association was found between both the intake amount and plasma concentration of vitamin E and preclinical carotid atherosclerosis

plasma levels were 20–21.5 μM. The authors also estimated that the threshold risk for cardiovascular disease would be <25 μM, which, in this particular population, corresponds to < 4.3 μmol vitamin E/mmol cholesterol. This finding is consistent with other studies showing an inverse correlation between vitamin E plasma levels and cardiovascular mortality.[41] It was noticed, in particular, that in persons with high risk for cardiovascular mortality the vitamin E/cholesterol ratio was 3.5, while in persons with low risk the ratio was almost 5.[42] The inverse correlation between vitamin E levels and CHD was also noted in another observational study in which 110 people with angina were compared with 394 controls.[43] The study demonstrated that patients with a history of angina had a lower vitamin E/cholesterol ratio than controls (3.66 *vs*. 3.86 μmol/mol, $P <$ 0.01) with a significant adjusted odds ratio for angina between patients in the lowest and highest quartile.

In a cross-sectional survey within a random sample of a single urban setting in India, the relation between risk of cardiovascular disease (CVD) and plasma levels of vitamin E was examined in 595 elderly subjects. Plasma levels of vitamin E appeared significantly inversely related to CVD. The adjusted odds ratios for CVD between the lowest and the highest quintiles of vitamin E levels were 2.53, after adjustment for confounding variables.[44]

Another study, designed to assess the degree of association between vitamin E and CHD in a sample of the Tunisian population, included 62 angiographically confirmed coronary atherosclerotic patients and 65 age- and sex-matched controls. A trend toward a meaningful decrease of plasma tocopherol was observed in affected patients compared with controls ($P = 0.06$). Vitamin E concentrations standardised for cholesterol and lipid concentrations were significantly lower ($P < 0.02$) in coronary patients than in controls (4.35 ± 1.03 *vs*. 4.82 ± 1.23 mmol/mol) for cholesterol-adjusted vitamin E. This association between vitamin E and CHD remained unchanged independent of age, sex, smoking habit, hypertension and diabetes.[45]

These findings have been further corroborated by another study in which 102 apparently healthy subjects were followed up for 47.4 months.[46] A higher risk of cardiovascular events in subjects in the lowest quartile of vitamin E plasma levels compared with those in the highest was found.

In a recent study the association between preclinical carotid atherosclerosis and both the intake and plasma concentrations of antioxidant vitamins was evaluated. Among 5062 participants in Progetto Atena, a population-based study on the aetiology of cardiovascular disease and cancer in women, 310 women were examined by B-mode ultrasound to detect early signs of carotid atherosclerosis. The participants answered a food-frequency questionnaire, and their plasma concentrations of vitamin E, vitamin A and carotenoids were measured. The occurrence of atherosclerotic plaques at the carotid bifurcation was inversely associated with tertiles of vitamin E intake. Similarly, the ratio of plasma vitamin E to plasma cholesterol was inversely related to the presence of plaques at the carotid bifurcation. No association was found between the intake of other antioxidant vitamins (vitamins A and C and carotenoids) or their plasma

concentrations and the presence of carotid plaques.[47] The results of a few similar previous studies were instead unclear.[48,49]

Taken together, these data suggest that vitamin E is an important predictor of CHD and may represent an independent risk factor for atherosclerosis and its complication. Owing to the lack of standardization and a somewhat large dispersion of vitamin E/cholesterol ratio values, accurate analysis of vitamin E levels in patients and healthy subjects is crucial in developing clinical practice and interventional trials. Very recently, for instance, healthy subjects[50] were shown to have values of vitamin E of 3.6 μmol/mmol cholesterol, which is much less than that reported in control population.[51] This finding also raises serious concerns on the methodology used for measuring vitamin and strongly suggests the need for standardization of the assay.

5.4 Dietary antioxidants and the prevention of CHD: evidence from clinical trials

While most epidemiological studies have demonstrated that dietary intake of vitamin E is inversely related to coronary heart complications, supplementation studies gave conflicting results. Clinical trials with antioxidants have been done in patients with or without previous history of cardiovascular disease (Table 5.2). Surrogate endpoints, such as analysis of atherosclerosis progression, or 'hard' endpoints, such as vascular death and MI, have been used to evaluate the clinical benefits of antioxidant vitamins. The Alpha-Tocopherol-Beta-Carotene-Cancer (ATBC)[52] prevention study was a randomized, double-blind, placebo-controlled primary-prevention trial to determine whether daily supplementation with alpha-tocopherol, beta-carotene or both reduced the incidence of lung cancer and other cancers. A total of 29 133 male smokers, 50–69 years of age, were randomly assigned to one of four regimens: alpha-tocopherol (50 mg per day) alone, beta-carotene (20 mg per day) alone, both alpha-tocopherol and beta-carotene, or placebo for 5–8 years of follow up. The results of this trial showed no beneficial effect of supplemental vitamin E (alpha-tocopherol) or beta-carotene in terms of the prevention of lung cancer, but the authors observed a reduction for death due to cardiovascular events in the group treated with alpha-tocopherol. For this reason, the authors made a sub-analysis analysing the clinical efficacy of 50 mg/day vitamin E in a population suffering from coronary heart disease, and showed no change in cardiovascular events during the follow up.[53]

The Cambridge Heart Antioxidant Study (CHAOS) tested whether treatment with a high dose of alpha-tocopherol would reduce subsequent risk of MI and cardiovascular death in patients with established ischaemic heart disease. In this double-blind, placebo-controlled study with stratified randomization, 2002 people with angiographically proven coronary atherosclerosis were enrolled and followed up for a median of 510 days (range 3–981). Alpha-tocopherol was assigned to 1035 people (capsules containing 800 IU daily for first 546 patients;

Table 5.2 Summary of randomized trials of vitamin E treatment

Trial	Patients' characteristics	Location of study population	Number in treatment group		Dose	Follow up (years)	Results
			Vitamin E	Control			
ATBC[53]	1862 men, smokers, aged between 50 and 69 years, who had a previous MI	Finland	963	799	50 mg	5.3	Vitamin E showed no effect
CHAOS[54]	Median age 62 years; angiographically proven CVD; 84% male (n = 2002)	UK, single centre	1035	967	400–800 IU	1.4	Vitamin E treatment substantially reduced the rate of nonfatal MI
GISSI[56]	Survivors of recent MI (<3 months); 85% male (n = 11 324)	Italy, multicentre	5660	5664	300 mg	3.5	Vitamin E showed no effect
HOPE[58]	Mean age 66 years; known cardiovascular disease or diabetes; 73% male (n = 9541)	Canada, USA, Europe, South America	4761	4780	400 IU	4.5	Vitamin E showed no effect
SPACE[59]	Haemodialysis patients with pre-existing cardiovascular disease (n = 196) aged 40–75 years	Israel, single centre	97	99	800 IU	1.4	Vitamin E reduced composite cardiovascular disease endpoints and MI

HPS[62]	Age range 40–80 years; known vascular disease or at risk of vascular disease; 75% male (n = 20 536)	UK, multicentre	10 269	10 267	600 mg	5	Vitamin E showed no effect
PPP[63]	Primary prevention in patients with at least one risk factor; age range 55–80 years (n = 4495)	Italy, multicentre	2231	2264	300 mg	3.6	The results on vitamin E are not conclusive
Fang *et al.*[64]	40 patients (0–2 years after cardiac transplantation)	USA, single centre	19	21	800 IU	1	Supplementation with antioxidant retarded the early progression of transplant-associated coronary arteriosclerosis
ASAP[65]	520 men and postmenopausal women aged 45 to 69 years with serum, cholesterol $\geq$ 5.0 mmol/L	Finland	390	130	272 IU	6	Supplementation with combination of vitamin E and slow-release vitamin C slows down atherosclerotic progression in hypercholesteroaemic person

400 IU daily for remainder); 967 received identical placebo capsules. The primary endpoints were a combination of cardiovascular death and nonfatal MI as well as nonfatal MI alone. This trial showed that in patients with symptomatic coronary atherosclerosis, alpha-tocopherol treatment substantially reduced the rate of nonfatal MI, with beneficial apparent effects after 1 year of treatment. No significant reduction in fatal MI was recorded; on the contrary, a non-significant increase in cardiovascular death was detected in patients receiving vitamin E.[54] In a further analysis of mortality, however, it became clear that only 6 of 72 cardiovascular-disease deaths occurred in patients compliant with vitamin E treatment.[55]

The GISSI-Prevenzione trial[56] assessed the efficacy of vitamin E and n-3 polyunsaturated fatty acids (PUFA) on cardiovascular death, nonfatal MI, or stroke in patients with recent MI. Some 11 324 people surviving recent (<3 months) MI were randomly assigned supplements of n-3 PUFA (1 g daily, n = 2836), vitamin E (300 mg daily, n = 2830), both (n = 2830), or none (control, n = 2828) for 3.5 years. The primary combined efficacy endpoint was death, nonfatal MI, and stroke. Intention-to-treat analyses were done according to a factorial design (two-way) and by treatment group (four-way). Treatment with n-3 PUFA significantly lowered the risk of the primary endpoint in the two- and four-way analysis. By contrast with n-3 PUFA, the results for vitamin E did not support the evidence of its efficacy, although it was possible to see a significant decrease of cardiovascular deaths in the four-way analysis. Moreover 300 mg/day of synthetic vitamin E daily (which is equivalent to about 150 mg natural vitamin E[57]) is below the range in which clinical trials report positive results.

Similarly the Heart Outcomes Prevention Evaluation (HOPE) Study was a double-blind, randomised trial with a two-by-two factorial design, conducted to evaluate the effects of ramipril and vitamin E in 9541 people at high risk for cardiovascular events. Participants were randomly assigned to receive either 400 IU of vitamin E from natural sources or an equivalent placebo daily for 4–6 years. The primary outcome of this study was measured by MI, stroke or death from cardiovascular causes. Secondary and other outcomes were death from any cause; unstable angina; hospitalization for heart failure with clinical and radiological signs of congestion; revascularization or limb amputation; the development of overt nephropathy; and the development of heart failure. In this study vitamin E did not reduce the incidence of cardiovascular events, as compared with the incidence among patients assigned to placebo, during the follow-up period. There were also no significant differences in the incidence of secondary cardiovascular outcomes or in death from any cause. There were no significant adverse effects of vitamin E. The investigators believed that perhaps longer follow-up was needed to detect benefit of vitamin E, although their data do not suggest a trend in that direction.[58]

The SPACE trial[59] investigated the effect of high-dose vitamin E supplementation on cardiovascular disease outcomes in haemodialysis patients with pre-existing cardiovascular disease. This population was chosen because it is well established that patients undergoing chronic haemodialysis are exposed to

increased oxidative stress induced by the membranes used in dialysis.[60,61] Some 196 patients were enrolled and randomized to receive 800 IU/day vitamin E or matching placebo. Patients were followed for a median 519 days. The primary endpoint was a composite variable consisting of: MI (fatal and nonfatal), ischaemic stroke, peripheral vascular disease and unstable angina. Among haemodialysis patients treated with high-dose vitamin E, a 54 per cent reduction was attained in the primary endpoint, contributed to largely by the reduction in total MI (70 per cent). The study was small, but the results are suggestive. Antioxidant therapy would be expected to have a greater treatment effect on patients in greater oxidative stress, and haemodialysis patients are in greater oxidative stress than other patient groups.[59] The accelerated cardiovascular disease event rate observed in haemodialysis patients, contributed to by increased oxidative stress, showed to be reduced by antioxidant therapy.

A negative result came from the HPS trial, in which 20 536 UK adults (aged 40–80) with coronary disease, other occlusive arterial disease or diabetes were randomly allocated to receive antioxidant vitamin supplementation (600 mg vitamin E, 250 mg vitamin C and 20 mg β-carotene daily) or matching placebo. Intention-to-treat comparisons of outcome were conducted between all vitamin-allocated and all placebo-allocated participants. Allocation to this vitamin regimen approximately doubled the plasma concentration of α-tocopherol, increased that of vitamin C by one-third, and quadrupled that of β-carotene. Primary outcomes measured were major coronary events (for overall analyses) and fatal or nonfatal vascular events (for subcategory analyses), with subsidiary assessments of cancer and of other major causes of morbidity. After a 5-year treatment period there were no significant difference in all cause mortality or in deaths due to vascular on nonvascular causes, in nonfatal MI or coronary death, or in nonfatal or fatal stroke between the two groups of participants.[62]

In addition to secondary prevention studies (which included patients with documented or known vascular disease), the Primary Prevention Project (PPP) studied the efficacy of vitamin E among people who had one or more cardiovascular risk factors (hypertension, diabetes or early family history of coronary disease). Some 4495 people were randomly allocated to receive aspirin (100 mg) or no aspirin, and vitamin E or no vitamin E. The main combined efficacy endpoint was the cumulative rate of cardiovascular death, nonfatal MI, and nonfatal stroke. After a mean follow-up period of 3.6 years the trial was prematurely stopped on ethical grounds when newly available evidence from other trials documented the benefit of aspirin in primary prevention. Vitamin E showed no effect on any prespecified endpoint. Findings for vitamin E could be regarded as a false negative result, because the trial was interrupted.[63]

The effect of antioxidant vitamins were also investigated using surrogate endpoints such as carotid atherosclerotic progression. Fang *et al.*[64] tested the effect of vitamin E (400 UI $\times$ 2) plus vitamin C (500 mg $\times$ 2) in a 40 people 0–2 years after cardiac transplantation. The primary endpoint was the change in average intimal index (plaque area divided by vessel area) measured by intravascular ultrasonography (IVUS). During 1 year of treatment, the intimal index increased

in the placebo group by 8 per cent (SE 2) but did not change significantly in the treatment group. Despite the small sample size, because of the limited number of people that undergo this procedure, the study was of particular interest because cardiac transplantation is associated to oxidative stress, which may contribute to the development of accelerated coronary arteriosclerosis.

The ASAP study[65] demonstrated that a combination of 136 IU vitamin E plus 250 mg of slow-release vitamin C slows atherosclerotic progression in hypercholesterolaemic patients. The subjects were 520 smoking and nonsmoking men and postmenopausal women aged 45–69 years with serum cholesterol < 5.0 mmol/L. The progression of common carotid artery (CCA) atherosclerosis was carried out by high-resolution ultrasonography. After 6 years of follow-up in covariance analysis in both sexes, supplementation reduced the main study outcome, the slope of mean CCA intima–media thickness by 26 per cent. The treatment was more effective in patients with low baseline values of vitamin C.

Most trials with antioxidants used vitamin E because epidemiological studies documented that regular assumption of this vitamin reduced the risk of cardiovascular events.[6] Patient selection of these trials was therefore based on the hypothesis that anyone at risk of cardiovascular disease would benefit from supplementation with this vitamin. Many primary and secondary interventional trials, such the GISSI-Prevenzione, the HOPE and the PPP studies did not consider the antioxidant status of participants as an entry criterion and did not report any data related to bioavailability of vitamin E.[56,58,63] The lack of this information makes interpretation of the results of these trials difficult because the serious issues related to vitamin E bioavailability were completely ignored. We demonstrated, in fact, that about 30 per cent of subjects did not have any increase of vitamin E plasma levels unless vitamin E was consumed after food intake.[42] This finding has been recently supported by Carrol *et al.*[66] who showed a significant increment of plasma vitamin E when supplements were given immediately before meals. Among antioxidant trials, five reported plasma values of vitamin E in the control population. Patients included in these trials suffered from cardiovascular disease or had classic risk factors for atherosclerosis[53,54,62] renal insufficiency[59] or had undergone heart transplantation.[64] In four of these trials a combination of vitamins was given: the ATBC[52] and the HPS[62] studies administered vitamins E, C and beta-carotene, the ASAP[65] and the study including patients undergoing heart transplantation[64] vitamins C and E. Vitamin E values of study populations were extremely wide ranging, from 5.6 (the CHAOS and the HPS studies) to 4.3 (heart-transplant atherosclerosis). Assuming that values of vitamin E <5 μmol/mmol cholesterol identify people at risk for cardiovascular disease,[57] we argue that only three studies included people with low antioxidant status. Among these trails the ATBC provided negative results while the other two studies demonstrated that vitamin E alone or in combination with vitamin C significantly reduced cardiovascular events.

5.5 Conclusion and future trends: reconciling the evidence

On the basis of these considerations we can conclude that there is compelling evidence that enhanced oxidative stress is detectable in patients with classic risk factors for atherosclerosis, but its impact in the context of atherosclerosis, progression is still unclear. The reason for this uncertainty is due to the lack of clear evidence indicating that markers of oxidative stress, such as blood lipid peroxides or urinary F2-isoprostane, are of some value for predicting the progression of atherosclerosis, even if there is some evidence suggesting that antibodies against oxidized LDL may be of some utility.[67] Conversely epidemiological studies seem to indicate that low antioxidant status increases the risk of cardiovascular disease.

Clinical characteristics of patients with low antioxidant status have not been defined and should be studied in the near future. So far, clinical trials with antioxidants included patients without evaluating either oxidative stress or antioxidant status and such indiscriminate enrolment could perhaps account for the negative results of antioxidant trials recently emphasized by meta-analysis.[68] A recent report by Meagher *et al.*[69] is highly relevant to this discussion. They fed normal subjects doses of vitamin E ranging from 200 to 2000 mg/day for 8 weeks. The highest dose increased plasma vitamin E levels 5-fold, but urinary excretion of isoprostanes and 4-hydroxynonenal (breakdown products of fatty acid auto-oxidation) was unaffected. The results suggest that in normally nourished subjects, additional vitamin E will not necessarily confer any additional antioxidant protection. Earlier studies in cigarette smokers, in contrast, did show a vitamin E effect on plasma isoprostane levels, suggesting that only in subjects under some oxidative stress will a vitamin E effect be obtained.[70] The protective effect of vitamin E against coronary events in the SPACE study may reflect the fact that the subjects were under the oxidant stress known to accompany haemodialysis.[59] In the same way, other people under increased oxidative stress (such as smokers, diabetics) could be also constitute a population more likely to benefit from antioxidants.

Moreover, as Steinberg and Witzum suggested,[5] the antioxidants might be effective in inhibiting the initial stages of human atherosclerosis and yet ineffective or much less effective in reducing plaque instability and rupture. If this were the case, it might be necessary to find some way to assess early stages of lesion development (e.g. high-resolution ultrasound or magnetic resonance imaging) rather than relying on the usual late clinical endpoints. Of course, if the development of early lesions were successfully inhibited, there should eventually be a decrease in the frequency of clinical events, but in that case, the trials might need to extend beyond the conventional 5 years.

Another issue that deserves further attention is the choice of appropriate antioxidant treatment. So far several mechanisms, including enzymatic and nonenzymatic oxidation of LDL, have been proposed, but the exact process leading to LDL accumulation within vessel wall is still unclear. This fact creates uncertainty in the type of antioxidants that could be relevant for inhibiting

atherosclerotic progress. Thus future trials with antioxidants should not be discouraged; conversely better identification of criteria *identifying* potential candidates for antioxidant treatment, together with the choice of an adequate daily regimen of antioxidants, should be studied.

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6

Iron intake and cardiovascular disease

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6.1 Introduction

Iron is an essential dietary component, necessary for a number of cellular functions including respiration and immune response. Since there is no physiological iron excretion, the element is reutilised in the body, and only a small fraction of the body's iron is gained or lost each day. The daily iron losses are mostly from desquamation of epithelia, such as skin and the lining of gastrointestinal tract. Greater iron losses may occur during growth in childhood, haemorrhages, menstruation and pregnancy in women.

Besides the fact that it is a vital element in life, iron may participate in diverse pathological processes. This may be due to its involvement in the production of reactive oxygen species. These species play important roles in immune response. However, they have also been shown to be involved in the pathogenesis of several diseases, such as Alzheimer's, Parkinson's, Crohn's disease, diabetes, cancer and arthritis. Furthermore, considerable evidence has supported the role of oxidative stress in atherogenesis. It has been hypothesised that iron-mediated oxidation is involved in this process (Sullivan 1981). Several epidemiological studies as well as *in vivo* and *in vitro* experiments are in favour of this iron hypothesis, although some studies have yielded conflicting results. This chapter describes the regulation of daily iron intake, the physiological, cellular and molecular metabolism of iron, the abnormal conditions of body iron balance, and the potential role of iron in the development of atherosclerosis and cardiovascular diseases.

6.2 Dietary iron intake, absorption and metabolism

Although there is no physiological means of iron excretion, a well-balanced diet containing sufficient iron is needed. Only about 10 per cent of ingested iron is absorbed in the gut. Therefore, around 10–20 mg of dietary iron intake is needed to balance the 1 or 2 mg of daily losses. The normal amount of total body iron is about 40–50 mg/kg body weight. In the body, iron is mainly needed to form the porphyrin complex of haemoglobin (30 mg/kg), myoglobin in muscle cells (4–8 mg/kg) and also iron-containing enzymes, such as cytochromes, oxidases and peroxidases. Up to 30 per cent of body iron (12 mg/kg) may be stored as ferritin and hemosiderin in the bone marrow, spleen and liver.

The efficiency of iron absorption is mainly regulated by body requirements to maintain iron homeostasis. Iron deficiency causes an increase in iron absorption, while iron overload reduces but does not eliminate absorption. Fertile women, for example, need to absorb up to 2–5 mg of iron each day to compensate for the menstrual blood loss. Many conditions causing a greater body iron demand may increase the efficiency of dietary iron absorption up to 20 per cent. A feedback mechanism exists to enhance or down-regulate iron absorption. Excretions from the liver, gall bladder and pancreas to the duodenum influence the uptake of iron.

6.2.1 Mechanism of iron uptake

Iron is mainly absorbed in the duodenum and the upper jejunum of the small intestine (Fig. 6.1). Both haeme iron and soluble complexes of iron are absorbable. Iron absorption from the gut lumen across the enterocytes to the circulation occurs in two stages: uptake across the apical membrane and transfer across the basolateral membrane (Fig. 6.2). The mechanisms of apical iron uptake from intestinal lumen to the enterocytes depend on the source of iron: Fe(II) or Fe(III) complexes. Haeme is absorbed in receptor-mediated fashion by the enterocytes. The haeme oxygenase-1 releases iron which then is reduced intracellularly. Fe(II) complexes are readily absorbed through a transporter called DMT-1. Fe(III) complexes are first reduced by a membrane-bound iron reductase called Dcytb before absorbed into the enterocytes, or bound to mucin and intracellularly reduced by either β-integrin, mobilferrin or flavin monooxygenase. The reduced intracellular iron may be stored in ferritin. The basolateral iron transfer is mediated by Ireg-1. After transfer, Fe(II) is immediately oxidised by the membrane-bound iron oxidase, hephaestin, or by the circulating oxidase, ceruloplasmin. Fe(III) is then bound to transferrin, an iron carrier protein in the circulation. Like any other cells, the enterocytes can take up transferrin by expressing transferrin receptor when iron is needed. Hepcidin is a signalling protein produced by the liver when iron level is high in the body. This molecule may regulate iron absorption through the enterocytes via an iron-sensitive protein, IRP. IRP can bind to iron-responsive element (IRE) present in transcription products of several genes including Dcytb, DMT-1, Ireg-1 and transferrin receptor regulating the expression of these corresponding proteins. HFE acts to facilitate TfR-1-mediated iron uptake from plasma into crypt cells.

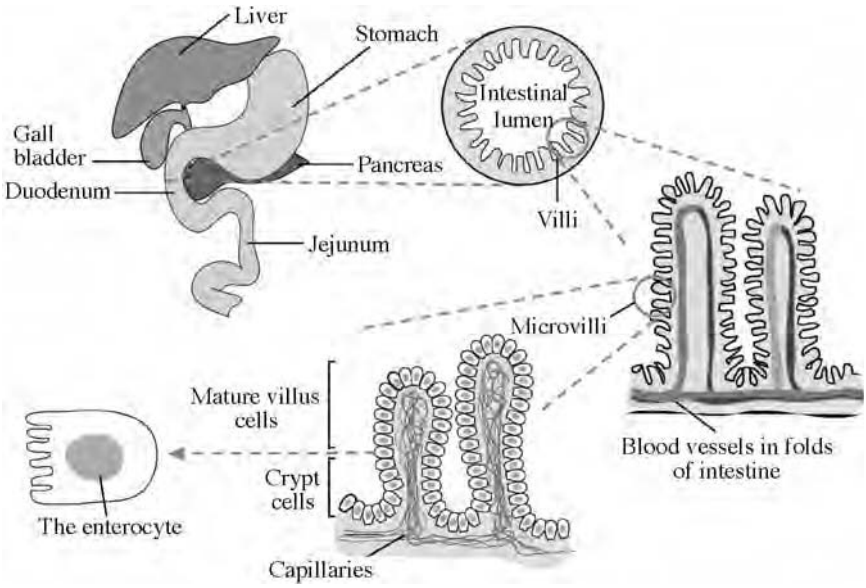

Fig. 6.1 Duodenal enterocytes within the gastrointestinal tract. Iron is absorbed from intestinal lumen, through the enterocytes lining the duodenum, to the circulation. The young enterocytes called the crypt cells move to the tip of microvilli and develop into mature villus cells. These two types of cells differentially regulate the uptake of iron.

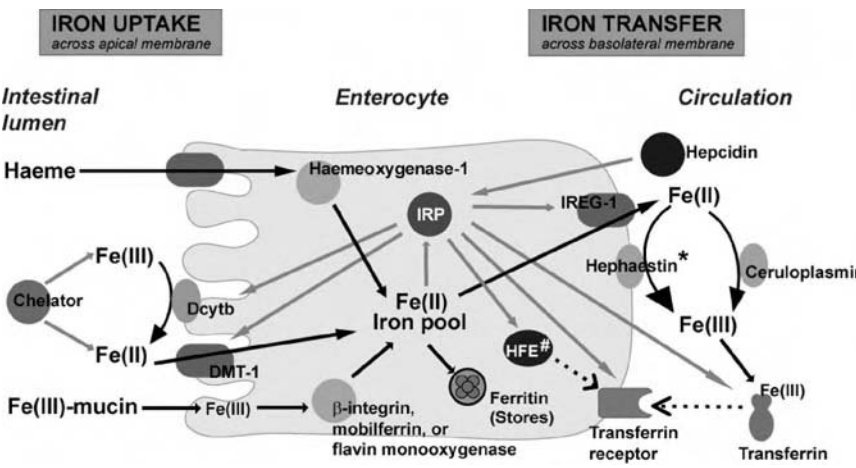

Fig. 6.2 Schematic representation of iron absorption and metabolism in normal enterocytes. # = in crypt cells only, * = mostly in mature villus enterocytes and hardly found in crypt cells. Fe(II) = divalent iron. Fe(III) = trivalent iron. Dcytb = duodenal cytochrome-b. DMT-1 = divalent metal transporter-1. IRP = iron responsive protein. IREG-1 = iron-regulated protein-1.

Apical iron uptake

The apical iron uptake is the absorption of iron from the intestinal lumen across the apical membrane to the enterocytes. The mechanisms of apical iron uptake from haeme and non-haeme iron sources are different.

Haeme iron derived from digested hemoproteins is able to interact with the membrane of the intestinal epithelial cells. This haeme is taken up by the enterocytes through a distinct receptor-mediated transport (Grasbeck *et al*. 1979). Inside the enterocytes, the haeme is broken down by haemeoxygenase-1. The released iron then enters the intracellular iron pool.

Non-haeme iron complexes can exist in two oxidation states: divalent iron, Fe(II), and trivalent iron, Fe(III). The soluble Fe(II) complexes can be taken up more easily than the almost insoluble Fe(III) derivatives by the cell membrane. However, in physiological condition, Fe(II) is rapidly oxidised to Fe(III). The presence of either endogenous chelators, such as mucin, lactoferrin or bile acids, or exogenous chelators, such as fatty acids, amino acids (e.g. asparagine, glycine, histidine) or organic acids (e.g. lactic, citric, succinic, ascorbic, malic, pyruvic) may increase the solubility and hence the uptake of iron.

Secretion of gastric acid lowers the pH of the gut. This increases the solubility and enhances the uptake of iron as well. When gastric acid production is impaired, for instance by the consumption of antacids and in the conditions of achlorhydria, iron absorption is reduced.

The soluble Fe(II) is taken up by the enterocytes through a specialised divalent metal transporter called Nramp-2, DCT-1 (divalent cation transporter) or DMT-1 (divalent metal transporter) (Gunshin *et al*. 1997). DMT-1 is highly expressed in the duodenum of normal individuals, and even strongly upregulated in anaemic conditions (Gunshin *et al*. 2001). This protein, however, is also capable of transporting other metal ions, such as zinc, lead, cadmium, manganese and copper. This lack of iron specificity may cause an increase in the absorption of toxic metals in iron deficiency.

The insoluble Fe(III) can only be taken up by the enterocytes after a reduction step catalysed by an iron-reductase. The iron-reductase, called Dcytb (duodenal cytochrome-b) has highest activity in the duodenum and lowest in the ileum (McKie *et al*. 2001), which is well-matched with the profile of iron absorption along the gut.

Mucin, a stomach glycoprotein, may also help the uptake of Fe(III) complexes in the duodenum. The mucin–Fe(III) complex, termed gastroferrin, readily traverses the mucus layer and acid microclimate at the mucosal surface. Within the enterocytes, Fe(III) is dissociated from the mucin. This released iron is readily reduced by several reductases including β-integrin, mobilferrin and flavin monooxygenase (Conrad *et al*. 1999), contributing to the intracellular iron pool.

Basolateral iron transfer

The basolateral iron transfer is the transport of iron from the enterocytes across the basolateral membrane to the circulation. Ireg-1, an iron-regulated protein, also called Ferroportin-1 or MTP-1, is responsible for this transfer (McKie *et al*. 2000). The expression of the protein is localised in the duodenum and also in

several other organs, such as the macrophages and the placenta where iron transfers between maternal and fetal circulations.

6.2.2 Regulation of iron uptake

The extent of iron absorption is mainly affected by the level of body iron, the degree of erythropoiesis, the amount of iron in the diet, and the composition of the diet itself. Other conditions, such as hypoxia, pregnancy and inflammation, may also alter the absorption. Furthermore, iron absorption is inappropriately increased in primary haemochromatosis.

The iron stores regulator induces a moderate increase in iron absorption as the body iron stores fall, and vice versa. It is still an unresolved question in how the duodenal mucosa is able to sense the level and changes in demand for iron. Iron content of the enterocytes is likely to be an indicating factor for this regulator. A central role of this regulatory process is assigned to the recently discovered hepcidin, a protein that is secreted into the plasma from the liver (Nicolas *et al*. 2001).

Approximately 70 per cent of body iron is incorporated into haemoglobin. In average, an adult person produces 2×10^{11} red blood cells daily containing 2×10^{20} atoms (20 mg) of iron. To meet this daily requirement, the body develops regulatory mechanisms whereby erythropoiesis profoundly influences iron absorption. This regulator would balance the rate of erythropoiesis in the bone marrow with the duodenal iron absorption.

Iron absorption is also modulated by the amount of iron in the diet and the composition of the diet itself. When increasing amounts of iron are ingested, the relative amount of iron absorbed decreases owing to the feedback mechanism of the absorption machinery; however, the absolute amount may still increase. Several chelators present in the diet, such as citrate from citrus fruits, can promote an increase in iron absorption, by increasing the solubility of iron in the duodenum. In contrast, phytates in wheat and some other cereals, as well as tannins in teas, chelate iron but prevent its uptake. Several metal ions, such as lead, cobalt, manganese and zinc, which are taken up by the same absorption machinery, may also block the iron uptake through competitive inhibition.

Haeme iron found in meats is more readily absorbed than non-haeme iron. The absorption is independent of duodenal pH. Experimental data indicate that haeme iron absorption is less responsive to the store regulator than that of non-haeme iron. Consequently, meat is an excellent nutrient source of iron. Lack of meat in the diet can be a cause of iron deficiency.

6.2.3 Metabolism of iron

Iron absorption, plasma iron transport, iron incorporation into cells and iron storage are meticulously regulated in the body to maintain iron homeostasis. Only a small fraction of body iron actually circulates, while most of body iron is prominently represented in haemoglobin, ferritin and haemosiderin.

Organs and cells communicate their needs for iron via the plasma as the central compartment of iron metabolism. Essentially, the body contains three types of cells: (1) those that need to obtain iron from the plasma (iron-requiring cells), (2) those that need to export iron towards the plasma (iron-donor cells), and (3) those that are able to take up and release iron for the protection of other more vulnerable cells in the body (hepatocytes). Transport and storage of iron in these cells are modified in situations of iron deficiency or overload.

Iron trafficking and uptake into cells

The iron-donor cells, mainly the macrophages and the intestinal mucosal cells, release iron to the plasma as Fe(II). The majority of this iron is rapidly oxidised by hephaestin or ceruloplasmin, then bound to transferrin. Hephaestin is found in the basolateral membrane of the mature villus enterocytes along the gut, while ceruloplasmin is a humoral protein produced and secreted by the liver to the plasma. Transferrin-bound iron is offered to iron-requiring cells, with majority going to the erythroblasts in the bone marrow for haemoglobin synthesis.

Transferrin is a 80 kDa single-chain glycoprotein containing two structurally similar subunits, each with one iron binding site. Therefore, one transferrin molecule can bind two Fe(III) atoms. Upon binding to iron the subunit undergoes a rigid rotation to enclose the iron atom. A distinctive feature of transferrin is its dependence on a synergistic anion, normally carbonate or bicarbonate for Fe(III) binding. When this anion is protonated, iron will be expelled from this harbouring protein.

Normally, all the non-haeme iron in the circulation is bound to transferrin. The liver synthesises transferrin and secretes it to the plasma. Transferrins are also produced locally in the testes and the central nervous system. Only around 20–45 per cent of transferrin binding sites are occupied in the circulation, so that most of available transferrins are free from iron. Nevertheless, non-transferrin-bound iron (NTBI) can be detected in some iron-overload conditions (de Valk *et al*. 2000) and may be attached to a variety of ligands. Most NTBI is taken up by the hepatocytes via the portal venous system. Furthermore, NTBI may also enter many other cell types promoting tissue damage.

Both monoferric and diferric transferrins are internalised by receptor-mediated endocytosis. Differic transferrin binds with higher affinity than monoferric transferrin. Two transferrin receptors have been described, i.e. TfR-1 and TfR-2. TfR-1 is expressed far more abundantly in iron-requiring cells than TfR-2, while TfR-2 is constitutively expressed in the liver (Gatter *et al*. 1983).

After binding to its receptor on the cell surface, transferrin is rapidly internalised through the formation of a clathrin-coated pit, which further develops into an endocytotic vesicle. This endosome undergoes acidification to pH 5.5 weakening the association between iron and transferrin. A membrane iron-reductase may help to completely dissociate iron from transferrin (McKie *et al*. 2001). Iron is then transported to the cytosol by DMT-1. The intact receptor-apotransferrin then recycles to the cell surface, where neutral pH promotes

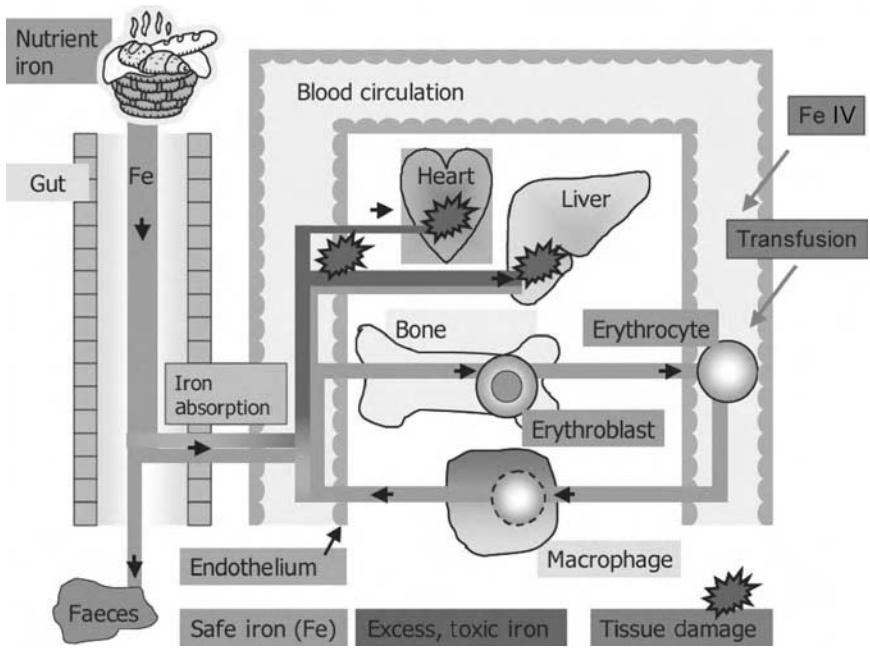

Fig. 6.3 Schematic representation of iron metabolism and its toxic potential (adapted from Marx and Hider, *Eur. J. Clin. Invest.* March 2002).

detachment of apotransferrin into the circulation. Exported apotransferrin can undergo further cycles of iron delivery into cells. The average transferrin molecule with a half-life of 8 days may be used up to one hundred times for iron delivery.

In iron overload, because of excessive iron intake, genetic defects, or repeated blood transfusions, considerable amounts of NTBI may be present in plasma. This iron can be weakly complexed to citrate, albumin, amino acids or sugars (Loreal *et al*. 2000). Most of NTBI is found in the complex form of Fe(III) to citrate, as shown by nuclear magnetic resonance (NMR) spectroscopy of serum from patients with iron overload (Grootveld *et al*. 1989). Non-haematopoietic tissues, mainly the liver, and also endocrine organs, kidneys, heart and the endothelium lining the blood vessels, preferentially take up NTBI through a transferrin-receptor independent mechanism. This mechanism may explain the continuous uptake of iron by the hepatocytes, in which iron overload has suppressed TfR1 expression beyond detectability. Furthermore, NTBI may generate toxic oxygen radicals and promote tissue damage in these organs (Fig. 6.3). Normal amount of iron, obtained from dietary iron or recycled body iron released by macrophages, is required for normal body functioning, especially for haemoglobin formation in the bone marrow. In the case of increased total body iron, either from increased dietary iron absorption, regular blood transfusion or intravenous iron injection, excess toxic iron may enter the circulation. Toxic iron is readily taken up by several organs, including the liver, the heart and the endothelium lining the blood vessels, and may cause further tissue damage in these organs.

Molecular metabolism and storage of iron

Inside the cell, iron first enters the intracellular iron pool in the form of Fe(II), which is soluble and biologically available. This iron is able to enter various intracellular locations, including mitochondria (especially for haeme biosynthesis, and ferritin (for storage).

Haeme biosynthesis occurs in all tissues, although the principal sites of synthesis are erythroid cells (~85 per cent) and hepatocytes (accounting for nearly all the rest of haeme synthesis). In hepatocytes, haeme is incorporated into cytochromes, in particular the P_{450} class which is important for detoxification. In erythroid cells, almost all of the haeme is synthesised for incorporation into haemoglobin. When the red cells mature, both haeme and haemoglobin synthesis cease. Normally after 120 days, senescent red blood cells are engulfed by macrophages. The globin is recycled or converted into amino acids, which in turn are recycled or catabolised as required. The haeme is oxidised by haemoxygenase, which results in the production of linear tetrapyrrole biliverdin, iron and carbon monoxide (CO). Most of the CO is excreted through the lungs, while the erythrocytic iron is then either stored as ferritin or released into the plasma via the iron export protein, ferroportin-1. The released iron is oxidised to Fe(III) by ceruloplasmin and is bound to circulating transferrin.

Sequestering of iron is necessary in all cells to avoid its tendency to form oxygen radicals that may damage cells. Ferritin and hemosiderin (reviewed by Harrison & Arosio 1996) are iron storage proteins that store iron within cells. Ferritin forms a hollow, spherical particle, in which 2000–4500 iron atoms can be stored as Fe(III). All ferritins are composed of 24 subunits associating to form a spherical particle. In animals, ferritin is found not only inside cells, but also circulating in the plasma. Plasma levels of ferritin are routinely been used as a measure for body iron.

Haemosiderin is another iron-storage complex. Its molecular nature is less defined than ferritin, but it is always found within cells and appears to be a complex of ferritin, denatured ferritin and other materials. Haemosiderin is most commonly found in macrophages and is especially abundant in tissues following internal haemorrhage, suggesting that its formation may be related to phagocytosis of red blood cells and haemoglobin.

Control mechanism for iron homeostasis in cells

Iron levels may regulate the expression of iron-related proteins, such as DMT-1, Ireg-1, ferritin, transferrin and TfR-1 (Fig. 6.2). This is shown by the presence of an iron responsive element (IRE) in the transcription product of the gene, which allows an cytoplasmic iron responsive protein (IRP) to bind in response to the level of intracellular iron (Hentze *et al*. 1987). In the case of ferritin production, for example, low intracellular iron conditions allow the binding of IRP to IRE, stopping protein synthesis. High intracellular iron level, on the other hand, prevents IRP–IRE binding, which results in increased ferritin synthesis. This IRP–IRE interaction provides control mechanism for intracellular iron homeostasis.

6.3 Iron homeostasis disorders: primary and secondary haemochromatosis

Disorders in the iron homeostasis may lead to either iron deficiency or iron overload. Iron deficiency is a condition where the iron intake does not meet the body's demands. Its manifestations are paleness, lethargy, palpitations and shortness of breath. Iron overload, also termed haemochromatosis, on the other hand, is characterised by a progressive increase in the total amount of body iron followed by an abnormal iron deposition in multiple organs (Fig. 6.3). In advanced cases, it also causes a bronze colour of the skin because of the deposition of iron-containing pigments in various tissues. The disease was once thought to be a singular disease with varying degrees of severity. Nowadays, it is known to be heterogeneous, resulting from defects in various genes.

6.3.1 Primary hereditary haemochromatosis

Several types of primary hereditary haemochromatosis have been described. Type-1 hereditary haemochromatosis (HH) is a common autosomal recessive disorder affecting mostly Caucasians. One in 200 (about 3.5 million) Europeans is homozygous for this, initially symptomless, chronic disease (Powell *et al*. 2000). Most individuals with primary haemochromatosis absorb excessive amount of dietary iron irrespective of the level of body iron, suggesting that the iron store regulator is dysfunctional. The excess iron accumulates over time, leading to tissue damage and organ failure. Clinical consequences include hepatic failure, liver carcinoma, arthritis, diabetes, impotence and cardiac failure.

This type-1 HH is associated with mutations in the *HFE* gene (Feder *et al*. 1996). The progression of iron overloading for this type of HH is quite slow, and affected individuals often start to have clinical symptoms only after the fifth or sixth decade of life. The initial symptoms include fatigue and joint complaints. As iron loading is progressing, patients develop skin hyperpigmentation and liver disease, which deteriorates gradually from fibrosis to cirrhosis. Cardiomyopathy and arrhythmias may develop from deposition of iron in the heart. Endocrine abnormalities, such as hypogonadism and diabetes mellitus, are also common.

HFE is strongly expressed by intestinal crypt cells and liver macrophages. The function of HFE protein itself is poorly understood. It appears to be a regulatory molecule that influences the efficiency of intestinal iron absorption, and may play an important role in iron homeostasis through its interaction with the transferrin receptor, TfR1. HFE facilitates TfR-1–mediated iron uptake from plasma into crypt cells, and its action is abrogated in *HFE*-linked HH in which there is functional loss of *HFE* protein. In the gut of HH, the cells behave as though they are relatively iron-deficient, causing an increase in intestinal iron absorption (Moura *et al*. 1998). HH patients have no iron-loading of macrophages, since wild type HFE also functions to inhibit iron release from these cells (Drakesmith *et al*. 2002), which results in increased release of low

molecular weight iron as Fe(II) from the cells to the circulation, and may further promote NTBI formation. The majority of type-1 HH patients carry a missense mutation (C282Y) in *HFE*. Other mutations and polymorphisms (H63D, S65C, I105T, G93R) have been identified, but their contributions to HH are not clearly understood. Treatment for type-1 HH is by phlebotomy, in order to keep serum ferritin levels below 50 μg/L. Initial treatment is 500 ml phlebotomy per week, followed by continuous treatment of one to four times a year. Cirrhosis usually occurs in HH patients when hepatic iron concentrations exceed 400 μmol/g dry weight liver (22.4 mg/g).

For type-2 HH, the juvenile haemochromatosis (Perkins *et al*. 1965) the responsible gene has not been identified. This type of HH is more severe than type-1 and it is characterised by rapid iron loading and clinical manifestations within the second decade of life. Cardiac and endocrine abnormalities dominate the clinical picture, although liver problems are also significant. Type-3 HH is associated with mutations in *TfR-2* (Camaschella *et al*. 2000) and is phenotypically similar to type-1 HH. Type-4 (Montosi *et al*. 2001; Njajou *et al*. 2001) and type-5 HH (Kato *et al*. 2001) are inherited in an autosomal dominant pattern. Type-4 is caused by missense mutations altering ferroportin-1. Patients accumulate large amounts of iron in the liver macrophages and have less transferrin-bound iron. However, they eventually also develop liver, heart and pancreatic complications. Type-5 HH, which has so far affected one Japanese family, affects the ferritin molecule, which in turn causes a defect in the iron-storing process.

6.3.2 Secondary haemochromatosis

Secondary haemochromatosis may be caused by several other conditions leading to iron overload. These include excess of dietary iron intake, chronic haemolysis and frequent blood transfusions. Phlebotomy is mostly impossible in these cases. The treatment most commonly used is a continuous administration of an iron-chelating agent.

Chronic anaemia such as aplastic anaemia, sickle cell anaemia, and thalassaemia cause iron overload mostly because of frequent blood transfusions. Each 250 ml transfused red cells adds about 250 mg elemental iron to the body. Frequent transfusions may promote diabetes mellitus and cardiac failure when iron concentrations exceed 268 μmol/g dry weight liver (15 mg/g). The end-organ manifestations of iron overload, such as cirrhosis, cardiac failure, hepatocellular carcinoma, diabetes mellitus and hypopituitarism resemble the manifestations in hereditary haemochromatosis patients.

6.4 The role of iron in cardiovascular disease

In significant parts of the modern world, iron overload is found in the population more often than iron deficiency. Consequently, the potential hazards of iron

excess are gaining more attention. Excessive iron may promote cardiomyopathy, arthropathy, infection, liver fibrosis, diabetes mellitus and malignancy, as well as endocrine and neurodegenerative disorders. A relatively new hypothesis has been postulated by Jerome Sullivan in 1981 (Sullivan 1981) that iron may play an important role in atherosclerosis and related cardiovascular diseases. Despite significant controversy and the negative results of several studies, de Valk and Marx (1999) concluded a strong epidemiological evidence for this iron hypothesis.

6.4.1 Epidemiological studies in favour of the iron hypothesis

Serum ferritin concentration as a measure of body iron has been shown to significantly correlate to the risk of myocardial infarction or carotid artherosclerosis (Haidari *et al*. 2001; Kiechl *et al*. 1994; Kiechl *et al*. 1997; Klipstein-Grobusch *et al*. 1999b; Salonen *et al*. 1992; Salonen *et al*. 1998; Tuomainen *et al*. 1997b, 1998). Ultimately, Lauffer (1991) showed significant correlation between iron stores, measured by liver biopsy, and cardiovascular mortality. Additionally, low prevalence of CHD has been observed in areas with high prevalence of iron deficiency (Sullivan 1981).

The lower incidence of coronary heart disease in premenopausal women compared with men of the same ages and with postmenopausal women was shown to be due to the lower total body iron caused by menstrual blood loss (Sullivan 1989). In men, body iron assessed by ferritin concentration, rose after adolescence, while in women, ferritin began to rise only after the age of 45 years (Burt *et al*. 1993). The Framingham study showed that the risk of heart disease in women increased equally by natural or surgical menopause (Gordon *et al*. 1978; Hjortland *et al*. 1976; Kannel *et al*. 1976). In heterozygotes of familial hyperlipoproteinaemia, the premenopausal women had a lower risk of coronary heart disease than men (Ascherio & Hunter 1994; Slack 1969; Stone *et al*. 1974).

The iron hypothesis may also explain the association between frequent blood donations and reduced risk of myocardial infarction (Meyers *et al*. 2002, Salonen *et al*. 1998, Sullivan 1991, Tuomainen *et al*. 1997b). Additionally, a community-based prospective cohort study showed that haem iron intake was positively associated with the total body iron and the risk of cardiovascular diseases (Ascherio *et al*. 1994; Klipstein-Grobusch *et al*. 1999a; Salonen *et al*.1992; Snowdon *et al*. 1984; Tzonou *et al*. 1998).

Recent studies have identified carriers of hereditary haemochromatosis (*HH*) gene to have significantly higher catalytically active iron than the normal population (de Valk *et al*. 2000). Some reports, moreover, demonstrated an association between heterozygous HFE gene mutation and the risk of cardiovascular events (Battiloro *et al*. 2000; Hetet *et al*. 2001; Rasmussen *et al*. 2001; Roest *et al*. 1999, Tuomainen *et al*. 1999). As for β-thalassemia, the clinical evidence of vascular complications has been shown to match with higher levels of oxidative modification of LDL compared with healthy controls (Livrea *et al*. 1998).

6.4.2 Epidemiological studies weakening the iron hypothesis

A number of studies failed to correlate serum ferritin and the risk for cardiovascular diseases (Auer *et al*. 2002; Eichner *et al*. 1998; Frey & Krider 1994; Manttari *et al*. 1994; Moore *et al*. 1995; Solymoss *et al*. 1994). Several other studies used serum iron, transferrin saturation or total iron binding capacity (TIBC) as a measure for body iron stores and did not find a correlation with cardiovascular disease. Since there is a poor correlation between transferrin saturation in the normal range and the total body iron (Beaton *et al*. 1989; Cook *et al*. 1976), their arguments opposing the iron hypothesis are weakened.

Some studies failed to reveal an increased risk of atherosclerosis in heterozygous and homozygous haemochromatosis (Candore *et al*. 2003; Fox *et al*. 2002; Franco *et al*. 1998; Nassar *et al*. 1998; Powell *et al*. 1994). Blood donation also did not appear to correlate with reduced risk for cardiovascular disease in one study (Ascherio *et al*. 2001).

Furthermore, some authors found no correlation between diatery iron intake and carotid atherosclerosis (Rauramaa *et al*. 1994), CHD (Liao *et al*. 1994), or myocardial infarction (Morrison *et al*. 1994). However, these studies did not differentiate between haeme and non-haeme iron intake. A lack of correlation between non-haeme iron intake and cardiovascular diseases suggests that dietary non-haeme iron does not contribute to an increased cardiovascular risk, except perhaps among patients with haemochromatosis (Ascherio & Hunter 1994).

Possible confounding factors are important to be taken into account before concluding any results from epidemiological studies. For atherosclerosis studies, it is necessary to involve an older population, since atherosclerosis progresses with age and the effect of iron on the incidence of severe atherosclerotic events can only be expected in such a population. In addition, excess iron alone as a risk factor may not be sufficient to demonstrate statistically significant different in cardiovascular events, as atherosclerosis is a multifactorial disease. The most recent study (Wolff *et al*. 2004) identified a relationship between serum ferritin levels and carotid atherosclerosis, and this correlation was even stronger when low-density lipoprotein (LDL)-cholesterol levels were taken into account.

6.4.3 Pathogenic mechanisms of iron-induced atherosclerosis

Atherosclerosis is a slow disease, starting in childhood, and progressing during ageing. It is due to a chronic inflammatory process coupled with dyslipidaemia. Two major mechanisms initiating the plaque formation include the oxidation of LDL-cholesterol and the transendothelial migration of leucocytes to the intima underneath the endothelial layer of the arterial vessels. Other processes involved in atherogenesis include T-cell and monocyte-mediated inflammation reactions, macrophage foam cell formation and proliferation of smooth muscle cells (Dzau *et al*. 2002). There are several possible mechanisms of iron involvement in atherosclerosis (Fig. 6.4)

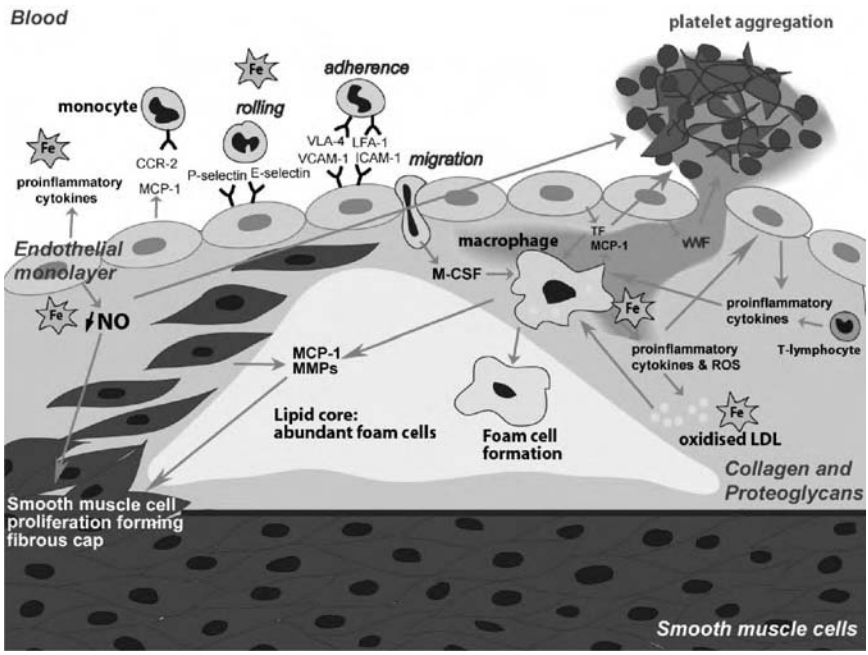

Fig. 6.4 Illustration of the cellular process and possible action of iron in the development of atherosclerosis. Fe = iron. P-selectin = platelet selectin. E-selectin = endothelial selectin.VLA-4 = very late activation antigen-4. VCAM-1 = vascular cell adhesion molecule-1. LFA-1 = lymphocyte function-associated antigen-1. ICAM-1 = intercellular adhesion molecule-1. MCP-1 = monocyte chemoattractant protein-1. CCR-2 = CC chemokine receptor-2. M-CSF = macrophage colony-stimulating factor. TF = tissue factor. vWF = von Willebrand factor. MMP = matrix metalloproteinases. NO = mononitrogen oxide. ROS = reactive oxygen species. LDL = low-density lipoprotein. These cellular processes may include: rolling, adherence and transendothelial migration of leucocytes, macrophage and T-cell mediated inflammation reaction, LDL oxidation, foam cell formation, decreased NO production, smooth muscle cell proliferation and platelet aggregation.

In vitro *studies*

The mechanism by which iron may stimulate atherogenesis is unclear. It is suggested that the catalytic role of iron in lipid peroxidation may influence the formation of atherosclerotic lesions. Iron-catalysed free radical formation may cause oxidation of LDL (Heinecke *et al*. 1984). The oxidised LDL is recognised by scavenger-receptors on macrophages, leading to accumulation of LDL in the cells. This is followed by the formation of foam cells, which are characteristic for the fatty-streak lesions of early atherosclerosis. Oxidised LDL also has chemotactic capacity providing recruitment of monocytes and macrophages to the site of lesions. The oxidised LDL also has cytotoxic capacity that induces changes in the endothelial cells with loss of endothelial integrity.

Transendothelial migration of leucocytes is also a fundamental inflammatory mechanism in atherogenesis (Gerrity 1981, Ross 1999). This process is partly

mediated by chemokines and the interaction between endothelial adhesion molecules and their ligands on monocytes (Meerschaert & Furie 1995; Navab *et al*. 1994; Shang & Issekutz 1998). Monocyte chemoattractant protein-1 (MCP-1) attracts monocytes bearing the chemokine receptor CCR-2 (Springer 1994). Several adhesion molecules have been shown to be present in human atherosclerotic plaques, including two members of the immunoglobulin superfamily of adhesion receptors, ICAM-1 (O'Brien *et al*. 1996; Printseva *et al*. 1992; van der Wal *et al*. 1992), VCAM-1 (O'Brien *et al*. 1993; O'Brien *et al*. 1996), as well as a member of the selectin family, E-Selectin (O'Brien *et al*. 1996; van der Wal *et al*. 1992). A significant correlation has been found between the degree of macrophage infiltration and endothelial ICAM-1, VCAM-1 and E-selectin expression in atherosclerotic lesions (O'Brien *et al*. 1996).

As a redox active metal, iron is capable of catalysing the formation of hydroxyl radicals in the Fenton reaction (Marx & van Asbeck 1996). Several antioxidants have been shown to protect against the endothelial dysfunction associated with atherosclerosis (Diaz *et al*. 1997). The oxygen radicals may involve in the regulation of nuclear factor KB (NF-$\kappa\beta$) DNA binding (Baldwin 2001), important for the transcription of a large number of genes, including the endothelial adhesion molecules (Collins *et al*. 1995; Neish *et al*. 1992).

The infiltration of leucocytes consists of consecutive adhesion-mediated events (Butcher 1991). The first step of adherence involves binding of selectins to carbohydrate ligands, which triggers tethering of the leucocytes to the activated endothelium along the vessel wall. After rolling and arrest, a firm adhesion of the leucocytes on activated endothelial cells may occur depending on the activation of the integrins including VLA-4 and LFA-1 (Adams & Shaw 1994; Luscinskas *et al*. 1994; Ross 1995; Springer 1994, 1995). Such activation may involve signalling initiated by inflammatory cytokines or signalling through binding of the integrins to their receptors (Adams & Lloyd 1997; Dedhar 1999; Ebnet *et al*. 1996; Ebnet & Vestweber 1999; Gahmberg 1997; Tuomainen *et al*. 1997a). Iron *in vitro* upregulates interleukin-6 (IL-6) production by endothelial cells (Visseren *et al*. 2002), while iron chelators inhibit the tumor necrosis factor-α (TNF-α mediated up regulation of endothelial adhesion molecules (Koo *et al*. 2003, Zhang & Frei 2003). Expression of IFN-κ-inducible genes in monocytic cells was affected by iron and iron-chelation (Oexle *et al*. 2003). Moreover, iron was shown to increase secretion of TNF-α (Lopez *et al*. 2003) and IL-1 (Szkaradkiewicz 1991) by monocytes.

In vivo *studies*

The *in vitro* experimental studies on generation of oxidised LDL by iron are supported by observation of the atherosclerotic lesions. The interior of advanced human atherosclerotic lesions is a highly pro-oxidant environment containing redox-active iron and copper ions which may induce lipid peroxidation (Smith *et al*. 1992). Ferritin was also found to be highly expressed in the atherosclerotic lesions (Pang *et al*. 1996). The iron is colocalised with ceroid, an insoluble complex of oxidised lipid and protein, extracellularly and also intracellularly in

the foam cells and smooth muscle cells (Lee *et al*. 1998). Iron deposits causing stimulation of macrophage infiltration to the atherosclerotic lesions and furthermore plaque rupture have recently been shown (Kolodgie *et al*. 2003). Further study by means of scanning and transmission electron microscopy revealed that erythrocytes containing haemoglobin were present in atherosclerotic lesions. Erythrophagocytosis by macrophages also occurred in the lesions (Lee *et al*. 1999a).

One study showed that patients with genetic haemochromatosis had significant eccentric hypertrophy of the radial artery, although none of them had arterial hypertension or evidence of cardiovascular diseases (Failla *et al*. 2000). The structural alteration leading to functional problems (stiffening), was largely reverted by iron depletion (Failla *et al*. 2000). Iron was also shown to induce early functional and structural vascular abnormalities due to endothelial dysfunction (Rooyakkers *et al*. 2002) which is associated with subsequent induction of oxidative stress. The radical species may also impair the mononitrogen oxide (NO) production, leading to the condition of arterial stiffness (Cheung *et al*. 2002). The vascular condition could be improved after administration of iron chelator (Duffy *et al*. 2001), which may indicate a reduced risk of cardiovascular events.

6.4.4 Animal studies

Several animal studies show the involvement of iron in the development of atherosclerosis and related cardiovascular diseases. Iron overload was shown to stimulate the formation of atherosclerotic lesions in hypercholesterolaemic rabbits (Araujo *et al*. 1995). Iron overload also increases the susceptibility of rat hearts to oxygen reperfusion damage (van der Kraaij *et al*. 1988; Voogd *et al*. 1992). Several studies showed a protective effect of iron chelators in the post-ischaemic cardiac injury period in animals, indicating that iron plays a role in reperfusion injury in tissues after ischaemeic insult (Badylak *et al*. 1987; Bolli *et al*. 1987; Reddy *et al*. 1989; van der Kraaij *et al*. 1988, 1989; Williams *et al*. 1991). Dietary iron restriction protected the apoE-deficient mice from developing the lesions (Lee *et al*. 1999b), and from having plaque rupture (Lee *et al*. 2003). Finally, iron chelation in experimental rabbits showed antiatherosclerotic effect by reducing plaque formation (Minqin *et al*. 2003).

6.5 Measuring iron toxicity

In the early course of iron overload, numerous homeostatic mechanisms prevent damage from accumulating iron. These include increased ferritin production needed to sequester the labile iron, and increment in individual antioxidants and/or antioxidant enzymes to protect against radical damage promoted by iron. However, these mechanisms might fail as more iron accumulates.

Measurement of iron toxicity is crucial for diagnosis and management of patients with iron overload from such disorders as hereditary haemochromatosis, thalassemia major, sickle cell disease, aplastic anaemia and myelodysplasia. Body iron can be measured by several parameters including serum ferritin concentration and transferrin saturation**.** The normal range of serum ferritin is 18–300 ng/ml. A decreased value of serum ferritin is associated with iron deficiency, while an increased value may indicate an increase of total body iron. However, it is also elevated in liver diseases, inflammatory conditions and malignant neoplasm. Another simple measure but insufficiently indicating total body iron (Beaton *et al*. 1989; Cook *et al*. 1976) is transferrin iron saturation, which is calculated as the concentration of serum iron divided by TIBC. Estimation of total body iron using this measure is less conclusive owing to high individual variation and strong influence of inflammation. The normal range of transferrin saturation is 15–55 per cent and the value increases in haemochromatosis. Furthermore, when moderate to severe iron overload is suspected, liver biopsy is necessary to be performed.

Magnetic resonance imaging (MRI) potentially provides the best available technique for examining the three-dimensional distribution of excess iron in the body; however, measurements and techniques must be calibrated for each individual machine. Biomagnetic susceptometry such as superconducting quantum interference device (SQUID) susceptometry (Brittenham *et al*. 1982) or, potentially, magnetic resonance susceptometry (Brittenham *et al*. 2001) provides the only noninvasive method to measure tissue iron stores that has been calibrated, validated and used in clinical studies, but the complexity, cost and technical demands of the liquid-helium-cooled superconducting instruments required have restricted clinical access to the method.

As previously mentioned, iron in the circulation is normally attached to transferrin. However in the case of iron-overload, NTBI is present (de Valk *et al*. 2000). Some species of NTBI may be safely bound to endogenous chelators; other species, however, may be catalytically active and capable of generating oxygen radicals, which is the major source of iron toxicity. This active NTBI is termed labile plasma iron (LPI) (Esposito *et al*. 2003). This iron species can also accumulate inside the cell and is termed labile iron pool (LIP). LIP may become catalytically active and is crucial for regulating the expression of many iron-related proteins. The findings mentioned above stress the need to identify the potentially toxic species of iron in both plasma and cells. Different means for small- and large-scale estimation of NTBI, LPI and LIP should be available with reliable and inexpensive methods to detect subjects at risk. Providentially, one of them is being developed (Breuer & Cabantchik 2001).

6.6 Methods of preventing iron damage

Screening for iron overload with biochemical methods and genotyping in patients who are suspected of having iron overload, such as type 2 diabetes

mellitus, atypical cardiac failure, early onset impotence in men and amenorrhea in women, early arthritis, high concentrations of liver enzymes, irritability, depression, joint pain and fatigue, is a recommended medical practice. Early detection of haemochromatosis is essential to prevent the potentially serious complications such as progression to severe organ damage. Currently, screening is not commonly done as part of routine medical care or check-ups, and many cases go undetected. A relatively inexpensive screening test for iron overload can actually be done by measuring the ferritin concentration together with the transferrin saturation from a blood sample.

Phlebotomy is the best solution for preventing iron damage in classical haemochromatosis. However, this treatment is not suitable for anaemia-related iron overload, and several other distinct forms of iron-overload. For these patients, iron damage can be prevented by administering an iron chelator. Desferoxamine (reviewed by Giardina & Grady 2001) commercially named Desferral®, is the only fully registered iron chelator that has been. However, the drug needs to be administered intravenously, is expensive and occasionally has some toxic side effects such as pain and swelling at the injection site, and rarely, impairment of vision and hearing or a general allergic or anaphylactic reaction. Patients may dislike wearing the pump, and fail to carry out the treatment. An alternative, safe, and effective oral iron chelator is urgently needed. An oral iron chelator, 1,2-dimethyl-3-hydroxypyrid-4-one (also known as L1, CP20 or Deferiprone) (Huehns *et al*. 1988), which is less expensive and relatively safe, has been recently recommended in Europe to be used in patients suffering from iron overload who cannot receive (too expensive) or cannot tolerate Desferral® (Kontoghiorghes *et al*. 2000). Experience with this drug has also been gained in Canada and India. Extensive research to develop safer and more effective oral iron chelators is in progress.

Furthermore, non-absorbable non-toxic oral iron chelators may also be beneficial to reduce iron absorption by the enterocytes of not only *HH* patients but also *HH* carriers. Additionally, since iron toxicity has been generally associated with a condition of free radical-mediated cell and tissue damage, the use of dietary antioxidants may help to protect against toxic effects of iron.

6.7 Conclusion and future trends

In summary, there is growing evidence for the role of iron in the development of atherosclerosis and related cardiovascular diseases. With all the possible confounding factors being taken into account, the epidemiological studies suggest a positive correlation between body iron level and this vascular disease. *In vitro* studies show several possible mechanisms in which iron may play a role in atherosclerosis. *In vivo* studies confirm the mechanisms of iron action in atherosclerotic plaque formation. The animal studies further validate the involvement of iron in this disease. Furthermore, elevated body iron level and NTBI have been observed in the carriers of hereditary haematochromatosis (de

Valk *et al*. 2000). The affected *HFE* gene has a prevalence of 10 per cent in Caucasian population. Together with the tendency of having iron overload in the modern world, many more people may thereby suffer from the risk of developing early cardiovascular disease.

Iron-deficiency anaemia remains a prevalent and debilitating illness in developing countries as well as in specific groups of the Western population. The disease mostly affects pregnant women and young children at levels of 79 per cent in South East Asia and 44 per cent in Sub-Saharan Africa, based on UN figures from the mid-1990s. In children, the illness may cause a permanent cognitive impairment. Identifying and caring for iron deficiency cases is certainly crucial for people especially from these regions.

As a consequence, iron supplementation is a common practice in many countries. WHO programmes also endorse this practice. Companies advertise their products, mentioning the benefits of iron fortification. However, these beneficial measures can have deleterious effects, especially in people with hereditary haemochromatosis and anaemias associated with iron overload.

There are many markers for iron deficiency, with serum ferritin and hypochromic red cell percentage currently the best markers available in clinical practice. Iron fortification is necessary in this easily diagnosed condition. Oral iron supplementation is inexpensive and safe, but poor patient compliance and reduced intestinal absorption may limit its efficacy. Intravenous iron, on the other hand, is effective, but it may have the potential of inducing iron overload.

The side effects of iron overload, such as infections, malignancies and vascular diseases, are well recognised. However, no guidelines exist for safe practice. A well-balanced diet containing a sufficient amount of iron is always necessary. In the Western world, however, the case of iron overload is increasing. Some dietary restriction would be useful to avoid iron accumulation in the body, for example by limiting the consumption of iron-rich foods such as liver, red meat and iron-fortified cereals, in subjects who are not prone to iron deficiency.

In the case of haemochromatosis, early screening has become cost effective, particularly for certain groups of people. Relatives, especially siblings, of patients with haemochromatosis should be tested for genes that indicate predisposition to the disease, allowing early treatment and prevention of the disease and tissue damage.

6.8 Sources of further information and advice

The sources mentioned below provide further relevant information. Two books provide more and up-to-date information on the process of atherosclerosis, the risk factors including nutrient iron, and some prevention advice.

1. *Immune mechanisms of atherogenesis*, Ming K. Heng and Madalene Heng (eds), Landes Bioscience; 1st edition (June 2003), ISBN: 1–58706–037–X,

2. *Atlas of atherosclerosis: risk factors and treatment*, Peter Wilson (ed.), Current Medicine; 3rd edition (June 2003), ISBN: 1573401870.

Another book explains more details of both primary and secondary haemochromatosis, the symptoms, screening strategies, available treatments and furthermore the complications including vascular problems.

3. *Hemochromatosis: genetics, pathophysiology, diagnosis and treatment*, James C. Barton and Corwin Q. Edwards (eds), University Press; 1st edition (March 2000), ISBN: 0521593808.

The last book provides more recent information on available iron chelators, especially for medical use.

4. *Iron chelators: new development strategies*, David G. Badman, Raymond J. Bergeron, Gary M. Brittenham (eds), Saratoga Publishing Group, Incorporated; (May 2000), ISBN: 1879894203.

Furthermore, we would like to introduce a website of a joined scientific project supported by the European Commission: http://www.nutrientirontoxicity.nl, which especially examines the deleterious effects of this essential iron nutrient, in different target organs.

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6.10 References

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7

Diet and diabetes: prevention and control

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7.1 Introduction: classifying diabetes

Diabetes mellitus is a heterogeneous metabolic syndrome with several different causes characterized by chronic hyperglycaemia with partial or total lack of insulin secretion and a reduced sensitivity to the hormone in peripheral tissues. If monitored inadequately and associated with other lipid and protein disorders, long-term complications may develop in several organs and systems, resulting in both high morbidity and mortality rates. Many of the long-term complications can be attributed to microangiopathy such as retinopathy, in the worst case leading to reduced sight or blindness; to nephropathy leading to insufficient kidney function; and to neuropathy, leading to motor-sensitivity deficit, a predisposing factor to the formation of ulcers and articular deformations of the feet. However, the most important epidemiological and clinical complications are those derived from macroangiopathy, primarily responsible for causing cardiovascular pathologies (chronic ischaemic cardiopathy, myocardial infarction and unexpected death) and cerebral-vascular diseases (stroke, transient ischaemic attack, multi-infarctual brain disease and peripheral vasculopathy).

The current classification of diabetes, proposed by ADA in 1997 and by WHO in 1999 is based on aethiopathogenetic criteria, an easier system when compared with previous classifications, but more difficult to use from the clinical point of view.[1]

Type 1 diabetes is the result of complete β-cell destruction. From an epidemiological perspective, the incidence of type 1 diabetes varies remarkably according to geographical location: the lowest incidence appearing in Asia and Oceania, while the highest ones are in Northern Europe, with the exception of

Sardinia. In only 10 per cent of cases can a family history of type I diabetes be documented.

Type 2 diabetes is a heterogeneous syndrome both in terms of aethiopathogenetic mechanisms and phenotypic aspects. Type 2 diabetes is the primary result of either insulin resistance or deficiency in insulin secretion, each having a completely different clinical perspective and presentation is usually characterized by a mixture of the two. A genetic predisposition is the most important aspect; environmental factors (eating disorders, reduced physical activity, overweight, obesity) precipitate and favour progression of the disease. It is very difficult to determine the incidence of type 2 diabetes because many recent onset cases of diabetes go undiagnosed owing to the absence of overt symptoms. However, current studies have shown that incidence varies from 1 case per 1000/year in the industrialized world to 25 cases per 1000/year in the Pima Indians. The observed differences among populations and ethnic groups reinforces the relevance of genetic and environmental factors.

The American Diabetes Association redefined in 1997 the diagnostic criteria for diabetes and lowered the previous classification of the threshold values of blood glucose. Diabetes is diagnosed with fasting blood glucoses >126 mg /dL on two separate occasions. Two hours after a glucose load, patients are diagnosed having diabetes with values >200 mg/dL, while values between 140 and 199 are defined as a person having impaired glucose tolerance.

7.2 Dietary strategies for preventing the onset of diabetes

The different natures of the two types of diabetes requires diverse dietary strategies in order to prevent their onset. For type 1 diabetes, while the exact causes are still being investigated, it is acknowledged that various environmental factors increase the risk of diabetes in genetically susceptible subjects. If these factors can be identified, there could be a good chance of decreasing the incidence of the disease.

It has been suggested that for type 1 diabetes an early exposure to cows' milk proteins may play a role in triggering the immune response that destroys pancreatic beta-cells.[2] Observational studies have shown that breastfeeding is associated with a lower incidence of type 1 diabetes.[3,4] It is hoped that the multicentre study, 'Trial to Reduce Type 1 Diabetes in the Genetically at Risk' (TRIGR) started in May 2002 will give a definite answer to this hypothesis. In this study, an offspring of someone with diabetes or first degree relative who possesses a high-risk genetic susceptibility to type 1 diabetes should be breastfed for at least 6 months of life. If the mother is unable to exclusively breastfeed, her child will then be randomly assigned to one of two groups. One group receives breastfeeding supplements of a special formula based on extensively hydrolysed cows' milk proteins; the other group receives a normal formula containing cows' milk with a small amount of hydrolysed proteins. In the hydrolysed cows' milk formula, the large molecular weight milk proteins are split into fragments too

small to stimulate an immune response. The rationale here is that the immune response in young infants genetically predisposed to type 1 diabetes is less mature and therefore unable to handle large intact food proteins. This sets up an immune response which can ultimately lead to the autoimmune destruction of insulin-producing cells.

Other dietary factors being investigated include the active form of vitamin D,[5] which is thought to help prevent the development of autoimmune diabetes and gluten since studies have shown that islet cell antibodies may disappear after a gluten-free diet in celiac patients.[6,7] However, time is needed before an answer on the efficacy of these dietary intervention trials is known.

There are various risk factors for developing type 2 diabetes. One of the primary ones being obesity as defined by a body mass index of over 30 (Table 7.1). Other risk factors include increased age, a family history of diabetes, prior history of gestational diabetes, impaired glucose tolerance, physical inactivity and race/ethnicity. For example, African Americans, Hispanic/Latino Americans, American Indians, some Asian Americans and Pacific Islanders are at particularly high risk of developing type 2 diabetes (Table 7.2). In recent years there has been an increase in type 2 diabetes related to changes in life style such as inactivity and diets rich in saturated fats. Approximately 80 per cent of people with type 2 diabetes are obese. Obesity is increasing both in the developed and in the developing countries. In the UK 20 per cent of the population is obese.[8] There is a worldwide trend towards obesity among children, and in the UK about 15–20 per cent of the teenage population is obese.[9]

The association between abdominal obesity (waist circumference > 102 cm in men, >88 cm in women[10]), high low-density lipoprotein (LDL) cholesterol, low high-density lipoprotein (HDL) cholesterol, hypertriglyceridaemia, high blood pressure, high fasting glucose (impaired glucose tolerance IGT $\geq$ 110 mg/dL and < 126 mg/dL), and insulin resistance (known as metabolic syndrome) is highly predictive of type 2 diabetes and potential coronary disease. Recently, a large clinical trial, the 'Diabetes Prevention Program' (DPP),[11] investigated whether diet and physical activity were more effective than metformin in preventing or delaying the onset of type 2 diabetes in subjects with impaired glucose tolerance and a family history of type 2 diabetes. The results of this study showed that the group that underwent lifestyle changes, intensive nutrition and exercise counselling (150 minutes a week) and behaviour modification lost 7 per cent of their body weight, with a 58 per cent reduction in the incidence of

Table 7.1 Body mass index and accepted criteria for defining body weight

<18.5	Underweight
18.5–25	Normal weight
25–29.99	Overweight
30–34.99	Obese (Class 1)
35–39.99	Obese (Class 2)
>40	Morbid obesity

Table 7.2 Risk factors for developing Type 2 diabetes

- Increased age
- BMI 25 or higher (overweight)
- Hypertension
- Very low HDL (high-density lipoprotein)-cholesterol levels (<40 mg/dL)
- Elevated triglyceride levels (>250 mg/dL or higher)
- Family history of diabetes
- Ethnicity (African American, American Indian, Asian American, Pacific Islander, or Hispanic American/Latino heritage)
- Gestational diabetes or giving birth to a baby weighing > 9 pounds (4.1 kg)
- Inactive lifestyle

diabetes versus 31 per cent in the metformin group. The clear message is that a realistic weight loss programme together with increased physical activity contributes to a reduction in weight and its maintenance with related positive health benefits.[12] According to the ADA Position Statement on the Prevention or Delay of Type 2 Diabetes 2003, 'Structured programs that emphasize lifestyle changes, including education, reduced fat and energy intake, regular physical activity and regular participant contact can produce long-term weight loss of 5–7 per cent of starting weight and reduce the risk for developing diabetes'.[13,14] Regular physical exercise is also advised to those who have a family history for diabetes in order to reduce their risk even if they do not have diabetes.[15,16] Improvements in fitness help keep glycaemic control and promote psychological well-being. The American Dietetic Association states that 'successful weight management to improve overall health for adults requires a lifelong commitment to healthful lifestyle behaviours emphasizing sustainable and enjoyable eating practices and daily physical activity.'[17]

A small caloric deficit of 500–800 kcal/day is sufficient to achieve a weight loss of about 1 or 2 pounds (1/2–1 kg) per week.[18] Total fat intake, particularly saturated fat, should be reduced as recent studies have reported the negative effect of dietary fat, especially saturated fats on insulin sensitivity. Moreover, reduction in fatty foods helps to lower the incidence of cardiovascular risk factors independent of weight reduction. People should be taught to select low-fat foods and their substitutes. They also have to reduce their portion sizes as it has been shown that overweight subjects tend to choose larger portions of high-fat foods and to underestimate their size.[19] A higher intake of dietary fibre has also been related to a decrease in the risk for type 2 diabetes. Some studies have also shown some positive effect of moderate alcohol intake in relation to improved insulin sensitivity and reduced risk for diabetes.[20] However, data supporting this evidence remain elusive.

Changing eating habits is very difficult and support is needed from health professionals who should develop individual therapeutic programmes for each patient according to his/her lifestyle requirements and tastes. In order to increase adherence every minimal success should be stressed to increase motivation and compliance with treatment. Nutritional counselling and visits should be very

close to each other to prevent relapse and to enable patients to make long-term lifestyle changes.[21]

7.3 Dietary strategies for the control of diabetes: carbohydrates and lipids

According to the American Diabetes Association, the goals of medical nutritional therapy for diabetes are to prevent and treat complications such as cardiovascular disease, hypertension, nephropathy, obesity and dislipidaemia, and include:

- achievement and maintenance of safe and near to normal or normal blood glucose levels;
- a normal lipoprotein profile'
- a change or improvement of health through food choices and physical activity'
- careful consideration of personal and cultural choices.

A healthy and balanced diet should provide enough calories for the daily energy requirement to maintain or achieve reasonable body weight, to provide for the needs of pregnant or lactating women, to allow for normal growth in children and adolescents and to satisfy the needs of ageing patients.

Attention should be paid to the daily energy requirement of children with type 1 diabetes, as they might lose weight at the onset of the disease. The weight–height charts used by paediatricians are useful to measure the adequacy of the energy intake. For adult type 1 diabetic patients, the daily energy requirement is not different from that of a normal individual. In this case it is also advisable to keep a desirable lean weight throughout life. However, for type 2 diabetes, since it usually occurs in overweight people, weight loss and behavioural changes are to be stressed.

In general, a healthy diet should provide 55 per cent of calories from carbohydrates, 10–20 per cent from protein and 30 per cent or less from fat.

7.3.1 Carbohydrates

Carbohydrates[22,23] are the body's main energy source; 50–55 per cent of the daily caloric intake should be provided by them. It is important that people with either type 1 or type 2 diabetes consume the right amount of carbohydrates, as they are the primary energy source for the central nervous system which depends on blood glucose. Carbohydrates also have the role of 'protein sparer', preventing the use of proteins for energy purposes, allowing them to perform their real role in tissue building and as metabolic primers for fat metabolism. The amount of carbohydrates in the diet regulates the levels of the intermediate products of fat metabolism, ketones. If the amount of carbohydrates is too low or unavailable, fat is oxidized for energy purposes with an increase of ketones

Table 7.3 Classification of major dietary carbohydrates

Class (degree of polymerization)	Sub-group	Components
Sugars (1–2)	Monosaccharides	Glucose, galactose, fructose
	Disaccharides	Sucrose, lactose
Oligosaccharides (3–9)	Malto-oligosaccharides	Maltodextrins
	Other oligosaccharides	Raffinose, stachyose, fructo-oligosaccharides
Polysaccharides (>9)	Starch	Amylose, amylopectine, modified starches
	Non-starch polysaccharides (inulin)	Cellulose, hemicelluloses, pectins, hydrocolloids

resulting in keto-acidosis, which is a common problem in patients with type 1 diabetes.

From a biochemical point of view, carbohydrates are divided into three groups: sugars, oligosaccharides and polysaccharides (Table 7.3). According to the American Diabetes Association terms such as simple sugars, complex carbohydrates and fast-acting carbohydrates should be discarded as 'they are not well defined and should be avoided'.

Traditionally simple sugars were thought to be absorbed quickly, thus rapidly increasing the level of blood sugars and so were forbidden. However, the ADA Position Statement 2002 has concluded that for people with diabetes it is the total amount of carbohydrates in meals and snacks, rather than the type, that determines the glycaemic response. Sugars, however, should be restricted in the diet, as a high intake usually increases triglyceride levels in the blood and may also contribute to the development of dental caries. Moreover, sugars are usually associated with high-calorie foods, which should be limited in order to maintain a healthy body weight. Their amount should not be more than 10 per cent of the daily total energy intake. Polysaccharides are preferred, especially those containing fibre, since the more fibre food contains, the more slowly it is digested, raising blood sugar levels at a slower rate.

A system for classifying carbohydrates, known as the glycaemic index[24] (Table 7.4), measures the effect that a food has on blood sugar levels. Foods that have a high glycaemic index cause a rapid and strong rise in blood sugar levels; diets filled with these foods have been linked to an increased risk for both diabetes and heart disease. Various factors, including the degree of processing, physical form and fibre content, determine a food's glycaemic index (Table 7.5).

Foods that contain complex carbohydrates, such as potatoes, quickly raise blood sugar levels, whereas foods that contain simple carbohydrates, such as whole fruit, raise blood sugar levels more slowly. However, the glycaemic index relates to the quality and not the quantity of carbohydrates consumed. Moreover, according to the ADA, 'although low glycaemic index foods may reduce postprandial hyperglycaemia, there is not sufficient evidence of long-term

Table 7.4 Glycaemic index of some common foods

Glucose	100
Cornflakes	83
French fries	75
White bread	70
Wholemeal bread	68
Rye	68
Table sugar	64
Potato – white boiled	63
Honey	62
Pizza (cheese)	60
Potato – new boiled	59
Rice (ex. basmati)	58
Kidney beans, canned	52
Custard	43
Yoghurt – fruit	36
Chocolate milk	35
Pasta (ex. spaghetti)	33
Skim milk	32
Whole milk	30
Beans, black boiled	30
Lentils green and brown	30
Yoghurt – plain	14

Extract from *The New Glucose Revolution Complete Guide to Glycemic Index Values* Jennie Brand-Miller, Kaye Foster-Powell, Johanna Burani, Susanna Holt, Marlowe & Company.

Table 7.5 Features that determine the glycaemic index of food

Factors	Explanation
Highly processed carbohydrates	White rice has a higher g!ycaemic index than brown rice because it contains less fibre due to technological processing.
Fibre content	Films influences the rate of sugar absorption, so the sugars in fibre-rich foods tend to be absorbed into the bloodstream more *slowly.*
Ripeness	A ripe fruit or vegetable has a higher sugar content than ones that are still green and, therefore, has a higher glycaemic index.
Fat content	The higher a food's fat content, the slower its carbohydrates are converted to sugar and absorbed into the bloodstream.
Physical form	Finely ground flour has a higher glycaemic index than more coarsely ground flour.

benefits to recommend use of low glycaemic index diets', stating that 'the total amount of carbohydrate in meals and snack is more important than the source or type'. Therefore, it is important to keep track of the quantity of carbohydrates consumed during a day. A good meal plan should provide the same amount of carbohydrates daily, divided evenly throughout the day to prevent changes in blood glucose levels. It is advisable to plan meals with a dietician who will take into account the exact daily caloric requirement and the right proportion between carbohydrates, fats and proteins.

The daily intake of carbohydrates will depend upon the type of exercise programme, the amount and timing of daily insulin requirement, the use of an insulin pump, the need to lose weight or for a special diet to reduce cholesterol or blood pressure levels. It is possible to monitor the intake of carbohydrates by calculating the grams eaten at meals and snacks with the help of food labels. This method enables a wider choice of foods. For those who take insulin, it is important to measure their pre-meal blood sugar levels and base the amount they will eat on it. If the measurement permits more carbohydrates to be eaten, then additional insulin will be required.

A similar method is the use of food exchange lists, designed by the American Diabetes Association and American Dietetic Association. A food exchange listing is shown in Table 7.6. Food can be exchanged within a certain list, but not between lists, even if they have the same calories. Choices will vary according to a person's recommended daily allowance.

Food such as honey, syrup, jam, jelly, candy, sweet rolls, regular gelatin, cake with icing, and pie should be avoided, as should soft drinks. The consumption of the so-called products for diabetics, such as chocolate, should also be restricted as they contain excess calories in fat.

7.3.2 Lipids

As people with diabetes have an increased risk of heart and blood vessel diseases, a normal to low-fat diet is advisable in order to reach and maintain good weight and health. As part of a healthy diet, 30 per cent of daily calories should come from fat, and of these less than 10 per cent should be saturated fat, less than 10 per cent polyunsaturated fat and 10–15 per cent monounsaturated fat. However, a diet with a low intake of fat can be dangerous since not only is fat a major source of energy for the body as it supplies 9 kcal/g, it is also the source of essential fatty acids (EFA), such as linoleic, linolenic and arachidonic acids. These are called 'essential' as the body cannot synthesize them and they are needed for a number of key functions such as transport and metabolism of cholesterol, precursors of prostaglandins and others. Among the EFA, omega-3 fatty acids found in fish, especially salmon, mackerel and herring, have been shown to lower plasma trygliceride levels in patients with type 2 diabetes. Moreover, fat is important because it helps the absorption of the fat-soluble vitamins A, D, E and K.

Monounsaturated fat is a type of unsaturated fat that lowers blood LDL cholesterol levels without altering those of HDL cholesterol.[25] It is also resistant

Table 7.6 Carbohydrate exchange list

Type of food	Carbo-hydrate (g)	Protein (g)	Fats (g)	Calories	Serving size
Starch	15	3	0	80	1/2 cup cereal, grain, or pasta; 1/3 cup of rice; 1 oz. (slice) of a bread product; 1/3 cup of beans, peas or lentils (cooked); potato baked 3 oz. (85 g), potato mashed 1/2 cup
Skim/very low-fat milk	12	8	0–3	90	Skim milk 1 cup; plain non-fat yogurt 8 oz. (225 g)
Low-fat milk	12	8	5	120	Low-fat milk 1 cup; Low-fat yogurt 8 oz. (225 g)
Whole milk	12	8	8	150	Whole milk 1 cup; Whole milk plain Yoghurt 8 oz. (225 g)
Fruit	15	0	0	60	1 small to medium fresh fruit 1/2 cup canned fruit; 1/2 cup fruit juice (ex. apple juice, orange juice, etc.)
Vegetable	5	2	0	25	1 cup raw vegetable; 1/2 cup cooked or canned vegetables; 1/2 cup veg. juice

American Diabetes Association Exchange Lists (Extracts)

to oxidation. This is important since oxidation enables cells in arteries to absorb fats and cholesterol and helps accelerate the formation of plaques. This type of fat is found in olive oil, avocados, canola oil, etc. In people with diabetes a diet higher in monounsaturated fats and lower in carbohydrates has been shown to reduce postprandial glycaemia and triglyceridaemia, but it is not recommended for someone with type 2 diabetes as it may cause weight gain. Monounsaturated fats and carbohydrate intake should be based on dietary goals and metabolic profile of each individual.

The effects of polyunsaturated fat on subjects with diabetes have not been well studied. Compared with monounsaturated fat, this vegetable fat lowers total blood cholesterol but is susceptible to oxidation, which as previously described may ultimately lead to coronary artery disease. Polyunsaturated fats are found in cottonseed, soybean, sunflower and safflower oils.

Saturated fat is an animal fat that raises total blood cholesterol and increases the risk for coronary artery disease.[26] It is found in hydrogenated vegetable fats, coconut and palm oils, cocoa butter, meat fat, whole milk, butter cream and fatty cheeses. Cholesterol blood levels should be kept to <200 mg/dL and the daily intake should be limited to 300 mg/day. According to the ADA recommendations, diabetics with dislypidaemia, such as those with high LDL cholesterol, should

lower saturated fats to less than 7 per cent and dietary cholesterol to 200 mg/day and increase soluble fibre to 10–25 g/day, together with physical activity. Also plant sterol and stanol esters have been shown to have a good effect on blocking the absorption of total and LDL cholesterol.

A healthy diet should be rich in fish, poultry (without skin), lean cuts of beef, lamb, veal, pork, skimmed or low-fat milk, low-fat cheeses, low-fat yogurt, olive oil, rice, pasta, bread, whole-grain cereals, legumes, vegetables and fruits, while intake of foods such as animal fats, bacon, sausages, commercially baked goods, cream, cheeses, butter, salad dressings made with cream and eggs and junk food such as potato chips, cookies should be cut down.

Particular attention must be made to margarine or shortening, which contain hydrogenated vegetable oils, which consequently form *trans*-fatty acids. These are highly related with artery disease, raising blood cholesterol and lowering HDL levels.

7.4 Dietary strategies for the control of diabetes: proteins, fibre and other dietary components

7.4.1 Proteins

Proteins[27] should account for 15–20 per cent of total caloric intake, 0.8–1 gram per kilogram of body weight. This amount should increase during pregnancy, breast feeding, infancy and childhood, illness and diseases.

Proteins, unlike fats and carbohydrates, contain nitrogen. They are usually large molecules composed of amino acid, nine of which are ‘essential’ (Table 7.7) as they cannot be synthesized and must be supplied by the diet. Foods that have all nine essential amino acids present are considered complete. These foods are of animal origin, e.g. meat, eggs, milk, fish and poultry.

Amino acids are the basic building blocks of the body and thus proteins are needed to build and maintain specific tissues. Proteins are normally not energy suppliers, but in cases of stress, diseases or fasting, when there is either an increased need of energy or the caloric intake is not sufficient to cover the body needs, they are used to provide energy. Thus, a diet with the right amount of

Table 7.7 The nine essential amino acids

Histidine
Isoleucine
Leucine
Lysine
Methionine
Phenylalanine
Threonine
Tryptophan
Valine

carbohydrate has a protein-sparing effect. However, dietary protein intake must be balanced, as large amounts can cause gout or kidney stones in some individuals, as purine breaks down to uric acid, and high concentrations can crystallize in the kidneys and joints. Moreover, excess nitrogen is a burden for the kidneys and animal food proteins are also rich in fat, especially saturated types, which cause cardiovascular disease and obesity. According to the ADA dietary guidelines, 'the intake of protein in the usual range does not appear to be associated with the development of diabetic nephropathy, although it is advisable to avoid intakes superior to 20 per cent of the total daily energy'.

7.4.2 Fibre

Fibre[28] is the indigestible part of plant foods. There are two types of fibre: soluble and insoluble. Soluble fibres, such as gums and mucilages, form gels with pectin or gums in the intestine and thus slow the rate of nutrient absorption, helping to reduce postprandial glucose levels. Insoluble fibres, such as lignins, celluloses and hemicelluloses, increase bulk and decrease transit time. Both types are useful as they prolong gastric emptying, shorten intestinal transit time, prevent constipation and bind cholesterol, thus limiting its absorption. Food rich in fibre include vegetables, fruits, whole wheat, bran, cereals and legumes. An adequate diet should provide 30 grams of fibre a day.

7.4.3 Vitamins and minerals

A well-balanced diet does not need supplementation of vitamins and minerals. Only in certain cases such as illness, stress or pregnancy is a higher intake required. High doses of dietary antioxidants such as vitamins C and E, selenium, beta-carotene and other carotenoids have been prescribed to people with diabetes.[29] Antioxidants are substances that neutralize the action of free radicals, which are molecules that damage cells, and increase the risk of cancer and heart diseases. In this case they are thought to protect LDL particles from oxidation. However, according to the ADA dietary guidelines, 'routine supplementation of the diet with antioxidants is not advised because of uncertainties related to long-term efficacy and safety'.

The role of microelements zinc and chromium in glycaemic control is also difficult to determine, as they are present in minute amounts and their deficiency is not easily assessed. The ADA dietary guidelines recommend the intake of 1.000/1.500 mg/day of calcium to older people to reduce the risk of osteoporosis.

7.4.4 Sodium

According to the ADA the amount of sodium in the diet should be limited as it helps reduce blood pressure and the tendency to retain fluids. The main source of sodium in the diet is common table salt. However, as natural foods contain the right amount for the body's need, the general consumption of salt is too high and

the recommended goal is to reduce sodium intake to 2400 mg or sodium chloride (salt) to 6000 mg/day. Snacks, pickles, bacon, sauces, olives, chips and processed foods in general contain too much salt and should be cut down.

7.4.5 Alcohol

Type 1 diabetes patients must pay particular attention to alcohol, as it can cause some problems such as lowering blood sugar levels. Otherwise patients with diabetes should take the same precautions as the general population. In fact according to the ADA dietary guidelines 2002, men should have no more than two alcohol-containing drinks daily, and women one. There should be no more than 15 g of alcohol per drink. Alcohol should be drunk at mealtimes and never in exchange for regular food in order to balance calories. Alcohol gives 7 kcal/g, stimulates appetite and also has an adverse effect on self-control, so people with type 2 diabetes should limit their alcohol consumption to special occasions only, especially when on a weight-loss programme. Alcohol also increases serum triglyceride levels. In certain conditions such as pregnancy or for medical problems such as pancreatitis, neuropathy and severe hypertriglyceridaemia, total abstention is advisable. An overall summary of a nutritional guideline is presented in Table 7.8. In general, a good nutritional diet that is low in fats and salt is important. For someone with type 1 diabetes, regular mealtimes and snacks and the right proportion of nutrients should be emphasized. Someone with type 2 diabetes, where in most cases weight reduction is necessary, not only should consider caloric intake, but also the component and type of food that is eaten.

7.5 Future trends

The trend is more and more towards prevention. The International Diabetes Federation (IDF) and other diabetes associations with the aid of the World Health Organization should join forces to put pressure on governments to pay more attention to diabetes. At the moment, however, not much can be done for type 1 diabetes, but IDF hopes that as subjects at high risk for type 2 diabetes are easily identifiable, in the future governments will collaborate in changing population behaviours, for example with advertisements and campaigns against junk food, inactivity and obesity. IDF also states that as good metabolic control can prevent the complications of diabetes, it will ask governments to push for better treatment as well.

In the United States the National Diabetes Education Program (NDEP), a joint effort of the National Institutes of Health (NIH) and Centers for Disease Control and Prevention (CDC) is directing a national awareness campaign based on the Diabetes Prevention Program clinical trial against type 2 diabetes called ‘Small Steps. Big Rewards. Prevent Type 2 Diabetes’. The message is that diabetes can be prevented through modest lifestyle changes and a loss of 5–7 per cent of body weight.

Table 7.8 Nutritional guidelines for diabetes

Type 1 diabetes	Type 2 diabetes
Non-obese patient	Overweight or obese patient
Normal caloric intake	Low-calorie diet
Match food intake to insulin	No
Regular times for meals and snacks	Advisable
Each meal must have the right proportion of nutrients	Not necessary
Extra food needed for exercise/illness to prevent ketosis	Not necessary as the aim is weight loss
Prefer lean cuts of beef, lamb, veal, pork, skimmed or low-fat milk, low-fat cheeses, low-fat yogurt, olive oil, rice, pasta, bread, wholegrain cereals, legumes, vegetables and fruits	Relevant
Cut down fats, bacon, sausages, commercially baked goods, cream, cheeses, butter, margarine, salad dressings made with cream and eggs, junk food such as potato chips, cookies, sour cream, sauces, gravies fried foods	Relevant
Use lemon or lime juice, vinegar, low-calorie salad dressings, spices or herbs	Relevant
Prefer broiling, grilling or baking foods	Relevant
Reduce salt intake	Relevant
Reduce alcohol	Relevant
Drink plenty of water	Relevant

According to the ADA Position Statement 2002, although there is evidence that type 2 diabetes can be prevented or delayed,

> it is not yet known whether the successful interventions will cost-effectively reduce the morbidity and mortality associated with diabetes. Diabetes prevention policies that focus on lifestyle modification, specifically modest weight loss and increased physical activity, are also very likely to have additional health benefits.

Furthermore, the statement encourages public health professionals and systems to encourage changes to achieve a healthy lifestyle.

7.6 Sources of further information and advice

7.6.1 Important Internet sites

American Diabetes Association – The American Diabetes Association is the nation's leading non-profit health organization providing diabetes research, diabetes education and advocacy for people. Their web link 'ADA store' lists excellent reference books and has the latest books about nutrition and diet related to diabetes.
www.diabetes.org

Children with Diabetes On-line community Children with Diabetes is the online community for parents, kids, adults, and families living with type 1 diabetes. It also contains other information about diabetes and activities specifically designed for diabetics and their families.
www.childrenwithdiabetes.com

National Institute of Diabetes & Digestive & Kidney Diseases National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health. We conduct and support biomedical research. The official site of the NIDDK of the National Institutes of Health.
www.niddk.nih.gov

Diabetes This is the official journal of the ADA. It contains current research and medical articles and reviews about diabetes.
www.diabetes.diabetesjournals.org

Juvenile Diabetes Research Foundation International JDF was founded in 1970 by parents of children with diabetes. Their mission: to find a cure for diabetes. This site is full of useful information.
www.jdrf.org

CDC Diabetes Public Health Resource CDC's Diabetes Public Health Resource is the official site of the Centers for Disease Control and Prevention's Division of Diabetes. It contains a wealth of information concerning diabetes.
www.cdc.gov/diabetes

Welcome to Diabetes UK Welcome to Diabetes UK is the leading UK charity working for people with diabetes.
www.diabetes.org.uk

7.6.2 Books

Mayo Clinic Diet Manual: A Handbook of Nutrition Practice, by Jennifer K. Nelson. W.B. Saunders.

Changing Therapies for Type 2 Diabetes, edited by R. David Leslie and Paolo Pozzilli. Martin Dunitz Ltd.

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8

Nutritional risk factors in the development of type 1 and type 2 diabetes

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8.1 Introduction

Type 1 and type 2 diabetes have been recognised as two different disease entities since the early 1970s. Quite different nutritional factors are thought to influence the development of these two types of diabetes. Type 1 diabetes is characterised by progressive beta-cell destruction, which leads to complete insulin deficiency. In children there is epidemiological evidence that an early introduction of cow's milk and/or cereals, high intake of nitrites and *N*-nitroso compounds or high linear height and weight gain increase the risk of type 1 diabetes, whereas breastfeeding, vitamins C, D and E, and zinc may protect from this disease. Type 2 diabetes is characterised by insulin resistance and impaired insulin secretion at the time of appearance of hyperglycaemia and clinical diabetes. Obesity, particularly of abdominal type, and sedentary lifestyle are well-known risk predictors of type 2 diabetes. High (saturated) fat intake seems to be related to insulin resistance, obesity and increased risk of type 2 diabetes, whereas diet high in fibre may protect from insulin resistance, glucose intolerance and diabetes.

8.2 Nutritional risk factors in the onset and prevention of type 1 diabetes

Type 1 diabetes is considered an immune-mediated disease, in which signs of beta-cell autoimmunity can be detected at variable times before the diagnosis of

clinical disease (Knip 2002). Large geographical differences in incidence and linearly increasing incidence seen in many countries during the last five decades cannot be explained solely by genetic factors (Onkamo *et al*. 1999; Karvonen *et al*. 2000; Green *et al*. 2001). The relatively low concordance of identical twins also confirm the important role of environmental factors in the aetiology of this disease (Barnett *et al*. 1981). So far there is little firm evidence on the role of nutritional factors. Breastfeeding, vitamins C, D and E, nicotinamide and zinc have been reported as possibly protecting from type 1 diabetes, whereas *N*-nitroso compounds, cow's milk, some cereals, increased linear growth, and obesity may increase the risk (Virtanen & Knip 2003).

8.2.1 Increased height and weight gain

Birth height and weight of children who later developed type 1 diabetes have been similar to the controls in most of the studies (Virtanen & Knip 2003), although in some studies cases have been longer and weighted more than controls at the time of birth (e.g. Dahlquist *et al*. 1999). In the only cohort study available, higher birth weight was related to an increased risk of type 1 diabetes (Stene *et al*. 2001). Increased height gain during childhood seems to be related to greater risk of type 1 diabetes (e.g. Blom *et al*. 1992; Price *et al*. 1992). Higher weight gain in infancy is consistently related to greater risk of type 1 diabetes according to case-control evidence (e.g. Baum *et al*. 1975; Hyppönen *et al*. 1999), whereas the findings on the role of weight gain after infancy are inconsistent (e.g. Blom *et al*. 1992; Hyppönen *et al*. 2000). Clearly results from cohort studies are awaited to settle the putative importance of height and weight gain in the development of this disease.

8.2.2 Maternal diet

Maternal diet and composition of breast milk may play a role in the development of immune-mediated diseases. It has been shown that small amounts of cow's milk proteins may be carried over to breast milk from the maternal diet (Axelsson *et al*. 1984), and sensitive infants may develop cow's milk allergy on exclusive breastfeeding (Høst 1994). Per capita coffee consumption correlated positively with incidence of type 1 diabetes in an international ecological comparison (Tuomilehto *et al*. 1990). However, maternal coffee or tea consumption during pregnancy was not related to the risk of type 1 diabetes in the offspring in two case-control series (Virtanen *et al*. 1994a; Soltész *et al*. 1994). A positive association was seen between maternal nitrite intake and the risk of diabetes in the child independently of the child's own intake and when adjusted for several sociodemographic factors (Virtanen *et al*. 1994b). Paternal use of coffee or tea or intake of nitrate or nitrite at the time of conception was not related to the risk of diabetes in the offspring (Virtanen *et al*. 1994a,b). In a case-control study maternal supplementation of cod liver oil during pregnancy was inversely related to the risk of type 1 diabetes in the offspring, suggesting that

either vitamin D, vitamin A or n-3 fatty acids, which are all abundant in cod liver oil, play a role in the development of this disease (Stene *et al*. 2000).

8.2.3 Infant feeding patterns

Whether breastfeeding protects from or early introduction of supplementary foods causes type 1 diabetes, remains unsolved, although these aspects of the diet have received more research attention than many other areas in the etiology of type 1 diabetes (Virtanen & Knip 2003). Findings from prospective studies with enough statistical power are awaited.

Putative protecting effects of breastfeeding could be due to protection against infections provided by breast milk through, for example, secretory IgA antibodies and enhancement of the infant's own immune responses, increased beta-cell proliferation (Juto 1985), or delayed exposure to foreign food antigens. Breast milk contains cytokines and growth factors, which affect the maturation of the gut-associated lymphoid tissue (GALT) (Srivastava *et al*. 1996).

An early introduction of cow's milk-based infant formulas and other cow's milk products may increase the risk of type 1 diabetes according to case-control evidence, although the results remain inconclusive (Virtanen *et al*. 1991; Virtanen & Knip 2003). An early introduction of cow's milk or a short exclusive breastfeeding were not related to early stages of beta-cell autoimmunity in birth cohort studies of individuals with increased genetic risk of type 1 diabetes (Norris *et al*. 1996; Couper *et al*. 1999; Hummel *et al*. 2000; Kimpimäki *et al*. 2001; Norris *et al*. 2003; Ziegler *et al*. 2003), but inversely to the development of four type 1 diabetes-associated autoantibodies out of the four studied (Kimpimäki *et al*. 2001). The findings from a pilot study of the only randomised trial available suggest that beta-cell autoimmunity can be prevented or delayed by giving hydrolysed infant formula instead of regular cow's milk-based one (Åkerblom *et al*. 1999).

Several theories try to explain the putative diabetogenicity of cow's milk (Knip & Åkerblom 1998). Early immunisation to bovine insulin may be related to the development of beta-cell autoimmunity (Vaarala *et al*. 1999). Abnormal tolerance development has been observed in those infants who develop early signs of beta-cell autoimmunity (Vaarala *et al*. 1999). The putative diabetes-promoting effects of dietary antigens may be mediated through GALT. Food proteins may induce beta-cell autoimmunity also because of changes in gut permeability due to microbial infections. Greater weight gain related to greater intake of energy has been observed in infant formula-fed compared with breast-fed infants from 3 months of age (Heinig *et al*. 1993). By increasing insulin demand, increased weight gain caused by supplementary feeding could be a contributory factor in the development of diabetes. An early exposure to cow's milk and rapid growth in infancy were both independent risk factors of childhood type 1 diabetes in one case-control series (Hyppönen *et al*. 1999).

Recently, it was suggested that exposure to gluten-containing cereals and rice at the age of 4 to 6 months would protect from development of early beta-cell

autoimmunity compared to earlier or later exposure (Norris *et al*. 2003). German birth cohort findings related early gluten exposure to development of early beta-cell autoimmunity (Ziegler *et al*. 2003).

8.2.4 Cow's milk and other foods

Cow's milk consumption may be diabetogenic also during childhood according to case-control and cohort findings (Verge *et al*. 1994; Virtanen *et al*. 1998, 2000). In an Australian case-control study, cereal consumption was positively related to the risk of diabetes, although the association disappeared after adjustment for other dietary factors (Verge *et al*. 1994). The cell-mediated immune response to gluten was detected more frequently among newly diagnosed children with type 1 diabetes than among controls (Klemetti *et al*. 1998).

8.2.5 Dietary toxins

Case-control studies suggest that dietary *N*-nitroso compounds (Dahlquist *et al*. 1990) and nitrite (Dahlquist *et al*. 1990; Virtanen *et al*. 1994b) increase the risk of type 1 diabetes in children. Also mother's intake of nitrite at the time of pregnancy was positively related to the risk of type 1 diabetes in children independently of child's nitrite intake (Virtanen *et al*. 1994b). Nitrate is a naturally occurring compound in vegetables. Nitrate and nitrite are both used as food additives in the processing of meat products. In food and the human gastrointestinal tract nitrate is reduced to nitrite by bacteria, and *N*-nitroso compounds are formed from nitrite in the chemical or bacterial nitrosation reaction with amino compounds (Slorach 1981). Vitamin C and alpha-tocopherol inhibit and thiocyanate ions accelerate the formation of *N*-nitroso compounds (Leaf *et al.* 1989).

Recently Bafilomycin A1, a toxin produced by *Streptomyces* species in soil, was shown to induce glucose intolerance and reduction in pancreatic islet size in mice (Myers *et al*. 2001). *Streptomyces* species can infest tuberous vegetables such as potatoes and beet.

8.2.6 Vitamins and minerals

Low groundwater zinc was associated with an increased risk of type 1 diabetes in a case-control series (Haglund *et al*. 1996). A low concentration of alfa-tocopherol in serum was related to increased risk of type 1 diabetes in a nested case-control study in adults (Knekt *et al*. 1999). Vitamin C intake from diet was not associated with type 1 diabetes in a Swedish case-control series (Dahlquist *et al*. 1990), whereas in an Australian one there were fewer users of vitamin C supplements among cases than among controls (Glatthaar *et al*. 1988). Vitamin D has been shown to prevent the development of insulitis and autoimmune diabetes in non-obese diabetic (NOD) mice (Mathieu *et al*. 1992, 1994). Vitamin D has immunosuppressive effects. Vitamin D supplementation during early infancy may protect from type 1 diabetes according to a recent case-control study from several European countries

(EURODIAB Substudy 2 Study Group 1999) and fish liver oil use during pregnancy may protect the child from type 1 diabetes (Stene *et al*. 2000). In the prospective Northern Finland mother–child cohort, both the use of vitamin D supplementation during infancy and the dose of supplementation were inversely associated with the risk of type 1 diabetes, whereas a positive association was observed between suspected rickets and risk of diabetes (Hyppönen *et al*. 2001). It should be noted that the recommended dose of vitamin D supplementation was high at that time (in 1966), 2500 IU i.e. five times the current recommendation.

8.3 Nutritional risk factors in the onset and prevention of type 2 diabetes

Obesity and sedentary lifestyle are well-established risk determinants of type 2 diabetes (Costacou & Mayer-Davis 2003). Recently two randomised trials provided evidence that type 2 diabetes can be prevented by changes in diet and in exercise pattern in men and women at high risk of the disease (Tuomilehto *et al*. 2001; Diabetes Prevention Program Research Group 2002). The beneficial role of dietary fibre and a harmful one of saturated fatty acids seem to be rather well shown, whereas evidence is contradictory as to whether dietary or supplementary antioxidant vitamins or minerals prevent the disease. Discussion on optimal proportions of fat and carbohydrate in the diet for the prevention of type 2 diabetes continues. Attention is increasingly paid to different types of fat and carbohydrate. Also, attempts to individualise the advice according to metabolic status have decreased the discrepancies in views (Grundy *et al*. 2002).

8.3.1 Energy balance

Both overall and abdominal obesity have deleterious effects on insulin sensitivity and insulin secretion and are risk predictors of impaired glucose tolerance and clinical type 2 diabetes (reviewed in Feskens 1992; Virtanen & Aro 1994; Costacou & Mayer-Davis 2003). Already a small, sustained decrease in weight improves insulin sensitivity and decreases the risk of type 2 diabetes (Tuomilehto *et al*. 2001; Diabetes Prevention Program Research Group 2002). The putative effects of dietary factors other than energy overload (such as fibre, glycaemic load, proportion of fat, type of dietary fatty acids) in the aetiology of overall and abdominal obesity remain to be settled.

Overweight and obesity have during the recent decades increased alarmingly in various populations worldwide, also among children and adolescents (e.g. Kautiainen *et al*. 2002; Jolliffe 2004).

8.3.2 Energy-yielding nutrients

High total and saturated fat intake has been linked to the development of impaired glucose tolerance and type 2 diabetes by the findings of several long-

term cohort studies (Marshall *et al*. 1994; Feskens *et al*. 1995; van Dam *et al*. 2002). In the Health Professionals' follow-up study, the significance disappeared after adjustment for body mass index (van Dam *et al*. 2002). However, obesity may well be in the causal pathway between fat intake and development of type 2 diabetes (Bray & Popkin 1998). In two large women cohorts, with adjustment for body mass index, total and saturated fat intake was not associated with the development of type 2 diabetes (Meyer *et al*. 2001; Salmerón *et al*. 2001). In US women with a high intake of vegetable fat the risk of developing type 2 diabetes was reduced (Colditz *et al*. 1992; Meyer *et al*. 2001). Monounsaturated fat has beneficial effects on glucose tolerance and insulin resistance. The putative protective effect of fish/fish oils on the risk of developing glucose intolerance remains to be settled (Feskens *et al*. 1991; Bhathena *et al*. 1991).

Compared with fat carbohydrate feeding consisting of the same amount of energy may induce higher postprandial plasma glucose, insulin and triglyceride and lower HDL cholesterol concentrations (Costacou & Mayer-Davis 2003). However, the effects of various types of carbohydrate are different and difficult to separate. The proportions of monounsaturated fat and carbohydrate in the diet can depend on an individual's cultural as well as on metabolic status, e.g. hyperlipidaemic individuals should receive more energy from monounsaturated fat than from carbohydrates, whereas carbohydrate intake should be increased and fat intake decreased in individuals attempting to lose weight (Costacou & Mayer-Davis 2003).

There is increasing evidence suggesting a beneficial effect of dietary fibre on insulin sensitivity (e.g. Manolio *et al*. 1991; Feskens *et al*. 1994; Vitelli *et al*. 1996; Ylönen *et al*. 2003a), whereas the findings on the effect of dietary fibre on glucose tolerance and risk of type 2 diabetes remain somewhat controversial (Marshall *et al*. 1991; Colditz *et al*. 1992; Feskens *et al*. 1995; Salmerón *et al*. 1997a,b). However, some recent cohort studies suggest that type 2 diabetes could be prevented by increasing intake of dietary fibre, especially non-soluble cereal fibre (Salmerón *et al*. 1997a,b; Meyer *et al*. 2000; Montonen *et al*. 2003).

The glycaemic responses of foods and meals have been suggested to play a role in the development of obesity and type 2 diabetes, although not consistently so (Salmerón *et al*. 1997a,b; Meyer *et al*. 2000; Pi-Sunyer 2002).

8.3.3 Vitamins and minerals

Oxidative stress may be implicated in the aetiology of diabetes (Ho & Bray 1999). Carotenoids and vitamins C and E are important components of the human defence system against oxidative stress (Stahl & Sies 1997). Studies in humans on the effects of vitamin E on glucose or insulin metabolism are controversial (e.g. Paolisso *et al*. 1993; Facchini *et al*. 2000; Sanchez-Lugo *et al*. 1997; Ylönen *et al*. 2003b). Two prospective cohorts have provided evidence for a protective effect of vitamin E in the development of type 2 diabetes in non-supplement users (Salonen *et al*. 1995; Mayer-Davis *et al*. 2002). Fruit and vegetable intake have been inversely related to the development of type 2

diabetes in three cohort studies (Colditz *et al*. 1992; Feskens *et al*. 1995; Ford *et al*. 2001), providing indirect evidence on the putative protective effect of carotenoids. A long-term randomised trial did not show any effect of beta-carotene supplementation on the incidence of type 2 diabetes (Liu *et al*. 1999). In a long-term cohort serum beta-carotene was not related to the incidence of type 2 diabetes (Reunanen *et al*. 1998).

Cross-sectional findings on the associations between dietary and serum carotenoids and measures of glucose and insulin metabolism are inconsistent (e.g. Ford *et al*. 1999; Facchini *et al*. 2000; Ylönen *et al*. 2003b). High dietary intake of vitamin C was related to a lower incidence of type 2 diabetes in a long-term cohort study (Feskens *et al*. 1995). Some minerals such as potassium, magnesium, chromium and zinc may affect glucose and insulin metabolism, but their putative effects on the development of glucose intolerance and type 2 diabetes remain to be elucidated (Costacou & Mayer-Davis 2003).

8.3.4 Foetal nutrition and early growth

According to the so-called thrifty phenotype hypothesis, conditions such as disturbed nutrition during foetal time or early infancy could cause structural or functional changes in muscles, liver or pancreas and therefore predispose to later disorders of glucose and insulin metabolism (Hales & Baker 1992). Low birth weight and birth thinness reflect foetal growth disturbance and have been related to an adverse profile of later glucose and insulin metabolism and to an increased risk of type 2 diabetes according to most of the studies (Hales & Baker 1992; Newsome *et al*. 2003). Catch-up growth may be detrimental to long-term survival and may increase the risk of type 2 diabetes (Forsen *et al*. 2000; Hales & Ozanne 2003).

8.4 Conclusions

Short, exclusive breastfeeding or an early age at introduction of supplementary feeding has been suggested as a risk determinant of both type 1 and type 2 diabetes (Pettitt *et al*. 1997). Increased weight gain may be a risk predictor of not only type 2, but also of type 1 diabetes (e.g., Hyppönen *et al*. 2000; Wilkin 2001; EURODIAB Substudy 2 Study Group 2002). Overweight was associated with the presence of autoantibodies to glutamate decarboxylase (GAD) antibodies in unaffected male first-degree relatives of subjects with type 1 diabetes (Weets *et al*. 2001) and among glucose-intolerant men and women (Rolandsson *et al*. 1999). Evidence is inconclusive whether type 1 and type 2 diabetes overlap within families (e.g. Dahlquist *et al*. 1989; Quatraro *et al*. 1990; Douek *et al*. 2002).

Both types of diabetes are increasing rapidly in many countries in various parts of the world. For type 2 diabetes we already have well-established means to prevent or at least to delay the onset of the disease. Large health programmes

are needed to promote exercise and diets high in fibre and low in saturated fatty acids and to prevent and decrease obesity in high-risk groups as well as in the total population. The preventive measures should start from childhood.

We cannot yet prevent type 1 diabetes. Research resources need to be allocated increasingly to study environmental risk determinants of this disease with high economical and human costs. If a dietary factor/s turned out to either prevent or cause type 1 diabetes, a safe and cost-effective way to prevent diabetes at the population level would be available.

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Part II

Phytochemicals and cardiovascular disease

9

Flavonoids and cardiovascular disease

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9.1 Introduction: classification, chemical structures and occurrence of flavonoids in plant foods

Clinical observations, basic science and several epidemiological studies have contributed to an emerging body of evidence for a potential role of flavonoids in the prevention of cardiovascular disease (CVD) (Hollman and Katan, 1999). Flavonoids have been shown to inhibit the oxidation of plasma low-density lipoprotein (LDL), decrease platelet function and to modulate cytokines and eicosanoids involved in inflammatory responses (De Whalley *et al.*, 1990; Murphy *et al.*, 2003). Several epidemiological studies suggest a protection of a high flavonoid intake on the mortality of coronary heart disease (CHD) (Hollman *et al.*, 1996a; Rimm *et al.*, 1996a; Knekt *et al.*, 1996; Yochum *et al.*, 1999). Some prospective studies on major flavonoid sources, such as tea, have, however, shown large discrepancies in the relative risk (RR) of death from CHD with RRs ranging from 0.42 (Hertog *et al.*, 1993a), 0.62 (Yochum *et al.*, 1999), 1.08 (Rimm *et al.*, 1996a), to 1.6 (Hertog *et al.*, 1997). Also a recent study on the risk of CVD in women failed to show a protective effect of flavonoid intake on the risk of CVD (Sesso *et al.*, 2003). Although the picture from epidemiological studies on the relationship between risk of CVD and intake of flavonoids is inconsistent, the majority of the studies suggest an inverse association between intake of flavonoids and the risk of CVD, which is supported by basic and clinical studies on flavonoids, indicating that flavonoids may have a protective action that deserves further investigations before final conclusions can be drawn.

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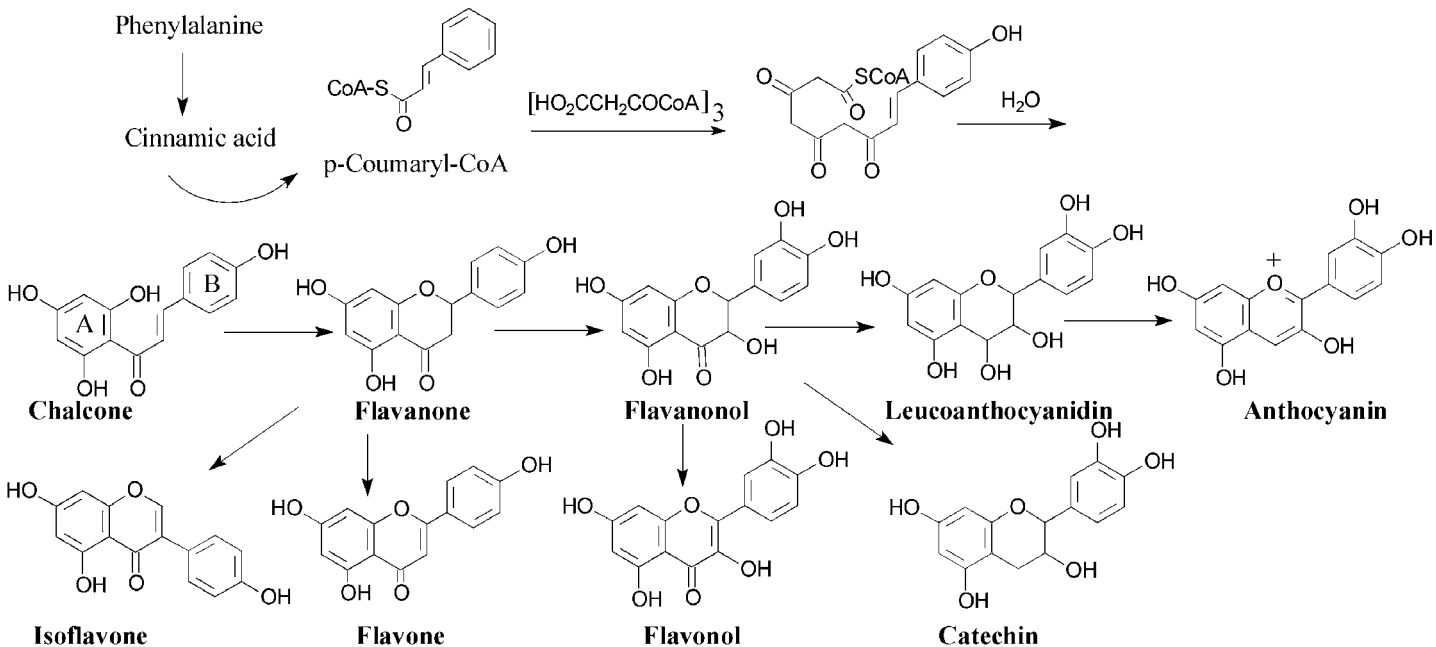


Fig. 9.1 Biosynthesis pathways of dietary flavonoids in plants.

The flavonoids constitute a large class of polyphenols that are found ubiquitously in the plant kingdom and are thus present in fruits and vegetables regularly consumed by humans. They account for a variety of colours in flowers, berries and fruits, from yellow to red and dark purple. The flavonoids are biosynthesised from phenylalanine (ring B) and three acetate units (ring A), giving the *chalcones* as the first identifiable intermediate (see Fig. 9.1) (Herbert, 1989). Ring closure of the chalcones gives rise to the *flavanones*, which can be further oxidised or derivatised to *flavanonols*, *flavones* or *flavonols*. Reduction of the carbonyl group in the 4-position and subsequent removal of the hydroxyl group result in the formation of the *catechins*, whereas oxidation of the C-ring affords the *anthocyanins*. Rearrangement of the flavonoid skeleton by an intramolecular 1,2-shift of the B-ring gives the *isoflavonoids*. Substituents such as hydroxyl, methoxyl and sugar moieties give rise to a multitude of different compounds, and more than 4000 different naturally occurring flavonoids have so far been described (Middleton and Kandaswami, 1994).

In plants, the majority of the flavonoids are found as glycosides with different sugar groups linked to one or more of the hydroxyl groups. They are mainly found in the outer parts of the plants, such as leaves, flowers and fruits, whereas the content in stalks and roots is usually very limited. The flavonoids located in the upper surface of the leaf or in the epidermal cells, have a role to play in the physiological survival of plants. They contribute to the disease resistance of the plant, either as constitutive antifungal agents or as phytoalexins (Harborne and Williams 2000). The flavonoids have also UV-B-protecting properties. They absorb light in the 280 ± 315 nm region, and with their almost universal presence in green leaves, they protect the underlying photosynthetic tissues from damage (Harborne and Williams, 2000). Also in the peel of fruits, the flavonoids can act as UV-B filters, e.g. in apple skin, quercetin glycosides have UV-B protective capacity and accumulates in the skin of certain apple sorts, when exposed to UV-B radiation (Solovchenko and Schmitz-Eiberger, 2003).

9.2 Dietary sources and intake levels of flavonoids

Only a few of the thousands of different flavonoids identified in plants are present in considerable amounts in the human diet, and the intake of these dietary flavonoids varies among countries and cultures (Table 9.1) The *dihydrochalcone* phloretin and its glycoside phloridzin are found in large amounts in apple, but no estimates of intake levels have been reported. The *flavanones* are present mainly in citrus fruits, predominated by naringin (the glycoside of naringenin), responsible for the bitter taste of grapefruit, and hesperidin (the glycoside of hesperetin) found in oranges. Since the intake of orange juice is extensive in many Western countries, e.g. Denmark and Finland, the intake of in particular the citrus flavanone hesperetin is very high (Kumpulainen *et al.*, 1999; Justesen *et al.*, 2000).

Table 9.1 Dietary sources and intake of flavonoids

Flavonoid subgroup	Major dietary sources	Major flavonoids	Estimated intake (mg/day)				
			Denmark[b]	U.S.[d]	Holland[e]	Finland[g]	Japan[i]
Flavanones	Citrus fruits, orange and grapefruit juice	Naringenin Hesperetin	7.1 9.3	n.d.	n.d.	8.3 28.3	n.d.
Flavones	Parsley, celery, red pepper, spices	Apigenin Luteolin	1–2	n.d.	2	n.d.	0.3
Flavonols	Onions, kale, broccoli, apples, berries, tea, wine	Quercetin Kaempferol Myricetin	8.6 3.4 1.5	16 1 5	17 4 1	7 2.2 1.1	16.4
Isoflavones	Soybeans, legumes	Genistein Daidzein	<1[c]	n.d.	n.d.	n.d.	47.2
Total flavonoid[a]			~31.5	22	24	46.9	63.9
Catechins	Tea	Epigallocatechin	45[c]	n.d.	50[f]	8.3	~40
Anthocyanins	Berries, red wine	Cyanidin	6–60[c]	n.d.	n.d.	82.5[h]	n.d.

[a] Total flavonoid are often given as a sum of the subgroups listed excl. the catechins and anthocyanins.
[b] Data from Justesen *et al.* (2000).
[c] Dragsted *et al.* (1997) based on Danish consumption levels.
[d] Sampson *et al.* (2002).
[e] Hertog *et al.* (1993c).
[f] Arts *et al.* (2001c).
[g] Kumpulainen *et al.* (1999).
[h] Average daily intake in Finland, Heinonen (2001).
[i] Data obtained from Arai *et al.* (2000).
n.d. = no data available.

The *flavanonols*, also called dihydroflavonols, are found only in trace amounts in plants, and are therefore not important constituents in the human diet (Pierpoint, 1986). The contribution of *flavones* to the human intake of flavonoids is generally limited; however, some spices and herbs contain high amounts of flavones. Parsley, for instance, contains large amounts of apigenin (Justesen *et al.*, 1998), and the highly methylated flavone tangeretin is found in the peel of citrus fruits, and can thus occur in juices made from whole fruits (Pierpoint, 1986).

The *flavonols* are one of the major groups of flavonoids present in the human diet. Quercetin is the most abundant flavonol, found ubiquitously in fruits and vegetables and is especially present in high amounts in onions, cruciferous, apples, wine and tea. Kaempferol, found in broccoli, kale and tea, and myricetin, found in tea and wine, are also major flavonols found in the human diet (Hertog *et al.*, 1992, 1993b).

The *isoflavones* are present only in legumes, especially in soybean. The European intake of isoflavones is therefore limited, but in Asia and especially in Japan, where soy are consumed in large amounts, the intake is considerable, and as seen in Table 9.1 it exceeds the intake of all other flavonoids.

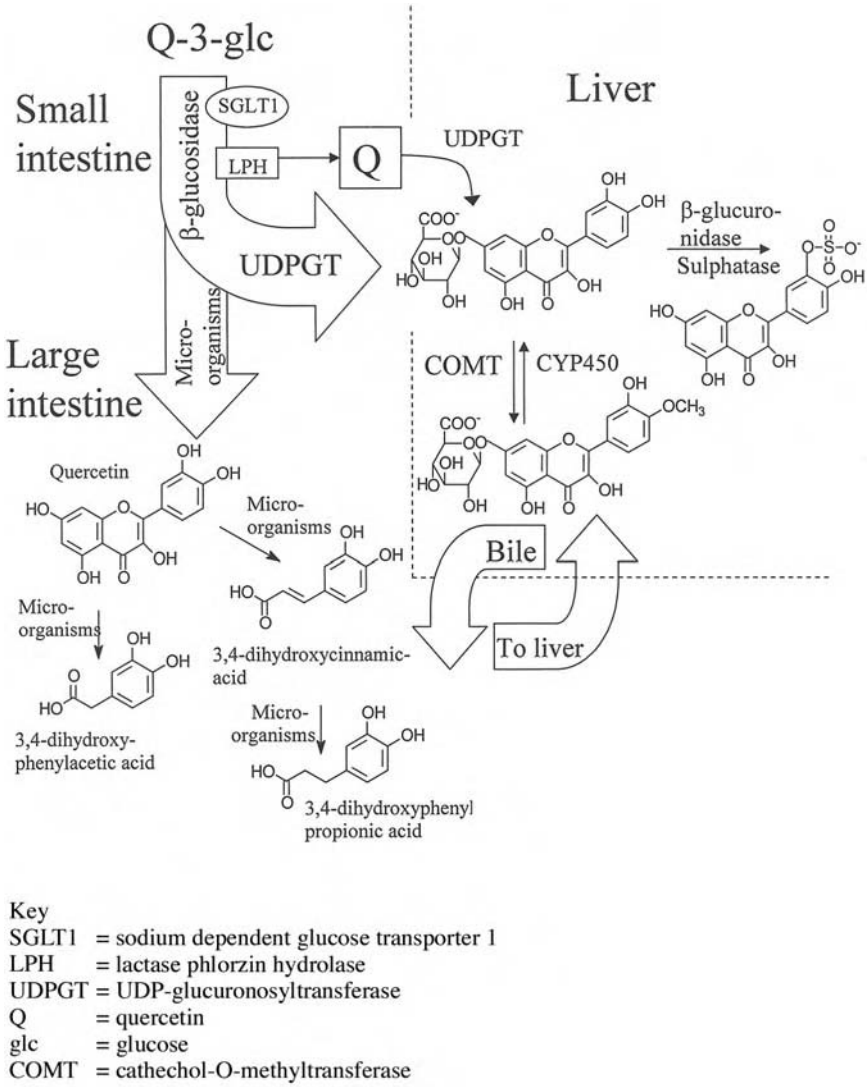
Catechins are quantitatively a quite large group within the human diet. They either occur as free catechins or are derivatised with gallic acid, and are found mainly in green and black tea, chocolate and wine (Forsyth, 1955; Rimm *et al.*, 1996a). In countries with a high intake of tea, such as Japan (mainly green tea) and the United Kingdom (black tea), the daily intake of catechins is substantial. As seen in Table 9.1, the average intake of the strongly coloured a*nthocyanins* may also be extensive, in particular for regular consumers of red wine, blackcurrant juice, berries, and red grapes.

The estimated average daily intake of flavonoids including catechins and anthocyanins is thus well above 50 mg for all countries presented in Table 9.1 and the real intake is probably higher than 100 mg/day if data on all flavonoid subgroups were available. The daily intake of other dietary antioxidants such as vitamin C (80 mg/day), vitamin E (8.5 mg/day) and β-carotene (1.9 mg/day) (Nielsen, 1999a) is comparable to or considerably lower than the intake of the flavonoids, so these compounds certainly constitute an important part of the daily intake of dietary antioxidants.

9.3 Bioavailability and metabolism of flavonoids

9.3.1 Bioavailability and absorption of the flavonoids

Until recently, it was generally accepted that the flavonoid aglycons had to be liberated from the glycosides in the large intestine, prior to absorption (Kühnau, 1976). It was thought that the hydrophilic nature of the glycosides precludes absorption in the small intestine, and that the flavonoid β-glycosides resists intestinal hydrolysis. Consequently, flavonoid glycosides would pass unaltered into the large intestine, being hydrolysed by micro-organisms to the free aglycon



Key
SGLT1 = sodium dependent glucose transporter 1
LPH = lactase phlorzin hydrolase
UDPGT = UDP-glucuronosyltransferase
Q = quercetin
glc = glucose
COMT = cathechol-O-methyltransferase

Fig. 9.2 *In vivo* metabolism of dietary flavonoids.

and sugar moiety (Hertog *et al.*, 1997) (see Fig. 9.2). This was supported by the findings that incubations of flavonoid glycosides with intestinal micro-flora resulted in the release of the free aglycons, and in germ-free rats the flavonoid glycosides were excreted unchanged with faeces (Griffiths, 1982). It was later demonstrated that human intestinal bacteria are in fact capable of hydrolysing flavonoid glycosides to the free aglycons and sugar moieties (Bokkenheuser and Winter, 1988). The intact flavonoid aglycons are then absorbed from the large intestine and enter the systemic circulation (see Fig. 9.2). The liver is the main

organ for metabolism, and here also the flavonoid aglycons are hydrolysed or demethylated by the cytochrome P450 enzyme system (CYP450) (Griffiths, 1982; Nielsen *et al.*, 1998, 2000a), methylated by catechol-*O*-methyl transferase (COMT) (Zhu *et al.*, 1994) and conjugated to glucuronic acid and sulphate esters by the Phase II enzymes (Gee *et al.*, 2000, Boersma *et al.*, 2002).

As seen in Fig. 9.2, the micro-organisms in the large intestine are also capable of degrading the flavonoid aglycons by ring fission of the C-ring, resulting in a variety of phenolic acids (e.g. phenylpropionic and phenylacetic acid derivatives) and phloroglucinol (from the A-ring) (Griffiths, 1982). These phenolic acids can thus also be absorbed from the gut, and further metabolised in the liver by the CYP450 enzyme system, resulting in either hydroxylation or methylation and further conjugated with glucuronic acid or sulphate.

During the past decade the absorption of flavonoid glycosides has been the subject of several controversies. Some research groups reported that intact flavonoid glycosides were being absorbed in humans after intake of a flavonoid-rich meal (Hollman *et al.*, 1995). However, the majority of the following studies on this matter were not able to verify any absorption of intact flavonoid glycosides in humans. In a study on naringin from grapefruit juice by Fuhr and Kummert (1995), naringin was not detectable in urine; only the aglycon naringenin was determined. Also in a study on diosmin, the 7-rutinoside of diosmetin (4′-OMe-Luteolin), failed to reveal any diosmin in urine or plasma after oral administration of diosmin to healthy volunteers (Cova *et al.*, 1992). Only the aglycone diosmetin was detected as glucuronic and sulphate conjugates. Furthermore, no apiin (apigenin-7-apiosylglycoside), the major apigenin glycoside in parsley, was detected in the urine after parsley consumption (Nielsen *et al.*, 1999b) and in a study with a fruit and vegetable mixture, only the flavonoid aglycons could be identified in urine by a liquid chromatography mass spectrometry (LC-MS) method able to reveal both flavonoid aglycons and glycosides if present (Nielsen *et al.*, 2000b, 2002). In a human study using onions as the quercetin source, Moon and colleagues (2000) were unable to determine quercetin glycosides in plasma by a very sensitive method using electrochemical detection, whereas quercetin was detectable, supporting the fact that no intact glycosides were absorbed.

Recently, several researchers have demonstrated the difficulties in distinguishing between the flavonoid glycosides and glucuronides when performing chemical analyses of urine and plasma samples. Both types of conjugates have UV-absorption spectra that are almost identical with quercetin itself (Day and Williamson 2001), and very similar retention times even with the most specific gradient systems. Using a highly sensitive and specific high-performance liquid chromatography (HPLC) method with coulometric detection and confirmation by LC-MS, it was demonstrated, that only the glucuronides and not the glucosides are present in human plasma after consumption of pure quercetin-3-glucoside or quercetin-4′-glucoside (Sesink *et al.*, 2001).

In 1995–1997 Hollman and co-workers performed several studies on the bioavailability of quercetin in humans (Hollman *et al.*, 1995, 1996b, 1997), and

it was observed that the amount of quercetin absorbed from quercetin *glucosides* was higher than from the free aglycon or other glycosides with different sugars than glucose as the terminal sugar, such as, for example, rutin. Furthermore, quercetin was also more rapidly absorbed when given as quercetin glucosides with peak plasma levels already within the first 30–60 min, whereas other quercetin glycosides from apple had peak plasma levels several hours later (9.3 h for pure rutin) (Hollman *et al.*, 1997). This was not in accordance with the general concept that the glycosides have to pass unaltered all the way to the large intestine prior to hydrolysis and absorption, since this would have required a much longer transit time than the 30–60 min observed for the quercetin mono-glucosides. Thus, the mono-glucosides must have already been absorbed in the small intestine. This was supported by the findings by Day *et al*. in 1998, demonstrating that both human and rat small intestine have *β-glucosidase* activities capable of hydrolysing flavonoid glucosides (see Fig. 9.2). Furthermore, studies by Gee *et al*. (1998, 2001) using isolated preparations of rat small intestine suggested a possible involvement of the sodium-dependent glucose transporter SGLT1 in the absorption of quercetin monoglucosides. These studies confirmed a more rapid absorption of the 3-or 4′-monoglucosides of quercetin compared to the 3,4′-diglucoside or quercetin itself. The authors concluded that there probably are two mechanisms for the transport of the quercetin monoglucosides as illustrated in Fig. 9.2

- the sugar transporter SGLT1 transport the intact quercetin glucoside into the epithelial cells, where the glucose moiety are released by β-glucosidases, and the free quercetin is then absorbed, or
- the extracellular enzyme LPH (lactase phlorizin hydrolase), which was also demonstrated to be able to hydrolyse quercetin glucosides (Day *et al.*, 2000), deglycosylate the flavonoid glucoside, prior to passive diffusion of the aglycon into the epithelial cells (see Fig. 9.2).

Furthermore, these studies verified that no intact quercetin glycosides were able to cross the intestinal epithelium, and only quercetin or quercetin glucuronides were determined after transfer. Recent studies on quercetin glucosides by the same research group has further showed that the major products produced in the small intestine are the 3- and 7-glucuronides of quercetin (O'Leary *et al.*, 2003). Based on their results, they suggest, that from a normal dietary intake of quercetin only glucuronic conjugates reach the liver via the hepatic portal vein (O'Leary *et al.*, 2003). They have furthermore demonstrated that in the liver, the flavonoid conjugates are further metabolised either by methylation by COMT or by intracellular deglucuronidation by β-glucuronidase followed by sulphation to the mono-sulphate conjugate as illustrated in Fig. 9.2. In this figure, all possible routes are illustrated for deglycosylation, absorption and metabolism of a flavonoid glucoside as it is presently thought to occur in the body. Flavonoids with other sugars attached than glucose, pass unaltered down to the large intestine, where the micro-flora can cleave the glycosidic bond and degrade the resulting flavonoid aglycone as seen in Fig. 9.2 and as reviewed by Kühnau (1976).

9.3.2 Metabolism of flavonoids

Although an extensive number of studies have reported effects of flavonoids on enzymatic, biological and physiological processes, only very few researchers have attempted to determine the actual compound/metabolite responsible for the observed effects. It has generally been assumed that the biological activities originated from the flavonoids investigated, although they may be bio-transformed into one or more structurally quite different compound *in vivo*. Investigations on *in vitro* metabolism of flavonoids have so far been limited. The synthetic flavonoids, α- and β-naphthoflavone, well-known inducers and inhibitors of monooxygenase activities, have been shown to be extensively hydroxylated by cytochrome P450 (Vyas *et al.*, 1983). The polymethoxylated flavone tangeretin was shown to be demethylated *in vitro* by the cytochrome P450 system, although the structures of the metabolites were not elucidated in this study (Canivenc-Lavier *et al.*, 1993).

The first systematic investigation of the structural requirements for metabolism of flavonoid aglycons by the cytochrome P450 enzyme system was provided from our laboratory only a few years ago in a study, where 16 different flavonoids were incubated with rat liver microsomes (Nielsen *et al.*, 1998). It was shown that the flavonoids naringenin, hesperetin, chrysin, apigenin, tangeretin, kaempferol, galangin and tamarixetin all were extensively metabolised by Aroclor-induced rat liver microsomes and to a minor extent by uninduced microsomes. All metabolites were isolated and their structures elucidated by LC-MS and ^{1}H NMR (nuclear magnetic resonance). The identity of the metabolites was consistent with a general metabolic pathway leading to the corresponding 3′,4′-dihydroxylated flavonoid either by hydroxylation or demethylation. No metabolites were, however, detected from eriodictyol, taxifolin, luteolin, quercetin, myricetin, fisetin, morin or isorhamnetin. Structural requirements for microsomal hydroxylation by the cytochrome P450 enzyme system thus appeared to be only a single or no hydroxyl group on the B-ring of the flavan nucleus. The presence of two or more hydroxyl groups on the B-ring seemed to abolish further hydroxylation. Furthermore, the results indicated that demethylation only occurs in the B-ring, when the methoxyl group is positioned at the 4′-position as in tamarixetin, and not in the 3′-position as in isorhamnetin. The CYP1A isozymes were found to be the main enzymes involved in flavonoid hydroxylation, whereas other cytochrome P450 isozymes seemed to be involved in the flavonoid demethylation (Nielsen *et al.*, 1998). These findings with rat liver microsomes were later verified in mouse and human liver microsomal preparations, where identical metabolic patterns were observed for the flavonoids (Breinholt *et al.*, 2002).

Most of the research on *in vivo* metabolism and disposition of flavonoids in experimental animals was performed in the 1960s and 1970s. These investigations have been thoroughly reviewed by Griffiths (1982), Hackett (1986) and by Hollman and Katan (1997). Except for the ring cleavage products, the only metabolites identified from flavonoid aglycons in rodents have been: isorhamnetin and tamarixetin (3′- or 4′-methoxyquercetin) from administration

of quercetin (Ueno *et al.*, 1983), 4′-hydroxy- and 3′,4′-dihydroxy-flavone from flavone, apigenin (3′-hydroxychrysin) from chrysin, and eriodictyol (3′-hydroxynaringenin) from naringenin (Hackett, 1986).

We recently investigated the *in vivo* metabolism of the polymethoxylated flavonoid tangeretin in order to evaluate the relevance of the identified *in vitro* metabolic pathways (Nielsen *et al.*, 2000a; Rasmussen and Breinholt 2003). Urine collected consecutively during 24 hours was enzymatically hydrolysed to release the flavonoid aglycons from glucuronic acid or sulphate ester conjugates, and, by means of LC-MS and proton NMR, the presence of ten metabolites of tangeretin were identified. The metabolites were either demethylated or hydroxylated derivatives of the parent compound. The changes were again found primarily to occur in the B-ring of the compound as also observed in the *in vitro* metabolic studies (Nielsen *et al.*, 1998, 2000a). Thus although the metabolism of flavonoids by CYP450 and COMT is limited for the majority of the dietary flavonoids, where only minor amounts of metabolites are produced, it is important that this endogenous metabolism is taken into account for some flavonoids, e.g. quercetin and tangeretin, where the metabolism is extensive. The metabolites produced are chemically very different from the parent compound and thus have the potential to exert biological effects other than those produced by the parent compound. The methylated derivatives of quercetin, isorhamnetin and tamarixetin, are both less polar and have a lower antioxidative potential than quercetin itself. On the contrary, the demethylated metabolites of tangeretin presumably have a higher antioxidative potential than the fully methoxylated compound, tangeretin.

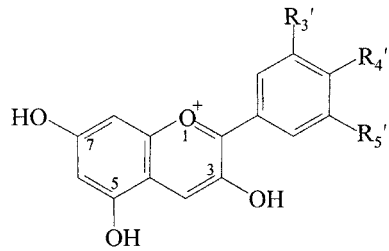
It is furthermore important to bear in mind that the majority of the flavonoids that reach the systemic circulation are conjugated by the Phase II enzyme system to glucuronic and sulphate conjugates, and that it thus are these compounds that have the potential to exert biological effects in the body.

9.4 Uptake and excretion of anthocyanins

9.4.1 Occurrence and dietary intake of anthocyanins

The red, violet or blue anthocyanins, found in most berries and fruits, belong to the group of flavonoids. The anthocyanins consist of an aglycon, the anthocyanidin, linked to a sugar moiety. The six most frequently found aglycons in fruits and berries are seen in Fig. 9.3. These aglycons may be glycosylated or acylated by different sugars and acids in different positions. The most common glycoside moieties found in anthocyanins are the 3-monosides, 3-biosides, 3-triosides and 3,5-diglycosides (Strack and Wray, 1986).

Anthocyanins are as the other classes of flavonoids present in most higher plants. They are biosynthesised in the vacuola with naringenin as the flavanone precursor (see Fig. 9.1). Naringenin is converted by a monooxygenase to the flavononol, dihydroxykaempherol, which can be hydroxylated further in the B-ring, forming the precursors of the 3′,4′ or 3′,4′,5′ hydroxylated or methoxylated



Aglycon	R_3'	R_4'	R_5'
Pelargonidin	OH	H	H
Cyanidin	OH	OH	H
Delphinidin	OH	OH	OH
Peonidin	OMe	OH	H
Petunidin	OMe	OH	OH
Malvidin	OMe	OH	OMe

Fig. 9.3 The major anthocyanin aglycons found in fruits and berries.

anthocyanins. The resulting dihydroxyflavonols are reduced in the 4-position, forming *leucoanthocyanins*. The leucoanthocyanins are transformed by anthocyanidin synthase to the corresponding *anthocyanidins*. The anthocyanidins is then glucosylated by 3-*O*-glucosyltransferase. This anthocyanidin-3-*O*-glucosides can further be either glycosylated by glycosyltransferases or methylated in the B-ring by methyltransferases (Delgado-Vargas *et al.*, 2000).

The content of anthocyanins in coloured fruits and berries varies a lot, from around 3.4 mg in reddish apples, 232 mg in strawberries, 1064 mg in blackcurrants up to 3090 mg in blueberries per 100 g dried fruit or berry (Kähkönen *et al.*, 2001). The anthocyanins are responsible for the colour difference between red and white wine (Mazza and Miniati, 1993). One glass of red wine can contain up to 80 mg anthocyanin, depending on the grape variety and processing of the wine (Waterhouse, 2002). As seen in Table 9.1, the average Danish dietary intake of anthocyanins has been estimated to around 6–60 mg per day (Dragsted *et al.*, 1997) and the Finnish average intake to be 82.5 mg per day (Heinonen, 2001). Other rich dietary sources, apart from red grapes and red wine, are cherries, elderberries, blueberries and blackcurrants (Macheix *et al.*, 1990).

9.4.2 Bioavailability and metabolism of anthocyanins

In contrast to the other classes of flavonoids, the anthocyanins are absorbed as intact glycosides in both animals and humans, although in very low amounts (Cao *et al.*, 2001; Nielsen *et al.*, 2001, 2003; Wu *et al.*, 2002). We recently investigated the absorption and excretion of blackcurrant anthocyanins in both

humans and rabbits (Nielsen *et al.*, 2003). Here we found as others also have reported, that the absorption of anthocyanins in humans is fast and proportional with dose, with peak plasma concentrations after about 1 hour (Bub *et al.*, 2001; Matsumoto *et al.*, 2001; Cao *et al.*, 2001; Nielsen *et al.*, 2003). A recent study by Passamonti *et al*. (2003) suggests that the anthocyanins are absorbed already in the stomach, and they demonstrate that malvidin 3-glucoside appeared in both portal and systemic plasma only 6 min after dosage.

In our study on blackcurrant anthocyanins, we found no aglycone-dependent differences in the absorption or excretion of the cyanindin and delphinidin anthocyanins in either rabbits or humans (Nielsen *et al.*, 2003). However, in both species a significantly larger absorption was observed for the anthocyanin *rutinosides* than for the anthocyanin *glucosides*. This was also reflected in the urinary data, where the rutinosides were excreted to a significantly higher extent than the glucosides. These observations were supported in the literature, although the investigators did not address this matter specifically (Matsumoto *et al.*, 2001; Netzel *et al.*, 2001). An explanation for the lower bioavailability of the two anthocyanin glucosides than of the rutinosides might be that part of the anthocyanin glucosides are cleaved by the β-glucosidases in the small intestine, resulting in the formation of their corresponding aglycone, as observed for other flavonoids (Day *et al.*, 1998). The aglycons of anthocyanins are quickly degraded at elevated pH and are absorbed only in small quantities, if at all (Cao *et al.*, 2001; Wu *et al.*, 2002). Cleavage of part of the anthocyanin glucoside*s* thus results in a larger proportion of the rutinosides being intact and accessible for absorption and distribution in plasma and urine.

Recently, Wu *et al*. (2002) observed trace amounts of anthocyanin metabolites in human urine after treatment of humans with large doses of an elderberry extract containing 720 mg anthocyanins. The detected metabolites were the methylated and glucuronidated derivatives of the ingested anthocyanins, which is similar to the metabolic products of other flavonoids (Sesink *et al.*, 2001, Zhu *et al.*, 1994). In the same study by Wu *et al*., high doses of blueberries containing 690 mg anthocyanin did not generate detectable amounts of anthocyanin metabolites. It was speculated by the authors that this was due to the large number of different anthocyanins (at least 25) in blueberries, resulting in lower doses of each anthocyanin and consequently a metabolite concentration below the limit of detection. It is thus likely that too low doses and the low absorption of anthocyanins are responsible for the lack of positive identification of anthocyanin metabolites in other studies. This is supported by a recent study on strawberry anthocyanins containing mainly pelargonidin-3-glucoside (Felgines *et al.*, 2003). In addition to pelargonidin-3-glucoside, HPLC-ESI-MS-MS (high-performance liquid chromatography – electron spray ionisation – mass spectroscopy – mass spectroscopy) studies revealed five anthocyanin metabolites in urine: three monoglucuronides of pelargonidin, one sulpho-conjugate of pelargonidin and pelargonidin itself. Total urinary excretion of strawberry anthocyanin metabolites corresponded to 1.80 ± 0.29 per cent (mean ± SEM, $n = 6$) of pelargonidin-3-glucoside ingested. More than 80 per cent of

this excretion was present as a monoglucuronide. As soon as 4 hours after the meal, more than two-thirds of the anthocyanin metabolites had been excreted in urine, although the excretion of the metabolites continued until the end of the 24-h experiment.

The urinary excretion of anthocyanins in humans has only been followed up to 24 h at the most (Cao *et al.*, 2001), showing a total excretion of intact unmetabolised anthocyanins between 0.033 and 0.28 per cent (Netzel *et al.*, 2001; Matsumoto *et al.*, 2001; Frank *et al.*, 2003). In animals, a total excretion of around 0.36–1 per cent has been observed (Morazzoni *et al.*, 1991; Nielsen *et al.*, 2003). We recently demonstrated that the biokinetics in both rabbits and humans were comparable, and that the urinary excretion correlated well with the amount determined in plasma (R^2 = 0.773) (Nielsen *et al.*, 2003). These data confirmed that very limited amounts of the anthocyanins are bioavailable in humans, presumably well below 0.5 per cent of a given anthocyanin dosage. If the metabolites, like the glucuronides observed by Felgines *et al*. (2003) are taken into account, the total absorption of anthocyanins is still around only a few per cent of the ingested dose. Based on this low bioavailability and rapid systemic elimination, a significant contribution to health protection of dietary anthocyanins thus seems questionable.

9.5 The use of flavonoids as biomarkers

9.5.1 Flavonoid intake data in epidemiological studies

The flavonoids have been shown to exert a number of health beneficial properties in *in vitro* studies and in animal studies (for review see Harborne and Williams 2000; Nijveldt *et al.*, 2001; Kris-Etherton *et al.*, 2002). Epidemiological studies have, however, not been able to give a clear picture of the protective impact of dietary flavonoids against such diseases as cancer or coronary heart disease. In most cases, the limitation in the outcome of epidemiological studies on the health protective effects of flavonoids is the lack of precise flavonoid intake data.

The first and also one of the best attempts to generate precise flavonoid intake data was the Zutphen Elderly Study investigating the association between flavonoid intakes and risk of coronary heart diseases (Hertog *et al.*, 1993a). This study was based on a thorough chemical analysis of the content of quercetin, kaempferol, myricetin, apigenin and luteolin in 28 vegetables and 9 fruits, tea infusions, wines, and fruit juices commonly consumed in the Netherlands (Hertog *et al.*, 1992, 1993a). However, since the study included only these five dietary flavonoids, it covered only a fraction of the total amount of flavonoids found in our diet (see Table 9.1). The authors found that the total average intake of the flavonoids determined was 23 mg/day, with the flavonol quercetin as the most important dietary flavonoid (mean intake 16 mg/day) and with tea as the major flavonoid source (48 per cent of total intake), followed by onions (29 per cent), and apples (7 per cent). It is seen from Table 9.1, that the inclusion of

Table 9.2 Epidemiological studies on flavonoids and cardiovascular disease.

Study design/country	Subjects (*n*), age, sex	Follow up (year)	Flavonoid intake (mg/day)[a]	Outcome (no. of cases) [b]	Association[c]	Rate ratios and significance[d] RR; [95% CI], *P*	Reference
Prospective cohort Zutphen, Netherlands	805 65–84 y, ♂	5	25.9 61% from tea	CHD-M (43)	↓	RR 0.42 [0.20–0.88] *P* = 0.015	Hertog *et al.* (1993a)
Cross-cultural correl 7 countries	16 cohorts, n = 12 763 40–59 y, ♂	25	2.6–68.2	CHD-M	↓[e]	–	Hertog *et al.* (1995)
Prospective cohort Zutphen, Netherlands	552 50–59 y, ♂	15	23.8 ± 7.6 70% from tea	Stroke (42)	↓	RR 0.27 [0.11–0.70][i]	Keli *et al.* (1996)
Prospective cohort Finland	5133 30–69 y, ♂,♀	20–25	3.4 (0-41.4), 95% quercetin onions, apples	CHD-M (♀: 149, ♂: 324)	→	♀: RR 0.54 [0.33–0.87] *P*<0.01 ♂: RR 0.78 [0.56–1.08] *P* = 0.24	Knekt *et al.* (1996)
Prospective cohort US	34 789 40–75 y, ♂	6	20.1 25% from tea, 25% from onion	CHD (496)	→	RR 1.08 [0.81–1.43][i]	Rimm *et al.* (1996a)
Prospective cohort Wales, UK	1900 45–59 y, ♂	14	26.3 ± 12.5 82% from tea	CHD (186) CHD-M (131)	→ ↑	RR 1.0 [0.6–1.6] *P* = 0.996 RR: 1.6 [0.9–2.9] *P* = 0.119	Hertog *et al.* (1997)
Prospective cohort Iowa, US	34 492 55–69 y, ♀	10		CHD-M (438)	↓	RR: 0.62 [0.44–0.87] *P* = 0.04	Yochum *et al.* (1999)

Prospective cohort Finland	26 593 50–69 y ♂ smokers	6.1	8.0	Stroke (736)	→	RR 0.98 [0.80–1.21] $P = 0.81$	Hirvonen *et al.* (2000)
Prospective cohort, Zutphen, Netherlands	806 65–84 ♂, ♀	10	72 ± 47.8 catechin, 87% from tea	CHD-M (90)	↓	RR 0.48 [0.28–0.82], $P = 0.007$	Arts *et al.* (2001a)
Prospective cohort Iowa, US	34 492 55–69 y, ♀	13	25.4 (0–278) catechin	CHD-M (767)	↓[g]	RR: 0.76 [0.58–1.03]	Arts *et al.* (2001b)
Prospective cohort, Finland	10 054 39.3 ± 15.8 y ♂,♀	28	24.2 ± 26.7 83% naringenin, hesperetin	CHD-M (681)	↓[h]	RR 0.79 [0.63–0.99] $P = 0.02$ (quercetin)	Knekt *et al.* (2002)
Prospective cohort, Rotterdam, Netherlands	7983 ≥ 55 y, ♂,♀	5.6	28.6 ± 12.3	CHD-M (146)	↓	RR 0.35 [0.13–0.98][i]	Geleijnse *et al.* (2002)
Prospective cohort, US	38445 45–89 y, ♀	6.9	24.6 ± 18.5 70% quercetin	CVD (729)	→	RR 0.87 [0.69–1.10], $P = 0.49$	Sesso *et al.* (2003)

[a] Data are given as average intake (mg/day) and SD or range is given when available.
[b] CHD = coronary heart disease, CVD = Total cardiovascular disease, M = mortality. Number of cases is given in parenthesis.
[c] ↓ = Inverse association between disease and flavonid intake.
[d] RR of the highest vs. the lowest category is given, P for trend is given when available.
[e] 25% of the variance in CHD-M in the 16 cohorts could be explained by flavonoid intake, $r = -0.50$, $P = 0.048$.
[g] Only inverse association with catechins from other sources than from tea. RR for tea catechins: 1.00 [0.77–1.29]
[h] Only quercetin and kaempferol intake (RR 0.82 [0.66–1.02]) lowered the risk for CHD-M.
[i] P value not reported.

especially the citrus flavonoids, but also of the catechins and anthocyanins in this study would have revealed other major flavonoid sources and altered the estimated flavonoid intake dramatically. Thus a stronger conclusion on the health effects of dietary flavonoid intake might have been drawn.

The majority of the epidemiological studies that followed this first attempt to investigate the health protective effects of dietary flavonoids have all been based on the same set-up or with even poorer tools to estimate the habitual flavonoid intake (see Table 9.2). For example, in the Seven Country Study, the average flavonoid intake was estimated by analyses of a few food samples that represented the average daily intake of flavonoid-containing foods in each country.

Additional flavonoid compounds, such as the citrus flavonoids and the catechins, were included in later epidemiological studies by Knekt *et al.* (2002) and Arts *et al*. (2001a,b), which investigated the association of these flavonoids with the risk of cardiovascular disease (see Table 9.2). However, in the study by Arts *et al*. on a cohort of postmenopausal women from Iowa, the estimated catechin intake was merely based on older analyses of catechin content in Dutch food (Arts *et al.*, 2001b). Furthermore, the semi-quantitative food frequency questionnaire (FFQ) used, with 127 items, was not designed to evaluate catechin intake. Some items in the FFQ referred to more than one food, e.g. 'fresh apples or pears', and evaluation of the FFQ's ability to asses catechin intake showed correlation coefficients between 0.45 and 0.83 for the main sources of catechins.

The drawback in prospective studies is thus often that the FFQ used at baseline is insufficient and too unspecific at the time of follow up, and this may cause misclassifications of the dietary exposure. Also the variation in the content of, for example, flavonoids in different types of foods due to different cultivars, seasonal variation, cooking and food production methods, is largely missed by the FFQs and this may also lead to misclassifications.

9.5.2 Biomarkers for flavonoid intake

An alternative to calculated estimates of the intake of flavonoids in epidemiological studies would be the use of a biomarker that could assess the flavonoid intake in each individual by a simple measure in a blood or urine sample.

We recently developed a very selective and sensitive LC-MS methodology that is able to quantify 12 dietary flavonoids simultaneously in urine from unsupplemented subjects (Nielsen *et al.*, 2000b). The methodology was applied on urine samples collected from 94 subjects on their habitual diet or eating a controlled diet either high or low in fruits, berries and vegetables for 6 weeks (Nielsen *et al.*, 2002). In the intervention period with controlled dietary intakes, we found highly significant differences between the urinary excretion of all measured flavonoid aglycons on the high fruit and vegetable diet compared with the low. The correlation between the habitual intake of fruits and vegetable, determined by 3 days of food registration, with the total excretion of flavonoids was 0.35, $p < 0.001$.

In the same study, the traditional biomarker for fruit and vegetable intakes, the plasma carotenoids, showed a correlation of only 0.213 with intake of fruits and vegetables (Nielsen *et al.*, 2002). Thus urinary flavonoids may not only be a valid marker for flavonoid intake, but also a useful biomarker for fruit and vegetable intake in general.

We are presently trying to further validate the use of the flavonoid biomarker, both as a marker for flavonoid intake and as a biomarker for fruit and vegetable intake in both dietary intervention studies and in cohort studies (Brevik *et al.*, 2004; Krogholm *et al.*, 2004). A study investigating the concentration of flavonoids in fasting plasma samples found that this parameter correlated significantly with the intake of flavonoids estimated by a 7-day dietary record (Radtke *et al.*, 2002). The authors in this study concluded that the combination of dietary estimates and biomarker determinations may be the best approach for epidemiological research on the health effects of flavonoids. Two other dietary intervention studies using high doses of onions and tea also investigated the use of flavonoid concentrations in plasma and urine as a biomarker of intake and both concluded that the dietary intake of flavonoids can be estimated by these parameters (de Vries *et al.*, 1998; Noroozi *et al.*, 2000).

The epidemiological studies listed in Table 9.2 mainly investigated the protective effect of flavonols predominated by the intake of quercetin from tea. However, our studies and the study by Radtke *et al*. demonstrate the importance of including other flavonoids than the flavonols, especially the citrus flavonoids when investigating the intake of flavonoids, since they account for a major part of the daily flavonoid intake (Nielsen *et al.*, 2002; Radtke *et al.*, 2002).

9.6 Flavonoids and the prevention of coronary heart disease

9.6.1 Development of atherosclerosis

Atherosclerosis is the primary cause of many cardiovascular diseases. Mortality from cardiovascular disease is the leading cause of death in the U.S., numbering 41.2 per cent of all deaths in 1997 (Reed, 2002). Atherosclerosis is an inflammatory disease that is characterised by the accumulation of lipids in the innermost layer, the intima, of the walls of large and medium-sized arteries (for reviews see Thompson, 1994; Steinberg, 1997). The process initiates by accumulation of modified low-density lipoproteins (LDL) in the intima and uptake of the modified LDL by macrophages. The LDL may be modified by oxidation by free radicals of enzyme-mediated oxidation resulting in aggregation within the intima (Brown and Goldstein, 1983). The trapped oxidised LDL initiates an inflammatory response of the endothelial cells that attracts monocytes to the area. The monocytes adhere to the endothelium, cross into the intima and differentiate into macrophages. Other macrophages engulf the oxidised LDL, leading to unregulated accumulation of LDL. These lipid-loaded macrophages are called foam cells and are the characteristic component of early atherosclerotic lesions called fatty streaks (Fuster, 1994). The foam cells

are incapable of escaping the intima and then they undergo cell death (apoptosis). This may result in an oxidative burst that further contributes to the oxidation of LDL, thus aggravating the inflammatory process. The progression of the lesion involves smooth muscle cells, collagen and platelets in addition to further lipid accumulation (Steinberg, 1997). The advanced lesion may also develop a fibrous cap. The atherosclerotic lesions or plaques reduce the endothelial function, limit the effective diameter of the vessels, and thereby restrict the blood flow and the supply of oxygen to tissues. When the plaques grow larger, they have an increased tendency to rupture and cause thrombosis and sudden death by myocardial infarction (Fuster, 1994).

9.6.2 Effects of flavonoids and anthocyanins on the oxidation of low-density lipoprotein

The uptake of oxidised LDL by macrophages and the subsequent formation of foam cells is an early event in the development of atherosclerosis. Antioxidants that can inhibit the oxidation of LDL may therefore potentially protect against atherosclerosis.

Flavonoids are potent antioxidants and have been shown to inhibit the oxidation of lipids both *in vitro* and *ex vivo* (De Whalley *et al.*, 1990; Miura *et al.*, 2000). Since the first study in 1990, where flavonoids were shown to be effective in protecting LDL from oxidation *in vitro* (De Whalley *et al.*, 1990), at least 130 studies of the effects of flavonoids on lipoprotein oxidation have been published (Leake, 2001). Some flavonoids have been shown to be active in inhibiting LDL oxidation even at low concentrations ($< 1\,\mu$M) that are physiologically achievable in plasma (De Whalley *et al.*, 1990; Miura *et al.*, 1994). Flavonoids and other antioxidants in black or green tea (Kasaoka *et al.*, 2002), fruit juices (Aviram *et al.*, 2002; Stein *et al.*, 1999) and cocoa (Osakabe *et al.*, 2000, 2001; Kondo *et al.*, 1996) have been shown to inhibit LDL oxidation. Anthocyanins and anthocyanin rich red wine polyphenol fractions have also been shown to protect LDL against oxidation *in vitro*, and red wine has been found to limit the uptake of oxidised LDL by macrophages (Satué-Gracia *et al.*, 1997; Kerry and Abbey, 1997).

The flavonoids probably act in part as chain-breaking antioxidants, thereby converting lipid peroxyl or alkoxyl radicals to lipid hydroperoxides or hydroxides, respectively (Leake, 2001). In addition to this effect, the flavonoids are thought to protect α-tocopherol, the main endogenous antioxidant in LDL, from being consumed during LDL oxidation. The flavonoids are also able to convert the α-tocopheroxyl radical back into α-tocopherol (Leake, 2001).

9.6.3 Inhibition of the inflammatory response in atherosclerosis

In the past decade there has been a major shift in the paradigm of our understanding of the pathogenesis of atherosclerosis. It is now generally accepted that inflammatory mechanisms play a central role in mediating all

phases of the development of atherosclerosis (Blake and Ridker, 2002). Since several of the potentially anti-atherogenic compounds in our diet have anti-inflammatory properties, their mechanisms of action may be by inhibiting or blocking the inflammatory processes of atherosclerosis.

Aggregation of platelets is known to contribute to the development of atherosclerosis by several mechanisms (Fuster *et al.*, 1992) and the inhibition of platelet aggregation is thus regarded as beneficial. Platelets produce the pro-inflammatory mediators such as thromboxane A2, PAF and serotonin, and are thus key participants in the atherogenesis (Ross, 1993).

Several studies have investigated the effect of flavonoids on platelet activation and aggregation. These studies have been reviewed by Middleton and Kandascami (1994) and later by Harborne and Williams (2000).

Recent studies on the flavonoids in cocoa have shown that epicatechin and its related oligomers, the procyanidins, also have potent anti-inflammatory properties (Steinberg *et al.*, 2003). The low-molecular weight procyanidins and epicatechin itself were shown to be a potent inhibitor of human 5-lipoxygenase (Schewe *et al.*, 2002) and procyanidins from cocoa were demonstrated to decrease platelet function significantly *in vivo* in humans (Murphy *et al.*, 2003). Furthermore, another study showed that the combination of quercetin and catechin synergistically inhibited platelet function in collagen-induced platelet aggregation by antagonising the intracellular production of hydrogen peroxide (Pignatelli *et al.*, 2000). A recent study on flavonoids and the platelet-activating factor (PAF) and related phospholipids in endothelial cells during oxidative stress showed that the flavonoids hesperedin, naringenin and quercetin were able to mediate these enzymes, and thereby limit the inflammatory response (Balestrieri *et al.*, 2003).

Studies on anthocyanins have also demonstrated, that they are able to inhibit platelet aggregation. Treatment of humans with blueberry anthocyanins for 60 days was found to reduce the *ex vivo* platelet aggregation (Pulliero *et al.*, 1989). This observation was supported in a study by Keevil *et al.* (2000) who after one week of treatment found a reduced platelet aggregation by red grape juice, but not by orange or grapefruit juice. Further indications of a beneficial effect were observed by Demrow *et al.* (1995) and Folts (1998) who found inhibitory effects on platelet aggregation in dogs and humans by red wine and red grape juice, but not by white wine, which could point to an anti-artherosclerotic effect of the anthocyanins.

The flavonoids may furthermore mediate other anti-inflammatory mechanisms involved in the development of cardiovascular disease. Studies indicate that they are implicated in the modulation of the monocyte adhesion in the inflammatory process of atherosclerosis. The expression of intercellular adhesion molecule-1 (ICAM-1), playing a pivotal role in the inflammatory response, was, for example, shown to be mediated by quercetin in human endothelial cells (Kobuchi *et al.*, 1999). Koga and Meydani (2001) investigated the effects of plasma metabolites of (+)-catechin and quercetin on the modulation of monocyte adhesion to human aortic endothelial cells, and found

that the plasma metabolites of catechin, but not of quercetin were potent inhibitors. This underlines the importance of investigating the biological effects of the metabolites present *in vivo*, e.g. the flavonoid glucuronic and sulphate conjugates instead of the parent compounds.

The endothelial function plays an important role in regulating the vascular function, and endothelial dysfunction is associated with increased cardiovascular disease risk. Several animal and human studies have shown that flavonoids also may have favourable effects on the vascular endothelial function as recently reviewed by Duffy and Vita (2003). Other biological effects of flavonoids in relation to cardiovascular disease have recently been reviewed by Nijveldt *et al.* (2001) and Kris-Etherton *et al.* (2002).

9.6.4 Epidemiological evidence for a preventive effect of flavonoids

Since the Zutphen Study by Hertog *et al.* (1993a), a number of epidemiological studies have been undertaken on the association between dietary flavonoid intake and the risk of cardiovascular disease (CVD). The epidemiological studies listed in Table 9.2 show that the majority of the studies revealed an inverse association with the risk of CVD, although the outcomes of some of the studies are conflicting. Overall, the protective effect of flavonoids was strongest against the mortality of coronary heart disease (CHD), whereas the effect on risk of nonfatal incidences of CVD was weaker or non-existing.

The average daily flavonoid intake in the studies in Table 9.2 ranged from 2.6 to 28.6 mg/day, with quercetin as the dominating flavonoid in most of the studies. However, as discussed in section 9.5.1, the flavonoid intake in these epidemiological studies was based mainly on the food composition tables generated by Hertog *et al.* (1992, 1993b), covering only the content of selected flavonols and flavones in the food. If intake data on additional flavonoids had been included in these studies, e.g. the citrus flavonoids, the catechins, the anthocyanins and the isoflavonoids, the flavonol quercetin would probably not have been the major dietary flavonoid in the cohorts listed in Table 9.2, tea would perhaps be a less important flavonoid source, and the outcome of these studies would then possibly have been different.

The quercetin intake originated mainly from tea intake, but apples and onions were also important sources of quercetin in some studies (Hertog *et al.*, 1993a; Rimm *et al.*, 1996a). The early studies by Hertog et al. showed a highly protective effect of both quercetin and tea against CVD (Hertog *et al.*, 1993a, 1995; Keli *et al.*, 1996). However, some of the later and larger cohort studies, trying to confirm these early studies, found no association or even aggravating effects of flavonoids and especially of tea consumption (Rimm *et al.*, 1996a; Hertog *et al.*, 1997; Hirvonen *et al.*, 2000; Sesso *et al.*, 2003).

The association of tea and incidences of CVD was later further investigated in several cohort studies. These studies have all been reviewed in a recent meta-analysis on the relationship between tea consumption and stroke, myocardial infarction and all coronary heart disease in 10 cohort studies and seven case-

control studies (Peters *et al.*, 2001). The incidence rate of myocardial infarction was concluded to be weakly inversely associated (11 per cent) with an increase in tea consumption of three cups per day. However, the authors stress that the heterogeneity of the studies and the risk of bias due to the larger number of smaller studies showing a protective effect, urge caution in interpreting this result. The mechanism of the protective effect of tea has recently been investigated and does, however, support a beneficial effects of tea intake. For example, consumption of 900 ml black tea for 4 weeks reversed the endothelial vasomotor dysfunction in patients with proven coronary artery disease (Duffy *et al.*, 2001).

It has been suggested that the catechin content in tea could be the protective factor, and Arts *et al.* (2001a) thus estimated the catechin intake to 72 ± 47.8 mg/day in the Zutphen Elderly Study and found a significant negative association between ischaemic heart disease and intake of tea catechins. However, in another study on catechin intake by the same authors, in postmenopausal women from Iowa, a protective effect of catechins was seen from only dietary sources other than tea (Arts *et al.*, 2001b).

The intake of red wine has been postulated to explain the French paradox, i.e. the low incidence of coronary heart disease in France despite the main risk factors for this disease being similar to those in northern European countries (Renaud and de Lorgeril, 1992). Anthocyanins are present in red wine, and several cohort studies have in fact suggested that wine drinkers have a lower mortality from CVD than others (Nanji, 1985; Renaud and de Lorgeril 1992; Gronbaek *et al.*, 1995; Theobald *et al.*, 2000). Other cohort studies have, however, found equally beneficial effects of all alcoholic beverages, and there is no general agreement on this matter as stated in the review by Rimm *et al.* (1996b). It has been proposed that the possible lower mortality by CVD in wine drinkers could be due in part to differences in lifestyle, e.g. in dietary habits and exercise, since factors such as dietary fat composition, little exercise and hypertension are major risk factors on the development of atherosclerosis (Tjonneland *et al.*, 1999).

Flavonoid and anthocyanin intake may thus be strongly influenced by socio-economic factors, and these factors are themselves strongly associated with coronary heart disease. Residual confounding may therefore be a major problem in epidemiological studies on flavonoids (Leake, 2001). Overall, the picture of the health effects of flavonoids in relation to cardiovascular disease is somewhat inconsistent, although there seems to be an emerging body of evidence for a protective effect of dietary flavonoids. However, attempts to reveal the specific food item or flavonoid compound that may exert the protective effect have yet failed to give conclusive results.

9.7 Future trends

The overall picture of the flavonoids as a protective agent against cardiovascular disease has been consolidated during the past decade. The mechanism of action

of the flavonoids is, however, still unknown, but recent studies have moved the focus away from the antioxidant properties of the compounds towards a broader view on the potential mechanisms of action including especially the anti-inflammatory effects of flavonoids.

Furthermore, several studies have shown the importance of investigating the metabolism of the flavonoids and of elucidating the biological significance of these metabolites rather than of the parent compounds, the flavonoid aglycons that are of minor importance *in vivo*.

The early research on the dietary protective action of flavonoids has mainly focused on the flavonols, especially on quercetin, in part because of limitations in the available analytical methods at that time, which merely restricted the investigations to this class of compounds. However, within the past few years, flavonoid research has produced evidence for the importance of other dietary flavonoid classes and subgroups with potential health protective properties and with a similar or even greater impact on our total daily flavonoid intake. Examples are the citrus flavonoids, the red-coloured anthocyanins, the tea catechins and the procyanidins present in cocoa and wine. Furthermore, there are indications of the importance of a diet rich in a range of different flavonoids, rather than containing a high concentration of an individual compound, since some studies have shown additive or even synergistically effects of flavonoids (Pignatelli *et al.*, 2000).

The inclusion of a broader range of the dietary flavonoids in future epidemiological studies, either by use of intake biomarkers and/or advanced food composition tables, will further elaborate on the importance of all these dietary compounds in relation to the risk of development of cardiovascular disease.

9.7.1 Further advice

The research on flavonoids in relation to cardiovascular disease merits more and improved epidemiological studies, including additional flavonoid compounds, than the few flavonols and flavones that have been extensively investigated during the past decade (Table 9.2). The majority of these studies found that the major dietary flavonoid was quercetin from tea, and these studies thus totally overlooked all the other flavonoids originating from fruits and vegetables. An increasing number of food composition tables on flavonoid content in foods and the development of new biomarkers would thus strengthen the epidemiological research on flavonoids and disease prevention. Biomarkers are very precise and often neglected tools for investigating biological effects of dietary compounds. A flavonoid biomarker would have the potential to reveal both the effect of the total intake of flavonoids, the effect of individual flavonoids, perhaps as markers of specific food items, and be useful to investigate the importance of flavonoids in combination with other dietary components.

Furthermore, disease-related biomarkers e.g. in relation to inflammatory mechanisms, should be included in future dietary intervention, case-control or

cohort studies on flavonoids to further elaborate on the potential disease preventive mechanisms of these compounds.

An important issue for future research is to gain more information on what developmental stages of the cardiovascular disease the flavonoids are able to prevent or delay. This is crucial information for the planning of future epidemiological studies on the disease preventive effects of flavonoids, and may explain part of the inconsistence in the outcome of previous studies.

9.8 References

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10

Isoflavones and coronary heart disease

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10.1 Introduction

The increase in coronary heart disease (CHD) incidence associated with decreased ovarian function at the menopause (McGrath *et al.,* 1998; Bittner, 2002) is in part attributable to a less favourable blood lipid profile and arterial dysfunction. Replacement of the natural hormones by exogenous oestrogen and progesterone, in the form of hormone replacement therapy (HRT), has been consistently shown to decrease plasma concentrations of low-density lipoprotein (LDL)-cholesterol and increase concentrations of the beneficial high-density lipoprotein (HDL)-cholesterol (Erberich *et al.*, 2002). As a result, HRT has been widely advocated as an effective means of delaying the progression of atherosclerosis in postmenopausal women. However, recent findings from long-term controlled intervention studies have proved disappointing, with no benefit, or increased incidence, of CHD reported in a number of well-controlled trials (Grady *et al.,* 2002; Skouby, 2002; Kuller, 2003). This lack of efficacy has been attributed to the fact that synthetic oestrogens are associated with increased likelihood of blood clot formation and a rise in circulating concentrations of triglycerides and the inflammatory marker C-reactive protein (CRP), which are independent risk factors for CHD (Manson *et al.,* 2003). Of concern is evidence from recent large-scale studies that demonstrate, conclusively, that HRT is associated with increased incidence of endometrial and breast cancer (Beral *et al.,* 1999). Lack of efficacy of HRT with respect to CHD progression, and clear evidence of increased risk of hormone-dependent cancers, has led to interest in the search for alternative therapies to counteract this loss of natural oestrogens at the menopause.

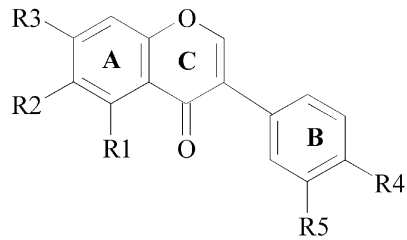
The oestrogenicity of isoflavones was first documented over 50 years ago, when isoflavones present in the diet of sheep were found to be responsible for the permanent infertility induced in these animals. Subsequent epidemiological evidence in humans suggested that high soy consumption, the main dietary source of isoflavones, was cardioprotective, in part attributed to the ability of the isoflavones in soy to act as oestrogen mimics. Demonstration of the ability of soy products to bring about a beneficial change in the blood lipoprotein profile led the US Food and Drug Administration (FDA, 1999) to approve a claim that '25g of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease'. It is currently uncertain whether soy isoflavones contribute to the cholesterol-lowering effects that are reported in controlled trials of soy and soy products. Recent studies have shown small hydrolysed soy peptides that may enter the circulation and up-regulate LDL receptors in the liver could be an important mechanism for the hypocholesterolaemic effects of soy and indicate the active cholesterol-lowering component to be the protein rather than isoflavones (Lovati *et al.*, 2000).

Furthermore, *in vitro* and animal data are emerging that suggest that isoflavones may be cardioprotective by mechanisms independent of blood lipids, but the underlying mechanisms are only partly understood. The recent discovery of a second oestrogen receptor (ERβ) (Gustaffson, 1999), which binds isoflavones with much higher affinity than ERα, and advances in analytical techniques allowing the measurement of a wide range of isoflavone metabolites in biological fluids, is greatly contributing to this knowledge.

10.2 Chemical structure of isoflavones

Isoflavones are non-nutrient plant components, which belong to the phytoestrogen family (Kurzer and Xu, 1997). Phytoestrogens are diphenolic compounds. Ring A and Ring B are separated by a heterocyclic pyrone ring and have a similar chemical structure to mammalian oestrogens (see Figs 10.1 and 10.2). Comparison of the chemical structure of 17β-estradiol and equol, a gut metabolite of daidzein (a major food isoflavone), indicates that the two compounds are almost super-imposable (Fig. 10.2).

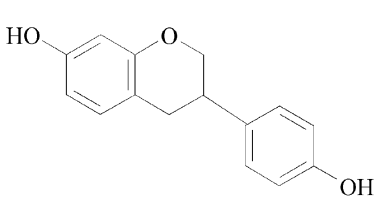
The first evidence that isoflavones have oestrogenic properties was documented in the 1940s when it was established that the infertility of Australian sheep was attributable to the high concentration of the precursor isoflavones, formononetin and biochanin A, found in clover-rich pastures (Bennett *et al.,* 1946). Although oestrogenicity assays reported low oestrogenic potency for dietary isoflavones (100–1000 times less than 17β-estradiol, with genistein being the most potent) (Miksicek, 1993), the fact that their high circulating levels could exceed endogenous estradiol concentrations by up to 10 000-fold (Adlercreutz *et al.*, 1993) was thought to explain their physiological effects.



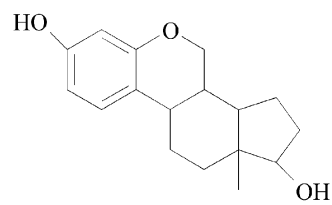
Isoflavone	R_1	R_2	R_3	R_4	R_5
Genistein	OH	H	OH	OH	H
Daidzein	H	H	OH	OH	H
Glycitein	H	OCH_3	OH	OH	H
Formononetin	H	H	OH	OCH_3	H
Biochanin A	OH	H	OH	OCH_3	H
Genistin	OH	H	O-G	OH	H
Daidzin	H	H	O-G	OH	H
Glycitin	H	OCH_3	O-G	OH	H

Fig. 10.1 Chemical structure of isoflavones found in plants.

The discovery and sequencing of the ERβ (oestrogen receptor β) gene in 1996 offered a further explanation for divergent actions of synthetic, endogenous and plant sources of oestrogens (Kuiper *et al.*, 1996). In contrast to the ERα, which is present in high concentrations in the ovaries and testes, ERβ is predominantly expressed in non-gonadal tissues including the blood vessels and bones. This receptor shows a much higher binding affinity for isoflavones compared with the α-receptor. Genistein demonstrates a binding affinity for ERα and ERβ of 4 and 87 per cent of oestrogen binding, whereas daidzein, the other major isoflavone, displays affinities of 0.1 and 0.5% respectively (Kuiper *et al.*, 1998). In addition X-ray crystallography data suggested that, despite the structural similarities to oestrogens, the ligand binding sites of natural oestrogens and isoflavones may be reversed, with the B ring hydroxyl group of isoflavones interacting with the ER (oestrogen receptor) in contrast to the A ring hydroxyl of 17β-estradiol (Setchell, 1998, 2001). This specificity of isoflavones for ERβ, and the orientation of isoflavones with the



Equol



Estradiol

Fig. 10.2 Structures of equol and estradiol.

ER, has led to the recent classification of isoflavones as selective oestrogen receptor modulators (SERMs) rather than phytoestrogens or oestrogen mimics. The specific tissue distribution of the two oestrogen receptor subtypes (ERα and ERβ) and interaction of isoflavones, particularly with the ERβ, will undoubtedly impact on their functional effects on cells and tissues. This may explain the suggested selective benefits of isoflavones on cardiovascular and bone health, without the associated negative effects in certain tissues such as the breast. However, as yet, such selective benefit is indicated largely from epidemiological evidence and more information from intervention trials and cell studies is required to substantiate these beneficial effects.

10.3 Dietary sources, bioavailability and metabolism of isoflavones

10.3.1 Dietary sources of isoflavones

Isoflavones are found naturally in soybeans and to a much lesser extent in legumes. Unprocessed soybeans contain 1–5 mg of isoflavones/g of dry weight (Wang and Murphy, 1994). In certain Asian countries such as parts of China and Japan, where soy is a traditional staple, intakes of isoflavones of 20–50 mg per day are common (Nagata *et al.*, 1998). In Westernised countries, where soy is not a commonly consumed food except in a small minority of the population, typical isoflavones intakes are less than 1 mg/day. Clover and Chinese vine are also rich in isoflavones, and although these plants do not form part of the human food chain, they serve as an important source of isoflavones for commercially available supplements.

Soybeans contain three main isoflavones, present in one of four chemical forms. The free isoflavone aglycones are genistein, daidzein and glycitein. Isoflavones predominantly occur in plants as the water-soluble β-glucosides (complexed to glucose) genistin, daidzin, glycitin, or as acetyl-β-glucosides or malonyl-β-glucosides (Song *et al.*, 1998). Formononetin and biochanin A are the precursors of daidzein and genistein respectively.

On harvesting the soybean the processing techniques used are important determinants of the total isoflavone content and chemical form in the consumed food (Kurzer and Xu, 1997; Coward *et al.*, 1998). Soy flour and texturised vegetable protein (TVP) generally contain about one-third of the isoflavone content (1–1.5 mg/g dry weight) of the original soybean. Because of harsh processing techniques, soy concentrate is a relatively poor isoflavone source. Secondary soy products such as tempeh burgers and tofu contain less than 20 per cent of the isoflavone content found in whole soybeans, because of the addition of large quantities of non-soybean ingredients to the products.

Processing is also known to influence the chemical form of the isoflavones (Coward *et al.*, 1998). Unprocessed soy foods contain mainly 6′ *O*-malonyldaidzin and *O*-malonylgenistin but the heating process used during the production of TVP converts these forms into the more stable β-glucosides.

Non-fermented foods (e.g. tofu) are generally rich in β-glucosides, whereas fermented soy (e.g. tempeh) is rich in aglycones, owing to enzymatic hydrolysis during fermentation.

The isoflavone form present in isoflavone supplements is highly variable and dependent on the source of the isoflavones and the manufacturing techniques. In addition a recent publication by Setchell and co-workers (2001) indicates that there are often considerable discrepancies between the true composition of supplements and the claims made on the label.

10.3.2 Isoflavone metabolism in the gut lumen

Isoflavones are thought to undergo extensive metabolism in the intestinal tract prior to absorption. Important factors that regulate and therefore determine isoflavone metabolism, bioavailability and subsequent biological activities are the presence and activity of intestinal microflora, the chemical forms in which isoflavones are ingested, and the presence of certain components in the diet. Adequate information exists for the metabolism of dietary isoflavones with limited information also available concerning their post-absorption metabolism (pharmacokinetics). However, information about the metabolism of the plethora of isoflavone extracts present in commercial supplements is distinctly lacking.

Isoflavones are present in food mostly in a conjugated form. After ingestion, isoflavone conjugates are hydrolysed by intestinal and bacterial β-glucosidases, thereby releasing the aglycones, genistein, daidzein and glycitin (Setchell *et al.*, 2002; Hur *et al.*, 2002). In addition to aglycones derived directly from the diet, these compounds can also be derived from dietary precursors. The precursors biochanin A and formononetin are transformed into genistein and daidzein in the gut lumen.

The aglycones may be absorbed directly from the small intestine by passive diffusion mechanisms or undergo further biotransformation to a range of metabolites, by specific enzymes produced by a limited range of as-yet largely unknown bacterial species in the colon. Genistein is transformed to dihydrogenistein, which is further metabolised to 6′-hydroxy-*O*-desmethylangolensin (DMA), whereas daidzein is transformed to dihydrodaidzein, which is further metabolised to both equol (70 per cent) and *O*-desmethylangolensin (O-DMA) (5–20 per cent) (see Fig. 10.3) (Setchell and Adlercreutz, 1988; Joannou *et al.,* 1995). As the oestrogenic potency of equol is 10-fold higher than its precursor daidzein, the transformation of daidzein to equol is considered to be a clinically relevant step in the therapeutic potential of soy isoflavones (Shutt and Cox, 1972; Cassidy *et al.*, 2000).

It has been widely acknowledged that the activity of intestinal microflora is an important factor in the metabolism of isoflavones (Lampe *et al.*, 1998; Rowland *et al.*, 2000, Cassidy *et al.*, 2000). The administration of antibiotics to laboratory animals blocks isoflavone metabolite production in the resultant germ-free animal. In addition in newborn infants, whose gut microflora is underdeveloped, no equol is detectable in the urine or plasma when soy formulas

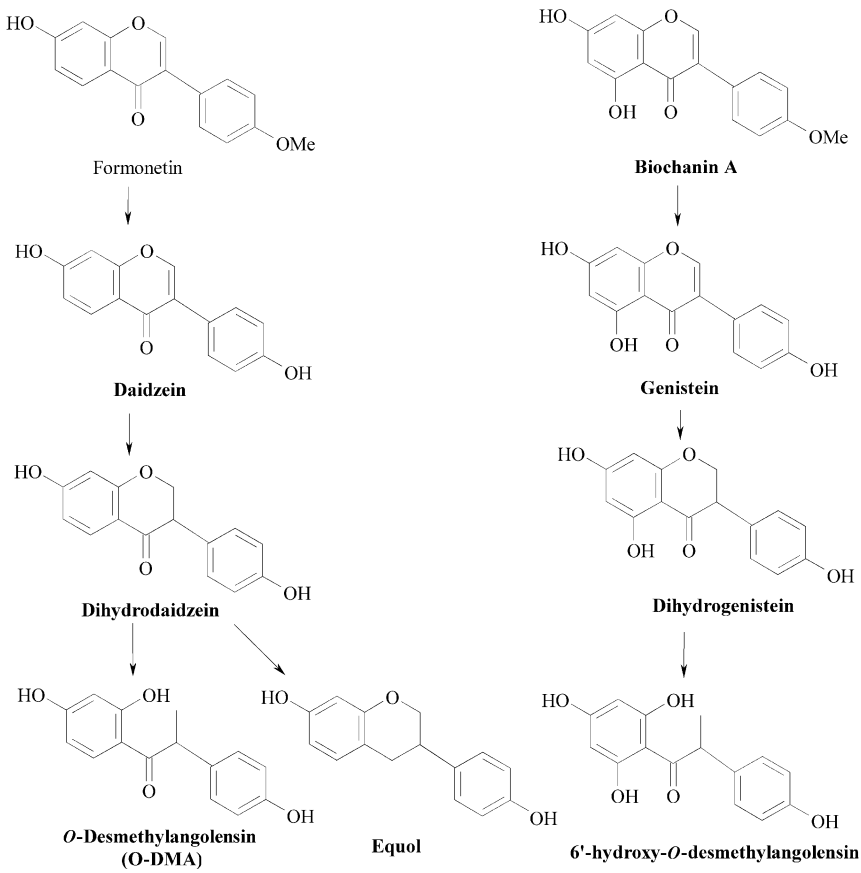


Fig. 10.3 Proposed metabolic pathway for daidzein and genistein by human gut bacteria (modified from Joannou *et al*., 1995).

are fed. Furthermore it is now well recognised that adults differ in their ability to produce equol. A number of investigators have concluded that only 30–40 per cent of individuals produce equol following daidzein ingestion (Lampe *et al.*, 1998). This lack of ability to synthesise equol has been attributed to an absence of specific bacterial enzymes in the large gut. Therefore, there is an increased interest in identifying the microorganisms that are responsible for isoflavone metabolism and furthermore for introducing specific dietary components such as fibre that may stimulate their growth (Bingham *et al.*, 2003). Three strains of bacteria that are capable of metabolising daidzein to equol have been identified, namely *Bacteroides ovatus*, *Ruminococcus productus* and *Streptococcus intermedius* (Ueno *et al.,* 2002). However, it is likely that other strains are involved.

Of total isoflavone ingested, approximately 20–50 per cent is absorbed when dietary intakes are less than 0.5 mg/kg body weight. Isoflavone intakes greater

than 0.5 mg/kg body weight can reduce absorption efficiency (Setchell *et al.*, 2001). This information, in combination with the fact that isoflavones are rapidly excreted in the urine (usually within 24 hours), suggests that consuming modest levels on a regular basis rather than large amounts intermittently can best achieve the maximum benefit from isoflavone consumption.

Rates of absorption and absorption efficiency are dependent on the chemical form in which the isoflavone is consumed and on the matrix of the food itself. Aglycones are generally absorbed more rapidly than the β-glycoside equivalent (Izumi *et al.*, 2000; Setchell *et al.*, 2001). However, the bioavailability of aglycones is thought to be less than the complexed isoflavones, as aglycones are more likely to be degraded in the gut lumen (Kelly *et al.*, 1993; Joannou *et al.*, 1995).

10.3.3 Post-absorption isoflavone metabolism and pharmacokinetics

In order for recommendations to be made regarding efficacious and safe long-term isoflavone intake, it is important to understand the post-absorptive metabolism of isoflavones and their metabolites. Once absorbed, isoflavones are mainly metabolised in the gut and liver where they are converted into either a glucuronidated (attached to glucose) or sulphated (attached to sulphate) form. There are two conjugation sites on genistein and on daidzein and each of these sites can be sulphated or glucuronidated (see Fig. 10.4) (Shelnutt *et al.*, 2002). These conjugates are more easily transported in the blood and excreted in bile or urine than the parent aglycones due to their greater water solubility.

The plasma concentration of individual isoflavones has been investigated in a limited number of studies. Higher plasma genistein concentrations are consistently observed when equal amounts of genistein and daidzein are consumed (King, 1998); this is because genistein is retained in the body to a greater extent owing to its higher lipid solubility, and daidzein has a wider body

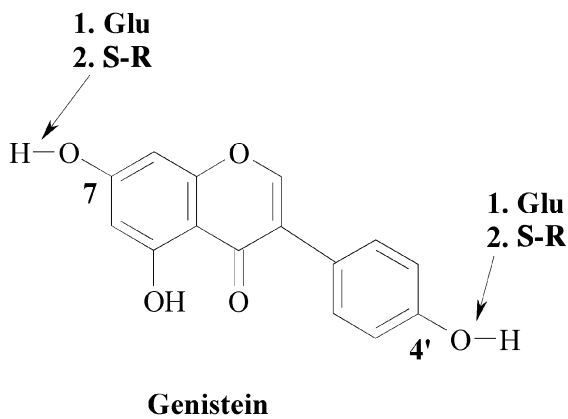


Fig. 10.4 Chemical structure of genistein showing the 7 and 4′ positions where either glucuronidation or sulphation can occur (modified from Shelnutt *et al.*, 2002).

tissue distribution. In a study investigating the effects of consuming a soy protein isolate beverage powder (60 g/day for 28 days) in 20 male subjects, plasma genistein and daidzein concentrations reached 0.9 μM and 0.5 μM, respectively (Gooderham *et al.*, 1996). Barnes (1995) reported the maximal physiologically achievable plasma isoflavone concentration to be 18.5 μM. In a study evaluating the pharmacokinetics and safety of purified unconjugated soy isoflavone preparations fed at a concentration of up to 16 mg/kg bodyweight in postmenopausal women, half-lives for free genistein, daidzein, and glycitein in the circulation averaged 3.8, 7.7 and 3.4 h, respectively (Bloedon *et al.*, 2002). The studies conducted to date have largely focused on the metabolism of the free parent aglycones, which represent a relatively small proportion of the total isoflavones present in plasma. Information on the metabolism of the sulphate and glucuronide conjugates are needed in order to get an accurate assessment of efficacy and safety (Shelnutt *et al.*, 2002). Also pharmacokinetic data have been accumulated using supplements as the isoflavone source, with little data available on isoflavone metabolism following soy ingestion.

10.4 The effect of isoflavones on coronary heart disease (CHD)

Isoflavones are suggested to have beneficial effects on a number of oestrogen-related conditions such as menopausal symptoms, osteoporosis, cancer and cardiovascular disease. Over the past two decades it is the potential cardioprotective benefits that have received most attention.

Cardiovascular disease is the primary cause of death in Western countries. The lower rates of cardiovascular disease in areas of South East Asia such as Japan compared with Western countries has been suggested to be partly attributable to the greater consumption of soy foods in these countries (Beaglehole, 1990; Adlercreutz *et al.*, 1992; Adlercreutz and Mazur, 1997). However, this observation is by no means conclusive as other lifestyle factors, such as lower dietary intakes of saturated fat or higher intakes of fresh vegetables and/or fish, may be in part responsible. In addition, isoflavones may not be the only constituent of soy responsible for the protective effects. Despite these uncertainties, this putative link has stimulated extensive research in the area of isoflavones and cardiovascular health. Proposed mechanisms of action include a beneficial effect on blood lipid metabolism, LDL oxidation, endothelial function and platelet aggregation.

10.4.1 Mechanisms of action: lipid metabolism

Raised total and LDL-cholesterol levels, low-HDL cholesterol and raised fasting triglyceride concentrations in the blood are known risk factors for the development of cardiovascular disease. It has been recognised for many years that reductions in circulating total cholesterol, LDL-cholesterol and

triglycerides, and increases in HDL-cholesterol can result from soy protein consumption. This has been shown in animals (Huff *et al.*, 1977; Anthony *et al.*, 1996, 1998; Balmir *et al.*, 1996; Wagner *et al.*, 1997; Clarkson *et al.*, 2001), and in many human intervention (see Table 10.1) and cross-sectional studies (Nagata *et al.*, 1998). In a meta-analysis of 38 placebo-controlled trials soy protein consumption was associated with an average 9 per cent decrease in total cholesterol concentrations in 34 of the studies (Anderson *et al.*, 1995). Furthermore, 26 out of 31 studies reported an average 13 per cent decrease in LDL-cholesterol, and 22 out of 30 studies reported an average 10 per cent decrease in plasma triglyceride levels. Significantly, this meta-analysis showed that reductions in plasma cholesterol were most likely to be seen in subjects with raised cholesterol concentrations. Since 1995, numerous further studies have been reported on the lipid-lowering effects of soy protein and soy isoflavones. (Table 10.1 summarises a representative selection of studies on soy protein and hypercholesterolaemics, and the less numerous studies on soy isoflavones in hyper- and normo-cholesterolaemics, and post-menopausal women.)

The possible mechanisms for the hypocholesterolaemic effects of soy are not yet clear, although soy isoflavones have been proposed as a putative cholesterol-lowering soy constituent. However, while the majority of soy protein studies have shown lipid-lowering effects, the small number of studies that have supplemented with isoflavones alone have to date produced negative results (e.g. Hodgson *et al.*, 1998; Simons *et al.*, 2000; Dewell *et al.*, 2002). This does not rule out a possible hypocholesterolaemic effect of isoflavones. Larger changes in lipid profiles have been observed following supplementation with intact soy protein compared to soy protein where the isoflavone content has been removed via ethanol extraction in monkeys (Anthony *et al.*, 1996, 1998) and postmenopausal women (Gardner *et al.*, 2001). Concern does, however, exist regarding the impact of the harsh ethanol extraction conditions on the composition of the resultant isoflavone-free product. It is also possible that other constituents of soy may have cholesterol-lowering properties or that isoflavones may act in conjunction with soy components such as saponins, fibre or specific amino acids (Erdman, 2000). It has been suggested that isoflavones may directly increase bile excretion (Lissin and Cooke, 2000), which would reduce the hepatic cholesterol pool and increase the receptor-mediated uptake of LDL from the circulation. Saponins also increase bile excretion, and there may be a greater hypocholesterolaemic effect through isoflavones and saponins acting together. *In vitro* studies have shown that specific subunits of 7S soy globulins increase LDL uptake and degradation by upregulation of LDL receptors in HepG2 cells (Lovati *et al.*, 1992, 2000; Manzoni *et al.*, 2003). Soy fibre may decrease intestinal cholesterol absorption. In addition, isoflavones may also reduce cholesterol levels by improving LDL receptor activity (Kirk *et al.*, 1998). Almost all isoflavone intervention studies to date have administered the treatment and placebo as a capsule. If isoflavones are active as part of a food,

Table 10.1 Soy and isoflavone intervention studies

Reference	Subjects	Duration	Isoflavone supplementation	Lipids and lipoproteins
Soy supplementation in subjects with hypercholesterolaemia				
Sirtori *et al.* (1977)	22 HC patients. CO	3 wks	Textured soy protein	↓ in T-C and LDL-C
Gaddi *et al.* (1991)	21 familial HC patients. Measurements before and after diet	4 wks	75 g/d textured soy protein	↓ in T-C and LDL-C
Baum *et al.* (1998); Potter *et al.* (1998)	66 HC PMW. P	6 mo	3 treatments – 55.6 mg/d, or 90 mg/d in soy protein, or casein	↓ in non-HDL-C ↑ in HDL-C
Crouse *et al.* (1999)	156 HC M and W. P	9 wks	3, 27, 37 or 62 mg/d in soy protein, or casein	↓ in T-C and LDL NC in TAG or HDL-C
Sirtori *et al.* (1999)	21 HC patients. CO	4 wks	32 mg/d in soymilk compared to cows' milk	↓ T-C and LDL-C
Teixeira *et al.* (2000)	81 HC M. P	6 wks	38, 57, 76 or 95 mg/d in soy/casein protein	↓ in T-C and non-HDL-C NC in HDL-C or TAG
Vigna *et al.* (2000)	77 HC and NC PMW. P	12 wks	60 g isolated soy protein	↓ in T-C, LDL-C, and LDL-C:HDL-C ratio
Gardner *et al.* (2001)	94 HC PMW. P	12 wks	42 g/d protein: milk, alcohol-extracted soy, or soy with 80 mg/d isoflavones	↓ LDL-C and T-C NC in HDL-C or TAG
Yildirir *et al.* (2001)	20 HC M. Measurements before and after diet	6 wks	Soy flour, soy beans and soy bean sprouts replacing 30% of total protein intake (but lower in cholesterol)	↓ in T-C, LDL-C, and TAG. NC in HDL-C
Jenkins *et al.* (2002)	41 HC M and PMW. CO	1 month	10 or 73 mg/d in soymilk and soy foods (tofu, burgers etc.) or control dairy and egg protein diet	↓ in T-C, T-C:HDL-C ratio, and LDL-C to HDL-C ratio ↓ in LDL-C
Lichtenstein *et al.* (2002)	42 HC M and PMW. CO	35 days	46.21 mg/d in soy protein or isoflavone-enriched animal protein, versus isoflavone-depleted soy protein or animal protein	↓ T-C, LDL-C, and TAG and ↑ HDL-C after soy protein but NC after isoflavones

Puska *et al.* (2002)	60 HC M and W. P	6 wks	192 mg/d in soy protein drink or casein/milk drink	↓ in T-C and LDL-C in both groups, but decrease greater in soy group NC in HDL-C or TAG
Tonstad *et al.* (2002)	108 HC M and 22 HC PMW. P	16 wks	30 g (111 mg/d) or 50 g (185 mg/d) soy or casein	↓ in T-C and LDL-C in both soy groups NC in HDL-C or TAG
Isoflavone supplementation normal and hypercholesterolaemic subjects				
Hodgson *et al.* (1998)	46 middle-aged M and 13 PMW. P	8 wks	55 mg/d in tablets	NC in T-C, LDL-C, HDL-C, or TAG
Nestel *et al.* (1997, 1999)	21 menopausal and perimenopausal W. CO	5 wks	80 mg/d	↓ in LDL-C and ↑ in HDL-C NC in T-C or TAG
Samman *et al.* (1999)	14 premenopausal W. CO	2 mo on each	86 mg/d in tablets	NC in T-C, HDL-C or TAG
Howes *et al.* (2000)	66 HC PMW. 66 on increasing dose treatment, 9 on placebo	5 wks on each dose	Placebo, then 43.5, then 87 mg/d in tablets (60% biochanin A, 37% formononetin, 2% genistein, 1% daidzein)	NC in T-C, TAG, HDL-C or LDL-C
Simons *et al.* (2000)	20 PMW. CO	8 wks	80 mg/d in tablets	NC in T-C, LDL-C, HDL-C, or TAG
Wangen *et al.* (2001)	18 PMW. CO	93 d	7.1, 65, or 132 mg/d as beverage powders	↓ in LDL-C and LDL-C:HDL-C ratio NC in T-C, HDL-C, TAG
Dewell *et al.* (2002)	36 HC PMW. P	24 wks	150 mg/d in tablets	NC in T-C, HDL-C or TAG
Han *et al.* (2002)	80 menopausal W. P	16 wks	100 mg/d in capsules	↓ in T-C and LDL-C NC in TAG or HDL-C

HC hypercholesterolaemic; M men; W women; PMW post-menopausal women; P placebo-controlled; CO crossover design; T-C total cholesterol; LDL-C low-density lipoprotein cholesterol; HDL-C high-density lipoprotein cholesterol; TAG triacylglycerides.

then more studies are needed to investigate the absorption, biokinetics and lipid-lowering effects of isoflavones incorporated into various foods.

10.4.2 Antioxidant function

The oxidation of LDL in the arterial wall leads to its uptake by macrophages and to the formation of lipid-filled foam cells, a key feature of atherosclerosis. Soy protein and isoflavone treatment have been shown to reduce lipid peroxidation in monkeys and rabbits (Wagner *et al.*, 1997; Yamakoshi *et al.*, 2000). Many authors have reported on the antioxidant effect of soy protein supplementation in humans, mainly represented as a delay in copper-mediated LDL oxidation *ex vivo* (Tikkanen *et al.*, 1998; Ashton *et al.*, 2000; Wiseman *et al.*, 2000; Steinberg *et al.*, 2003) but also by other methods commonly used to assess lipid peroxidation/antioxidant status (Wiseman *et al.*, 2000; Scheiber *et al.*, 2001; Bazzoli *et al.*, 2002; Jenkins *et al.*, 2002; Fritz *et al.*, 2003). Again, however, the evidence for the effects of isoflavones alone is not strongly supported by the intervention studies conducted up until now. Samman and colleagues (1999) found no change in copper-mediated LDL oxidation in premenopausal women following two months of isoflavone supplementation, while Hodgson *et al.* (1999b) reported no effect of supplementation on urinary F_2-isoprostane concentrations (a marker of whole body lipid peroxidation).

In vitro studies have indicated that equol and genistein are more potent antioxidants than daidzein, genistin, biochanin A and formononetin, with equol being more potent than genistein (Wei *et al.*, 1993, 1995; Kapiotis *et al.*, 1997; Ruiz-Larrea *et al.*, 1997; Arora *et al.*, 1998; Mitchell *et al.*, 1998). It is thought that isoflavone structure is a major determinant of antioxidant potency, with the hydroxyl groups at the C4 and C5 positions of the molecule being integrally involved. The cellular processes responsible for the antioxidant activity of isoflavones are not clear. Scavenging of free radicals is a potential mechanism. However, a number of isoflavones showed no significant scavenging effects on a range of radicals in a recent study (Guo *et al.*, 2002). Other possible mechanisms include the inhibition of hydrogen peroxide production (a source of the destructive hydroxyl radical) and stimulation of antioxidant enzymes such as catalase (Wei *et al.*, 1995). An increase in endothelial cell glutathione concentrations was also observed following exposure of the cells to physiologically achievable concentrations of genistein and daidzein, which would increase the antioxidative effects of these isoflavones (Guo *et al.*, 2002). Kerry and colleagues (Kerry and Abbey, 1998) noted that genistein inhibited LDL oxidation, but that the hydrophilic isoflavone was poorly incorporated into the LDL particle. It was consequently shown (Meng *et al.*, 1999) that esterification of isoflavones increased incorporation into LDL with a number of isoflavone-fatty acid esters inhibiting LDL oxidation. *In vivo,* lipophilic isoflavones may therefore be more important with respect to LDL oxidation than the native hydrophilic form of the compounds (Kaamanen *et al.*, 2003).

As is the case for the pharmacokinetic data, most *in vitro* studies have investigated the effects of the aglycones, genistein and daidzein, for which there is a relatively low tissue exposure. Rimbach *et al.* (2003) compared the free radical-scavenging properties of the metabolites equol, 8-hydroxydaidzein, O-desmethylangiolensin and 1,3,5-trihydroxybenzene in comparison to their parent aglycones, genistein and daidzein. 8-Hydroxydaidzein was the most potent scavenger of hydroxyl and superoxide anion radical, and the isoflavone metabolites exhibited higher antioxidant activity than the parent compounds, indicating that the metabolism of isoflavones affects their free radical scavenging and antioxidant properties.

In conclusion, soy protein has significant antioxidant effects *in vivo* but this has not been shown after isoflavone supplementation. The evidence from *in vitro* studies is strongly supportive of an antioxidant role for isoflavones, but caution is needed in interpreting these results as pharmacological doses were employed in the majority of cases.

10.4.3 Blood pressure and endothelial function

Hypertension is a classical risk factor for cardiovascular disease, and is a result of reduced blood vessel flexibility and increased resistance to blood flow due to narrowing of the vessel. This causes endothelial injury and dysfunction, thereby potentiating the development of atherosclerosis and increasing the risk of CHD and stroke.

A number of human studies have shown a decrease in blood pressure after soy protein supplementation (Washburn *et al.*, 1999; Vigna *et al.*, 2000; Teede *et al.*, 2001; Jenkins *et al.*, 2002; Rivas *et al.*, 2002), but again this has not been the case when encapsulated isoflavones were administered (Hodgson *et al.*, 1999a; Han *et al.*, 2002). Vascular and endothelial function, as measured by systemic arterial compliance, pulse wave velocity and flow-mediated vasodilatation, have also been reported to improve after soy protein supplementation (Teede *et al.*, 2001; Yildirir *et al.*, 2001; Steinberg *et al.*, 2003). In contrast to the negative findings for cholesterol and LDL oxidation, isoflavone supplementation has been shown to improve vascular function (Nestel *et al.*, 1997, 1999; Squadrito *et al.*, 2003), although there are conflicting results (Hale *et al.*, 2002). The human data is supported by the animal data (Honore *et al.*, 1997; Williams and Clarkson, 1998; Karamsetty *et al.*, 2001; Nevala *et al.*, 2002).

The most likely mechanism by which isoflavones may improve endothelial function is via the oestrogen receptors, ERα and ERβ (Kuiper *et al.*, 1997; Register and Adams, 1998). ERα and ERβ both mediate non-genomic endothelial derived nitric oxide synthase (eNOS) activation, the key enzyme responsible for the production of nitric oxide, a major vasodilator (Chen *et al.*, 1999; Chambliss *et al.*, 2002). Furthermore, increased nitric oxide production was observed following isoflavone supplementation in animals (Squadrito *et al.*, 2000; Catania *et al.*, 2002) and in human volunteers (Squadrito *et al.*, 2003).

The evidence for the effect of isoflavones on vascular reactivity and dilatation is convincing, and may prove to be of greater physiological importance in cardiovascular disease than the lipid-lowering effects.

10.4.4 Platelet aggregation

Thrombus (large blood clot) formation in a vessel already narrowed by atherosclerosis can cause a complete occlusion, leading to tissue damage in the affected coronary tissue. Platelet aggregation is a key feature of thrombus formation. Studies in rats (Peluso *et al.*, 2000) and monkeys (Williams and Clarkson, 1998; Kondo *et al.*, 2002) have shown lower rates of platelet aggregation following consumption of soy protein or isoflavones; and both genistein and daidzein have been shown to inhibit *in vitro* platelet aggregation (Nakashima *et al.*, 1991; Gottstein *et al.*, 2003). However, soy protein supplementation had no effect on collagen-induced platelet aggregation in normocholesterolaemic men (Gooderham *et al.*, 1996), and therefore the importance of isoflavones in reducing platelet aggregation *in vivo* is not yet clear.

The mechanisms by which isoflavones affect platelet aggregation have not yet been established, but a number of mediators in the aggregatory process may be involved, e.g. cyclooxygenase (via protein tyrosine kinase), cyclic-3′,5′-adenosine monophosphate (cAMP), lipoxygenase or hydrogen peroxide, a second messenger in platelet activation (Beretz *et al.*, 1982; Landolfi *et al.*, 1984; Pignatelli *et al.*, 1998). Although demonstration of efficacy has been observed in animal and *in vitro* aggregation studies and the underlying mechanisms are partly understood, data on the impact of isoflavones on platelet aggregation in humans are lacking

10.4.5 Cell adhesion molecules and inflammatory cytokines

The production of inflammatory cytokines and cell adhesion molecules (CAMs) by the arterial endothelium are key cellular events involved in the development of atherosclerosis. Activation of the endothelium results in the release of vascular cytokines such as interleukin-1 (IL-1β) and tumour necrosis factor alpha (TNF-α). These cytokines induce the expression of CAMs such as intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), which, together with activated monocyte chemoattractant protein-1 (MCP-1), recruit monocytes through the vascular wall, where they are involved in foam cell formation.

CAMs are increasingly regarded as important molecular markers of atherosclerosis. The nuclear transcription factor, NFκB, is a mediator in TNF-α-induced expression of cell adhesion molecules. NFκB is activated by an atherogenic diet (Liao *et al.*, 1993) and oxidised LDL (Brand *et al.*, 1997), and activation is inhibited by various antioxidants (Kunsch and Medford, 1999). Therefore it is of great interest that genistein attenuated NF-κB DNA binding

and TNF-α release in human monocytes (Shames *et al.*, 1999). Genistein, but not daidzein, inhibited TNF-α-induced NF-κB activation in *ex vivo* human lymphocytes following consumption of 100 mg isoflavones/day for 3 weeks, as well as in cultured human lymphocytes (Davis *et al.*, 2001). However this small pilot study ($n = 6$) was not placebo-controlled.

Isoflavones have been found to have no effects on CAM expression *in vivo* (Blum *et al.*, 2003; Steinberg *et al.*, 2003). Nevertheless, genistein has been shown to inhibit CAM surface expression and monocyte cell adhesion in cultured endothelial cells (McGregor *et al.*, 1994; Weber *et al.*, 1995; May *et al.*, 1996). Therefore it appears that isoflavones have the potential to attenuate this inflammatory response, but whether this can be achieved at physiologically relevant isoflavone concentrations remains to be established.

10.4.6 Summary of cardioprotective benefits of soy isoflavones

Overall the evidence from human, animal and *in vitro* studies suggests that soy protein, and perhaps isoflavones, have potential as a dietary means of reducing cardiovascular risk. Evidence for a cholesterol lowering and antioxidant effect of soy is convincing. However, data are not currently available to suggest that isoflavones are the active ingredient. The vasodilatory effect of isoflavones has been repeatedly observed in human trials and it is likely that chronic isoflavone intake could positively increase the elasticity of blood vessels. Although there is an ever-increasing body of *in vitro* evidence to suggest that isoflavones are anti-inflammatory, improve endothelial cell function and decrease platelet aggregation, the concentrations used *in vitro* are higher than those that are physiologically achievable in human studies. Efficacy in humans remains to be established.

10.5 Potential risks of isoflavones

Recently, the safety of soy and its constituent isoflavones has been questioned. Concerns have arisen from animals and *in vitro* studies, which suggest that isoflavones may be involved in cancer development, thyroid dysfunction and reduced fertility.

10.5.1 Isoflavones and cancer risk

Although there is substantial epidemiological evidence to suggest that isoflavones may be anti-carcinogenic, data from a limited number of intervention studies have generated concerns regarding the safety of isoflavones. Short-term dietary soy supplementation (45 mg isoflavones/day) induced proliferation in breast tissue of premenopausal women with breast cancer (McMichael-Phillips *et al.*, 1998), raising concern that isoflavones may stimulate oestrogen-dependent tumours in the breast. In a further study,

consumption of soy protein isolate (38 mg isoflavones/day) was associated with the appearance of hyperplasic cells and increased secretion of breast fluid (Petrakis *et al.*, 1996). However, data are limited and require further investigation.

Animal studies have shown conflicting results. Some studies have shown that genistein has a suppressive effect on chemically induced tumours (Constantinou *et al.*, 1996; Fritz *et al.*, 1998), whereas other studies showed that genistein could stimulate the growth of mammary implanted tumours (Hilakivi-Clarke *et al.*, 1999). So far there is no direct association between isoflavone consumption and other cancer types.

10.5.2 Isoflavones and thyroid function

Results from several studies have raised speculation on the effect of isoflavones on thyroid function. Goitrogenic effects in infants fed soy-based infant formula were first reported in the 1960s. The subsequent substitution of soy flour with soy protein isolate and the supplementation of the formula with iodine overcame the goitrogenic effects of soy-based infant formula (Fomon, 1993). Since then there have been no reports of goitre in children fed soy formula.

There have been a few intervention studies investigating the effects of isoflavones on thyroid function. In these studies, supplementation of isoflavones in pre-menopausal and post-menopausal women led to an alteration in the levels of thyroxine, triiodothyronine and thyroid binding globulin (TBG). However, the changes in hormone concentrations were considered to be of too small a magnitude to be clinically important (Duncan *et al.*, 1999a,b; Persky *et al.*, 2002). Unlike human studies, some cell studies posed concerns on isoflavone safety and indicated that isoflavones can interfere with thyroid function, although high levels of isoflavones were generally used. In these studies isoflavones inhibited thyroid peroxidase, a key enzyme in the production of thyroid hormones (Divi and Doerge, 1996, 1997). Some animal studies have also suggested the interference of isoflavones with thyroid function (Ikeda *et al.*, 2000; Balmir *et al.*, 1996).

Because of the potential interactions between isoflavones and thyroid function, chronic high-dose isoflavone consumption may not be advisable in individuals with reduced thyroid function.

10.5.3 Isoflavones, fertility and development

Concerns that phytoestrogens may have adverse effects on mammalian development and fertility were initially posed when animals fed phytoestrogen-rich plants displayed loss of fertility (Bennett *et al.*, 1946; Moersch *et al.*, 1967; Obst and Seamark, 1975). Subsequent animal studies provided evidence that dietary exposure to phytoestrogens can adversely affect reproduction (Adams, 1995; Kallela *et al.*, 1984). The fact that exposure to potent oestrogens *in utero* can have long-term adverse effects on human

development and fertility in males and in female offspring, has raised concerns that exposure to phytoestrogens, including isoflavones, may also give rise to similar effects later in life.

A main concern is the consumption of soy-based milk by infants since chemical compounds in milk are of particular significance for the differentiation and development progress throughout the neonatal period. Furthermore, the concentrations of isoflavones in soy infant formula and the exposure levels for infants fed on soy infant formulas (per kg body weight), are much higher than the dose consumed by Asian adults (Setchell *et al.*, 1997, 1998).

Human studies examining the effect of phytoestrogens on fertility and development are limited and the majority of information is derived from animal studies. One human study examined the effect of soy-based formula feeding on subsequent sexual development and fertility. Apart from small increases in the duration and discomfort of menstruation in young adult females fed soy formula as infants, there was no evidence for adverse clinical effects on sexual development or reproductive health (Strom *et al.*, 2001). An association between consumption of soy infant formula and premature thelarche (breast development before 8 years of age) has also been suggested (Freni-Titulaer *et al.*, 1986). Rodent and primate studies suggest that isoflavone consumption can affect tissue differentiation and reproductive function as well as causing hormonal changes (Awoniyi *et al.*, 1997; Harrison *et al.*, 1999; Lewis *et al.*, 2003). However, the extrapolation of the data from rodent and primate studies to humans is very difficult because there are significant species differences in sexual development and reproductive function, and in the concentration and tissue distribution of oestrogen receptors. Furthermore, many studies used the subcutaneous route of isoflavone administration that bypasses the gut and hepatic metabolism, which can affect phytoestrogen bioactivity. Additionally, many studies do not report doses on a body weight basis. Therefore, it is not meaningful to make direct comparisons of these studies

10.6 Future trends

Although plausible mechanisms exist that support the case for cardioprotective benefits of isoflavones, the evidence is largely based on isoflavone consumption in the form of soy. Adequately powered human intervention studies that can definitely establish the benefits of either encapsulated isoflavones or isoflavone-fortified foods are needed. This work needs to be supported by kinetic experiments examining the absorption and subsequent metabolism of different isoflavone sources.

Also little information exists on the inter-individual variability in the gut metabolism, absorption, pharmacokinetics and physiological impact of increased isoflavone intake. It is likely that there are subsets of the population that respond to isoflavone supplementation to a greater extent than others. For example, it has been speculated that those individuals who can metabolise

daidzein to equol in the colon may be more responsive to isoflavone supplementation relative to non-producers (Setchell *et al.*, 1984). In addition, the effectiveness of isoflavones on cardiovascular risk factors may be modulated by genotype. Polymorphisms in glucuronidation and sulphation enzymes and in oestrogen receptor-α and-β genes may impact on responsiveness. Although this would be of interest to public health, this information is currently lacking.

A wide range of cell culture work has already been carried out to establish mechanisms of action of isoflavones. Further work in this area needs to be conducted using the forms of isoflavones present in the plasma, rather than the native aglycones, at physiologically relevant concentrations.

Isoflavone safety remains an issue and needs further investigation. It is unlikely that chronic isoflavone consumption has any negative effect on cancer risk or thyroid function in the majority of individuals, but may be a concern in those with a family history of breast cancer or thyroid dysfunction, and those who already suffer from these conditions. The evidence suggesting that physiologically achievable circulating isoflavone concentrations impact on sexual development or fertility in adults is currently weak, although there is a substantial body of animal data showing adverse effects, albeit at very high doses. The impact of high *in utero* exposure to isoflavones, as well as soy milk consumption by infants, on sexual development in later life is of particular concern. Consequently, there are unresolved questions regarding the suitability of high isoflavone supplementation during pregnancy or the use of soy based infant formulas.

In conclusion, isoflavones offer a possible alternative to HRT therapy in postmenopausal women. However, further work is needed to fully establish the safety of chronic high intakes and efficacy with respect to cardiovascular risk.

10.7 Sources of further information and advice

10.7.1 Web sites

www.soyfood.com
www.soyonlineservice.nz

10.7.2 Books

Nutrition and Health edited by T Carr and K Descheemaeker. Oxford: Blackwell Science, 2002

Phytoestrogens in the Human Diet edited by P Holmes. London: Medical Research Council, 2000

Phytoestrogens and Health edited by JJB Anderson and JJ Baxter. Champaign Ill: AOCS Press, 2002

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11

Plant sterols and cholesterol reduction

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11.1 Introduction: cholesterol as a risk factor in cardiovascular disease

An increased blood cholesterol concentration is an established modifiable risk factor for coronary heart disease mortality (Stamler *et al*., 2000). Blood cholesterol concentrations may be modulated by changes in the intake of dietary fat and cholesterol (Tang *et al*., 1998). It has been known since the 1950s that plant sterols reduce blood cholesterol concentrations (Pollak, 1953; Farquhar *et al*., 1956).

Plant sterols, which are not synthesized by humans, are naturally occurring plant compounds. The most common plant sterols are β-sitosterol, campesterol and stigmasterol, from which β-sitosterol is the most abundant one (constituing 90 per cent of the total). The hydrogenation of unsaturated bonds in plant sterols results in plant stanols. Plant sterols and stanols are structurally related to cholesterol, but contain an extra methyl or ethyl group on the cholesterol side chain (Fig. 11.1). Plant sterols and stanols occur naturally in food as free alcohol, esterified with long-chain fatty acids (25–80 per cent of total plant sterols) or conjugated as glycosides (usually in small amounts).

The main sources of plant sterols in the basic diet are cooking oils and margarine. Bread and cereals can also contribute significantly to total plant sterol intake (Morton *et al*., 1995). The majority of plant oils contains 0.1–0.5 per cent, while some germ oils (rice bran, wheat germ, oats) contain up to 4 per cent total plant sterols. Reduced-fat health spreads on the market contain approximately 0.3–0.4 per cent plant sterols or stanols. Individual concentrations of plant sterols in cereal grains, vegetables and fruits are reviewed by Piironen *et al*. (2000). Vegetables and fruits contain <0.05 per cent (based on the edible

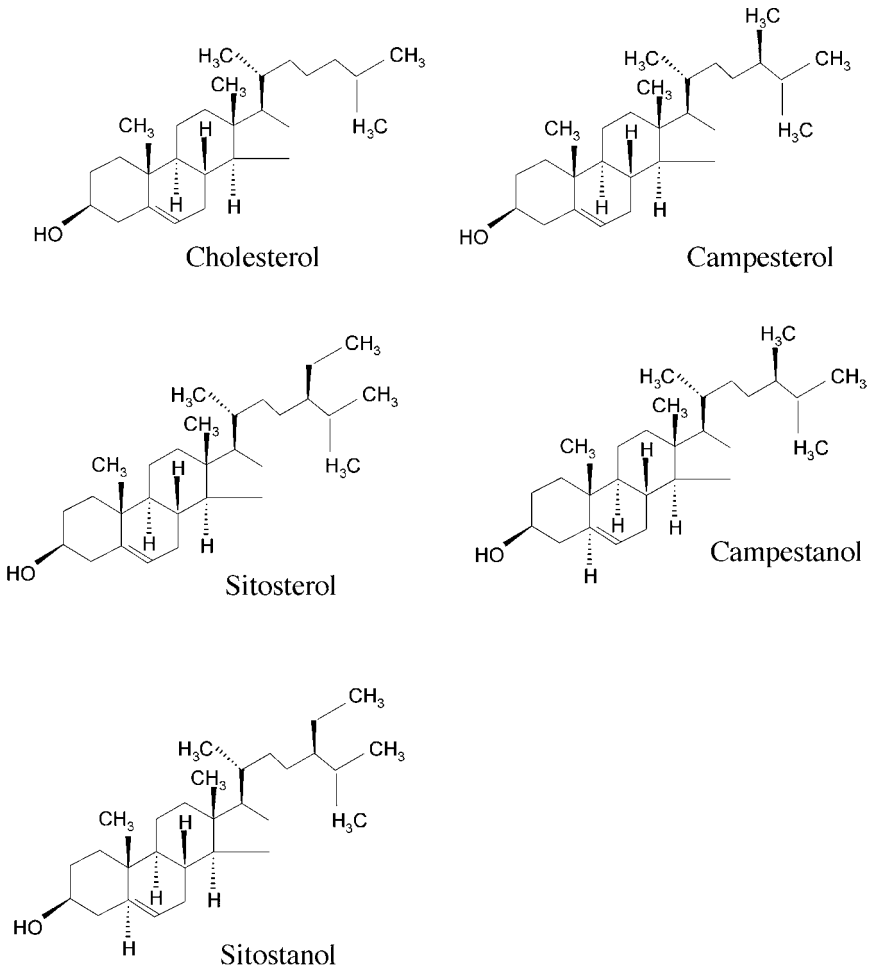

Fig. 11.1 Chemical structure of cholesterol, the plant sterols campesterol and sitosterol and the hydrogenated (saturated) forms campestenol and sitostanol.

portions), except for seedlings of barley, beans, and peas, which contain 0.1–0.2 per cent plant sterols. Some seeds are also rich sources: sunflower and sesame seeds contain 0.5–0.7 per cent and legumes can contain 0.22 per cent plant sterols.

In the Netherlands an average daily intake of plant sterols of 263 (women) to 307 mg (men) has been estimated (Normén *et al*., 2001). In the United Kingdom, this intake is estimated to vary between on average 150 and 200 mg (Morton *et al*., 1995). For the United States, average daily intakes of plant sterols are reported between *ca*. 180 and 250 mg (Connor, 1968) and for Japan about 400 mg (Hirai *et al*., 1986). The Tarahumara Indians of Mexico, who consume a diet containing unusually high amounts of beans and corn, ingest over 400 mg of phytosterols per day (Cerqueira *et al*., 1979). In general the intake of adult vegetarians and their

children is higher (up to 40 per cent) than the average for the population as a whole (Nair *et al*., 1984; Miettinen *et al*., 1990; Ling and Jones, 1995).

The plant sterols used in the 1950s were poorly soluble in fat and high amounts (10–20 g per day) were needed to obtain substantial reductions in serum cholesterol levels. This limited the practical use of plant sterols as cholesterol-lowering agents for many years. Esterification of plant sterols and stanols with fatty acids increases this solubility in fat, thus allowing lower concentrations to be incorporated in foods and increasing its functionality at lower dietary intakes. This renewed the interest in plant sterols and stanols as blood cholesterol-lowering agents. Plant sterol mixtures from various sources have been isolated and modified, i.e. hydrogenated and or esterified, for application as a functional food ingredient. Furthermore, new technologies have been developed to improve solubility and functionality of unesterified plant sterols and stanols. At present many plant sterol and plant stanol-enriched food products are on the market and claim cholesterol-lowering activity.

11.2 The effects of plant sterols and stanols on lowering cholesterol levels

Plant sterols and stanols are known to have a marked effect on human blood cholesterol, specifically a lowering of low-density lipoprotein (LDL) cholesterol which is known to be the risk factor for cardiovascular diseases. It has been shown that the main mechanism to lower blood cholesterol is by inhibiting dietary cholesterol absorption as well as biliary cholesterol absorption (Grundy and Mok, 1976; Heinemann *et al*., 1991; Gylling *et al*., 1997). The precise mode of action is not yet clear, but the postulated mechanism is that plant sterols and stanols are able to displace cholesterol in bile acid micelles due to competition for micellar solubilization (Grundy and Mok, 1976; Ikeda *et al*., 1989). As plant sterols and stanols are more hydrophobic than cholesterol, they have a higher affinity for micelles. As a consequence the micellar cholesterol concentration will be reduced, resulting in a decrease of cholesterol absorption.

In addition, some other mechanisms for the cholesterol-lowering effect have been proposed. It has been suggested that plant sterols and stanols influence the cholesterol metabolism in the enterocyte, resulting in an increased excretion of cholesterol by the enterocyte back into the intestinal lumen (Plat and Mensink, 2002). At present, scarce evidence for this effect is available. Also effects of plant sterols and stanols on cholesterol excretion via the enterohepatic cycle have been suggested. However, no consistent evidence is available. Bile salt excretion was reduced in transgenic mice after consumption of plant stanols (Volger *et al*., 2001) but remained unchanged after consumption of plant stanols by colectomized patients (Miettinen *et al*., 2000) or plant sterols in ileostomy patients (Normén *et al*., 2000).

In 1981 Pollak and Kritchevsky reviewed published studies on the clinical use of plant sterols. The authors estimated that clinical data on the cholesterol-

lowering action of plant sterols was available in about 1800 subjects at that time. A significant reduction of plasma cholesterol from the pre-treatment level was reported after consumption of extremely high amounts of plant sterols (10–20 g/day) in the unesterified form. From the time of this review until 2003 many clinical trials were performed on the cholesterol-lowering capacity of plant sterols and stanols at lower intake levels and mainly in its esterified form. Published papers on this topic, specifically from the mid-1990s until mid-2003, are discussed in this chapter.

11.2.1 Efficacy in healthy volunteers

Plant sterols have lowered serum total and LDL-cholesterol under a wide range of different study conditions. Table 11.1 summarizes part of the clinical studies of plant sterols and stanols published since the review by Pollak and Kritchevsky (1981). The dosages of plant sterols and plant stanols given in these studies ranged from 0.7 to 3.4 g per day. In four of the reviewed studies, plant sterols or stanols were in the unesterified form (Denke 1995; Christiansen *et al*., 2001; Vanstone *et al*., 2002; Jones *et al*., 2003). Table 11.1 shows that total cholesterol was decreased by up to 13.4 per cent and LDL-cholesterol by up to 16.0 per cent. Levels of serum HDL-cholesterol and triacylglycerols remained more or less unchanged. In two out of the 25 reviewed studies the cholesterol-lowering effect of either unesterified plant stanols (Denke, 1995) or unesterified plant sterols (Jones *et al*., 2003) could not be demonstrated.

Recently, a meta-analysis was performed by Katan *et al*. (2003). They demonstrated that at intake levels higher than 1.1 g/day the mean reduction in the LDL-cholesterol level was 10.1 per cent (95 per cent confidence interval 8.9–11.3 per cent) in 27 trials testing stanols (mean dosage 2.5 g/day) and 9.7 per cent (95 per cent confidence interval 8.5–10.8 per cent) in 21 trials testing sterols (mean dosage 2.3 g/day).

Table 11.1 shows that stanols and sterols are effective in lowering plasma lipoproteins within a few weeks, the effect remaining stable in studies during 1 year. Miettinen *et al*. (1995) reported a reduction 8.5 per cent of LDL-cholesterol in a hypercholesterolaemic Finnish population after consumption of 1.8 g/day plant stanol esters during one year. Another study of one year conducted by Hendriks *et al*. (2003) showed a sustained cholesterol-lowering effect of daily consumption of 20 g spread enriched with 1.6 g plant sterol esters during one year in Dutch normocholesterolaemic and mildly hypercholesterolaemic men and women. This cholesterol-lowering effect was consistent throughout the study: cholesterol was reduced on average by about 4 per cent and LDL-cholesterol on average by about 6 per cent. The differences in response between both studies are probably not caused by differences in efficacy between stanols and sterols but by differences in, for example, blood cholesterol levels between study populations.

It has been suggested that plant stanols are more effective in cholesterol lowering than plant sterols since β-sitostanol has shown to be more effective

Table 11.1 Overview of trials investigating the cholesterol-lowering effects of plant sterols and plant stanols in different populations

Reference	Design	Subjects	Number of subjects receiving treatment	Matrix	Duration	Dosage (g/day)	Effect on serum lipids (% change)	
							Total-cholesterol	LDL-cholesterol
Hendriks *et al.*, 2003	Randomized, double-blind, placebo-controlled, parallel	Normo- and mildly hypercholesterol-aemic adults	89 sterol esters	Spread	1 y	1.6	−4%	−6%
Jones *et al.*, 2003	Randomized, double-blind, placebo-controlled, crossover	Adults, moderately hypercholesterol-aemic	15 free sterols	Beverage, non-fat & low-fat	21 d	1.8	−3.1 % (non-fat) −1.6 % (low-fat) decrease not sign	−5.4% (non-fat) −3.5 % (low-fat) decrease not sign
Admundsen *et al.*, 2002	Randomized, double-blind, placebo-controlled, crossover	Children with familiar hypercholesterol-aemia	38 sterol esters	Spread	8 wk	1.6	−7.4%	−10.2%
Mensink *et al.*, 2002	Randomized, double-blind, placebo-controlled, crossover	Adults, normo-cholesterolaemic	20 stanol esters	Yoghurt, low-fat	4 wk	3	−8.7%	−13.7%
Mussner *et al.*, 2002	Randomized, double-blind, placebo-controlled, crossover	Normo- and mildly hypercholesterol-aemic adults	63 sterol esters	Margarine	3 wk	1.82	−3.4%	−5.4%
Noakes *et al.*, 2002	Randomized, double-blind, placebo-controlled, crossover	Adults, hypercholesterol-aemic	46 sterol esters	Spread	3 wk	2.3	−6.1%	−7.7%
			stanol esters			2.5	−7.3%	−9.5%

Table 11.1 Continued

Reference	Design	Subjects	Number of subjects receiving treatment	Matrix	Duration	Dosage (g/day)	Effect on serum lipids (% change) Total-cholesterol	LDL-cholesterol
Temme *et al.*, 2002	Randomized, double-blind, placebo-controlled, crossover	Adults, mildly hypercholesterol-aemic	42 sterol esters	Spread	4 wk	2	−7%	−10%
Vanstone *et al.*, 2002	Randomized, double-blind, placebo-controlled, crossover	Adults, hypercholesterol-aemic	15 free sterols	Butter	3 wk	1.8	−7.8%	−11.3%
			free stanols			1.8	−11.9%	−13.4%
			free stanols+ sterols			1.8	−13.1%	−16.0%
Christiansen *et al.*, 2001	Randomized, double-blind placebo controlled, parallel	Adults, hypercholesterol-aemic	46, free sterols	Spread	6 mo	1.5	−8.9%	−11.3%
			43, free sterols			3	−8.3%	−10.6%
Meguro *et al.*, 2001	Randomized, double-blind, placebo-controlled, crossover	Normo- and moderate hypercholesterol-aemic adults	12 free sterols	Mayon-naise	2 wk	0.5 in TAG	No change	No change
					2 wk	0.5 in DAG	−4.7%	−7.6%
Neil *et al.*, 2001	Randomized, double-blind, placebo-controlled, crossover	Adult, familiar hypercholesterol-aemic	29 sterol esters	Spread	8 wk	2.5	−7.8%	−10%
Hallikainen *et al.*, 2000	Randomized, double-blind, placebo-controlled, crossover	Adults, hypercholesteorl-aemic	22 stanol esters	Margarine	4 wk	0.8	−2.8%	−1.7%
						1.6	−6.8%	−5.6%
						2.4	−10.3%	−9.7%
						3.2	−11.3%	−10.4%

Jones *et al.*, 2000	Randomized, double-blind, placebo-controlled, crossover	Adults, hypercholesterol-aemic	15 sterol esters	Spread	21 d	1.84	−13.4%	−12.9%
			stanol esters			1.84	−10.2%	−7.9%
Plat *et al.*, 2000	Randomized, double-blind, placebo-controlled, crossover	Adults, normo-cholesterolaemic	39 stanol esters	Margarine	4 wk	2.5	−6.6%	−10.4%
Sierksma *et al.*, 1999	Randomized, double-blind, placebo-controlled, crossover	Adults, normo-cholesterolaemic	75 sheanut oil	Spread	3 wk	3.3	−1%	−2%
			soybean oil			0.8	−4%	−6%
Hendriks *et al.*, 1999	Randomized, double-blind, placebo-controlled, crossover	Normo- and mildly hypercholesterol-aemic adults	80 sterol esters	Spread	3.5 wk	0.83	−4.9%	−6.7%
						1.61	−5.9%	−8.5%
						3.24	−6.8%	−9.9%
Westrate and Meijer, 1998	Randomized, double-blind, placebo-controlled, crossover	Normo- and mildly hypercholesterol-aemic adults	80 soybean oil	Margarine	3.5 wk	3.2	−8.3%	−13%
			sheanut oil			3.0	−0.7%	−0.9%
			ricebran oil			1.7	−1.1%	−1.5%
			stanol esters			2.7	−7.3%	−13%
Hallikainen and Uusitupa, 1999	Randomized, double-blind, placebo-controlled, parallel	Adults, hypercholesterol-aemic	38 stanol esters	Margarine	8 wk	2.3	−10.6%	−13.7%
Gylling and Miettinen, 1996	Randomized, double-blind, placebo-controlled, crossover	Adults, NIDDM with hypercholesterol-aemia	8 stanol esters	Margarine	7 wk	3	−11%	−14%
Denke, 1995	Not blinded or controlled	Adults, moderate hypercholesterol-aemic	33 free stanols	Capsule	3 mo	3	No change	No change

Table 11.1 Continued

Reference	Design	Subjects	Number of subjects receiving treatment	Matrix	Duration	Dosage (g/day)	Effect on serum lipids (% change)	
							Total-cholesterol	LDL-cholesterol
Miettinen *et al.*, 1995	Randomized, double-blind, placebo-controlled, parallel	Adults, mildly hypercholesterol-aemic	51 stanol esters	Margarine	1 y	2.6	−10.2%	−14.1%
Pelletier *et al.*, 1995	Randomized, double-blind, placebo-controlled, crossover	Adults, normo-cholesterolaemic	12 sterol esters	Margarine	4 wk	0.740	−10%	−15%
Vanhanen *et al.*, 1994	Randomized, double-blind, placebo-controlled, parallel	Adults hyper-cholesterolaemic	15 stanol esters	Mayon-naise	9 wk	0.8	−4.1%	−10.3%
Gylling and Miettinen, 1994	Randomized, double-blind, placebo-controlled, crossover	Adults, NIDDM with hypercholesterol-aemia	11 stanol esters	Margarine	6 wk	3	−6%	−9%
Vanhanen *et al.*, 1993	Randomized, double-blind, placebo-controlled, parallel	Adults, hyper-cholesterolaemic	34 stanol esters	Mayon-naise	6 wk	3.4	−7.5%	−10%

than β-sitosterol in the inhibition of cholesterol absorption (Heineman *et al*., 1988, 1991; Ntanios and Jones, 1998). Also Katan *et al*. (2003) suggested a higher efficiency of plant stanols. They indicated that although the difference in LDL-lowering capacity between plant stanols (10.1 per cent) and sterols (9.7 per cent) was not statistically significant 'the comparison lacked the statistical power to detect a moderate difference'. As illustrated in Table 11.1, there is a wide range in efficacies over the various studies testing either sterols or stanols. Straightforward conclusions on the cholesterol-lowering effect of plant sterols and plant stanols can be drawn only from a direct comparison in the same study. Efficacy of plant sterols and plant stanols has been compared directly in several studies so far (Weststrate and Meijer, 1998; Hallikainen *et al*., 2000; Jones *et al*., 2000; Normén *et al*., 2000). These short-term human trials, either dietary controlled or in free-living conditions, showed a similar (Weststrate and Meijer, 1998; Hallikainen *et al*., 2000; Normén *et al*., 2000) or better (Jones *et al*., 2000) efficacy in cholesterol lowering for spread enriched with plant sterol esters than for spread enriched with plant stanol esters. Although it is difficult to draw firm conclusions from these results, most probably there is no difference in the cholesterol-lowering effect of plant sterols compared with plant stanols.

11.3 Factors influencing the effectiveness of plant sterols and stanols

Besides dosage and hydrogenation (sterols vs stanols), a number of factors have been suggested as influencing the efficacy of plant sterols and stanols. The possible influence of esterification, matrix, diet, blood cholesterol concentration and eating moment are discussed in the following sections.

11.3.1 Esterification and matrix

Esterification of plant sterols and stanols increases solubility, thus providing a technically feasible way of introducing plant sterols into edible fats and oils (Wester, 2000). The cholesterol-reducing effect of plant sterols and plant stanols may be dependent on the physical state (i.e. esterified or unesterified). Jones and Raeini-Sarjaz (2001) reviewed several studies in this field. They concluded that unesterified sterol and stanols can have the same effect on plasma cholesterol as sterol and stanol esters. Indeed Table 11.1 shows a great cholesterol-lowering potential of unesterified sterols and stanols when incorporated in high-fat products. Vanstone *et al*. (2002) examined the effects of unesterified plant sterols and stanols on total and LDL-cholesterol concentrations in 15 people with hypercholesterolaemia. When added to butter in a daily dosage of 1.8 g, total cholesterol decreased up to 13.1 per cent and LDL-cholesterol up to 16 per cent. Also Christiansen *et al*. (2001) showed that use of spreads enriched with unesterified plant sterols by people with hypercholesterolaema decreased total and LDL-cholesterol concentrations up to 11.3 per cent. However, especially for

unesterified sterols and stanols, the matrix seems to be important for their efficacy. For instance, Jones *et al*. (2003) incorporated unesterified plant sterols in a non-fat or low-fat beverage which were consumed by people with moderate hypercholesterolaemia on a controlled diet for 21 days. They concluded that intake of plant sterols as part of non-fat or low-fat beverages did not exert any greater hypocholesterolaemic effect than a non-fat placebo beverage. Furthermore, Meguro *et al*. (2001) compared the cholesterol-lowering effects of unesterified plant sterols dissolved in triacylglycerols and given with mayonnaise with the effects in diacylglycerols. No serum total cholesterol-lowering effect was observed when dissolved in triacylglycerol. Free plant sterols dissolved in diacylglycerols reduced LDL-cholesterol more than when dissolved in triacylglycerol (Meguro *et al*., 2001).

For plant sterol and stanol esters the matrix seems to be less important. Also when incorporated into low-fat products such as bread and cereals or low-fat yogurt they have shown to be effective in cholesterol lowering (Nestel *et al*., 2001). Also Mensink *et al*. (2002) demonstrated that daily intake of 3 g plant stanol acid esters reduced LDL-cholesterol by 14 per cent when incorporated into a low-fat yoghurt.

11.3.2 Diet

It has been suggested that composition of the diet might influence the cholesterol-lowering capacity of plant sterols and plant stanols. Mussner *et al*. (2002) demonstrated that the LDL-cholesterol-lowering capacity of margarine enriched with plant sterol esters enriched margarine was more pronounced at higher cholesterol and fat intake. Furthermore, Denke (1995) could not demonstrate a cholesterol-lowering effect of 3 g/day sitostanol in people with hypercholesterolaemia when supplemented to a diet low in cholesterol. However, evidence has became available in recent years that plant sterols and plant stanols are effective in cholesterol lowering in people with hypercholesterolaemia consuming low-cholesterol or low-fat diets (Cleghorn *et al*., 2003; Hallikainen *et al*., 2000; Maki *et al*., 2001; Shin *et al*., 2003).

11.3.3 Blood cholesterol concentrations

Efficacy of plant sterols and plant stanols may be higher at high blood cholesterol concentrations. Mussner *et al*. (2002) investigated the cholesterol-lowering effects of 3-week consumption of margarine enriched with plant sterol esters. They demonstrated that the efficacy was more pronounced at higher basal cholesterol absorption. The correlation between initial cholesterol concentration and the degree of cholesterol reduction was demonstrated by our own results (Hendriks *et al*., 2003). For both total cholesterol and LDL-cholesterol, the cholesterol-lowering after consumption of spread enriched with plant sterol esters tended to be higher when baseline cholesterol concentration was higher. Dividing the study population into quartiles based on baseline cholesterol

concentration mean total cholesterol-lowering during the study was 0.2 per cent for the lowest quartile and 5.1 per cent for the highest quartile. Percentage reduction of LDL-cholesterol was 1.5 per cent for the lowest quartile and 7.2 per cent for the highest quartile.

11.3.4 When to eat

Since the supposed mechanism for cholesterol-lowering is inhibition of cholesterol absorption from the intestine, it may be expected that a constant presence of plant sterols in the intestine may be needed for an optimal effect. It can therefore be hypothesized that for the best effect, plant sterols should be ingested simultaneously with meals containing cholesterol in order to block the absorption of exogenous cholesterol from the intestinal lumen (Pollak and Kritchevsky, 1981). In most trials the total daily intake of plant sterols and stanols is divided over two or three portions per day. In our study (Hendriks *et al*., 2003) it appeared that after 13 weeks the majority of the volunteers consumed all the spread with breakfast. Since we considered it relevant that the spread enriched with plant sterol esters should be consumed with at least two meals per day, a request was issued half way through the study to eat 10 g of spread with both breakfast and lunch. The request resulted in a clear increase in the proportion of volunteers consuming spread with two meals (from about 40 per cent to about 70 per cent), but the cholesterol-lowering efficacy did not dramatically change. This is in accordance with the results of Plat *et al*. (2000). They showed that 2.5 g plant stanols taken at lunch produced the same LDL-lowering effect as 2.5 g of plant stanols divided over the three meals. Although both results suggest that eating moment is not essential in the cholesterol-lowering effect of plant sterols and plant stanols, more research is required to draw firm conclusions with respect to this topic.

11.3.5 Efficacy in patients and role of genotype

Plant sterols and stanols are effective not only in people with normal and mild hypercholesterolaemia, but also in those receiving cholesterol-lowering therapies. Maki *et al*. (2001) studied the effect of daily consumption of low-fat spreads (40 per cent) enriched with 1.1 or 2.2 g plant sterol esters in people with low to mildly hypercholesterolaemia on a cholesterol-reducing diet. After 2 weeks, LDL-cholesterol was reduced with respectively 7.6 and 8.1 per cent compared with control treatment. Also Hallikainen *et al*. (2000) studied the cholesterol-lowering capacity of margarines enriched with plant sterols and plant stanols in people with hypercholesterolaemia. During 4 weeks, 34 people consumed a control spread, a spread enriched with plant sterols and a spread enriched with plant stanols. Sterols lowered LDL-cholesterol with 10.4 per cent and stanols with 12.7 per cent compared with the control product. Comparable cholesterol-lowering effects were found by Blair *et al*. (2000) and Vuori *et al*. (2000). Both groups demonstrated that

consumption of plant stanol esters in people with hypercholesterolaemia receiving statin therapy lowered LDL-cholesterol levels by 10 per cent or more compared with consumption of placebo. Neil *et al*. (2001) investigated the cholesterol-lowering effect of daily consumption of margarine enriched with 2.5 g plant sterols in people with heterozygous familial hypercholesterolaemia receiving either statin therapy or no therapy. In both groups, LDL-cholesterol concentrations decreased by 10–15 per cent. There was no difference in response between hypercholesterolaemic patients prescribed statins and those not taking lipid lowering drug.

In people with diabetes, plant stanols have also been shown to be effective in reducing LDL. Gylling and Miettinen (1994) showed a reduction in LDL-cholesterol of 9 per cent in people with diabetes after consumption of plant stanols, which was comparable with the observed effect in those without diabetes. In patients with type 2 diabetes receiving a statin therapy, LDL-cholesterol was lowered by an additional 14 per cent after consumption of stanol esters (Gylling and Miettinen, 1996).

The apolipoprotein E polymorphism may influence the absorption of cholesterol from the intestine and thus the response of serum cholesterol to diet. Some studies (Miettinen and Vanhanen, 1994; Vanhanen *et al*., 1993) indicated that subjects with *apoE4* genotype responded to sitostanol consumption with a greater reduction in total cholesterol and LDL-cholesterol than individuals with the *apoE3* genotype. Others concluded that the serum cholesterol response to plant sterols is not affected by the apolipoprotein E polymorphism in healthy people who consume a low-cholesterol diet (Geelen et al. 2002, Ishiwata *et al*., 2002).

11.4 Safety issues affecting plant sterols

Subjects suffering from the rare inherited metabolic disease phytosterolaemia (sitosterolaemia) have serum plant sterol concentrations approximately 20–100 times higher than healthy subjects (Bhattacharyya and Connor, 1974; Salen *et al*., 1985; Ling and Jones, 1995). These elevated concentrations of plant sterols have been implicated as a risk factor for premature atherosclerosis and coronary heart disease. The estimated frequency in the population of the homozygous state is in the order of one in 5 to 10 million. Increased dietary sitosterol absorption and decreased excretion are believed to be responsible for the accumulation of sitosterol in plasma and tissues. Since cholesterol absorption in people with phytosterolaemia seems to be normal, there may be an intestinal defect that discriminates between cholesterol and plant sterols, resulting in excessive plant sterol absorption. Recent literature suggests that intestinal ABCG5 and ABCG8 transporters are involved in the absorption of plant sterols but not cholesterol (Sehayek 2003). Mutations in these genes are suggested to be responsible for at least some forms of phystosterolaemia (Sehayek, 2003). Whether increased plasma concentrations of plant sterols or plant stanols might

be a risk factor for coronary heart disease (CHD) in people without phytosterolaemia has not been established.

11.4.1 Absorption and plasma concentration of plant sterols

Intestinal absorption of plant sterols in non-phytosterolaemic subjects is low compared with absorption of cholesterol. Heinemann *et al*. (1993) reported in a study with human volunteers percentage absorption values for campestanol, campesterol, stigmasterol and sitosterol which were, respectively 12.5 per cent, 9.6 per cent, 4.8 per cent and 4.2 per cent. Sitostanol absorption was negligible, whereas cholesterol absorption in the same study was 31 per cent. Ostlund *et al*. (2002) reported absorption of plant sterols varying from 0.4 to 3.5 per cent whereas intestinal absorption of plant stanols varied from 0.02 to 0.3 per cent. Sitosterol absorption in people suffering homozygous phytosterolaemia is typically 15–25 per cent (taken from Katan *et al*., 2003).

Plant sterols and stanols are cleared from the plasma and preferentially taken up by the liver and to a lesser extent distributed to other tissues. Results from rat studies indicate that of the other tissues, the adrenals showed the highest levels on the basis of tissue weight followed by the testes (Subbiah and Kuksis, 1973). No accumulation of plant sterols has been observed in dogs and rats (Shipley *et al*., 1958). For humans, only a little information is available regarding the distribution of plant sterols in various tissues of the body. Only trace amounts of plant sterols have been determined in the liver and liver microsomes of healthy people (Ling and Jones, 1995). High levels of plant sterols in liver microsomes have been found in people suffering from phytosterolaemia.

Consumption of plant sterols and stanols has been shown to increase plasma levels in healthy people. Weststrate and Meijer (1998) demonstrated that plant sterol esters from soy, which have a high content of both sitosterol and campesterol, raised the concentration of both plant sterols in the plasma. Hendriks *et al*. (2003) showed that after consumption of spread enriched with plant sterol esters during 1 year, the concentrations of campesterol and β-sitosterol in red blood cells were increased as compared with the control. The relative increase in red blood cells was similar to the increase in serum suggesting that plant sterols did not accumulate in cell membranes. Hallikainen *et al*. (2000) compared the effect of margarines enriched with plant sterol esters and plant stanol esters. Both plant sterol and plant stanol concentrations in plasma increased. Consumption of plant stanols reduced plasma sterol concentrations (Hallikainen *et al*., 2000). Concentrations of plant sterols in plasma in people who used margarine enriched with sterol esters are within the range of 11–30 μmol/L which is about 20–100 times lower than in people with phytosterolaemia. Whether such an increase in plasma plant sterol concentrations might be a risk factor for coronary heart disease is not known.

11.4.2 *In vitro* and animal experiments

Numerous studies on toxicity of plant sterols and plant stanols have been performed. The acute toxicity of plant sterols and plant stanols is low; an abstract by Robinson *et al*. (1998) reported an acute oral LD50 for plant stanols of $\geq$5000 mg/kg body weight. There are no reports in literature of allergic reactions associated with plant sterols and plant stanols. Test substances for sub-acute and semichronic toxicity of various plant sterols or plant stanols in general did not reveal any adverse toxicological effects. No observed adverse effect levels (NOAEL) for plant sterols and plant stanols were established by Turnbull *et al*. (1999a) and Hepburn *et al*. (1999). Turnbull *et al*. (1999a) conducted a 13-week oral toxicity test with two stanol esters in rats consuming dietary sterol concentrations from 0 up to 50 g/kg food. They concluded that the mid-dose level (10 g/kg food, equal to 0.5 g total stanols/kg bw/day) was the NOAEL for both stanol ester preparations. Hepburn *et al*. (1999) reported a 90-day oral toxicity study with plant sterol esters in rats. Plant sterols were obtained from a variety of common edible vegetable oil distillates (mainly soybean) and then re-esterified with fatty acids from sunflower oil. It was concluded by the authors that the highest dose level (81 g/kg food, equal to 6.6 g/kg bw/d and equivalent to a plant sterol concentration of 50 g/kg food, equal to 4.1 g/kg bw/day) was the NOAEL. Plant sterols and stanols are not genotoxic (Turnbull *et al*., 1999b) or teratogenic (Slesinski *et al*., 1999). It has been suggested that plant sterols or stanols have oestrogenic activity. However, *in vitro* and *in vivo* assays did not reveal any oestrogenic or uterotrophic activity of either stanol esters, free stanol fatty acids (Turnbull *et al*. (1999c) or plant sterols sourced from a variety of edible vegetable oil distillates (Baker *et al*., 1999).

11.4.3 Human studies

Clinical safety

As described in this chapter many clinical studies with plant sterols and stanols, with both sexes, in both children and adults, with both healthy volunteers and those with hypercholesterolaemia have been performed. The aim of most of these studies was mainly to demonstrate the reduction of serum cholesterol levels by the addition of plant sterols in the diet. In general, the occurrence of adverse events associated with the use of plant sterols and plant stanols is rare and the adverse events reported are mild and not considered to be treatment-related. Studies investigating effects of plant sterols or plant stanols on blood chemistry and haematology parameters did not show adverse effects in these parameters after short-term consumption (Weststrate and Meijer, 1998; Plat and Mensink, 1998; Plat *et al*., 1999). Two studies were conducted to evaluate long term effects of consumption of plant stanol esters and plant sterol esters. Miettinen *et al*. (1995) studied in a randomized double-blind placebo-controlled parallel trial the effects of daily consumption of a sitostanol-enriched margarine (1.8 g sitostanol/day) in mildly hypercholesterolaemic people for 1 year. No adverse effects on body weight were observed and no adverse clinical signs were

reported. Hendriks *et al*. (2003) evaluated the clinical and safety parameters after daily consumption of a spread enriched with plant sterol esters (1.6 g/day) during one year in a randomized double-blind, parallel placebo-controlled study in healthy volunteers. No adverse side effects, defined as reported adverse events or undesirable changes in clinical chemical parameters, haematological parameters and urinalysis, were observed. In addition, hormone levels in males and females were unaffected.

Nutritional safety

Several studies indicate that plant sterol enrichment may interfere with the uptake of fat-soluble vitamins and nutrients, primarily carotenoids, from the intestine. Table 11.2 summarizes part of the trials that studied the effects of plant sterols on fat-soluble vitamins and nutrients. Hendriks *et al*. (1999) observed a decrease (3–19 per cent) in plasma β-carotene, lycopene and α-tocopherol concentration after consumption of spread enriched with plant sterols (0.83–3.24 g/day). Correction for the reductions in the total plasma lipids, however, showed only plasma ($\alpha + \beta$)-carotene concentrations to be reduced by about 8 and 15 per cent after consumption of the low (0.83 g/day) and the high dose (3.24 g/day) of plant sterols, respectively. We concluded that these doses affect plasma carotenoid concentrations to a limited extent.

In another study (Weststrate and Meijer, 1998), plasma ($\alpha + \beta$)-carotene and lycopene concentrations were both reduced by 22 per cent after an intake of 3 g plant sterols per day. Expressed per plasma lipid concentration, however, lycopene concentrations were not affected, but ($\alpha + \beta$)-carotene concentration was decreased (19 per cent). Sierksma *et al*. (1999) found that plasma lipid-standardised concentrations of $\alpha + \beta$-carotene were not statistically significantly affected by the soybean-oil sterol spread (0.8 g/d), in contrast to lipid-standardised plasma lycopene levels which showed a statistically significant decrease (9.5 per cent). Hallikainen and Uusitupa (1999) did not observe a significant effect of lipid-standardized β-carotene levels from plant sterol ester consumption. However, Hallikainen *et al*. (1999) found that serum α-carotene concentration did not change significantly when subjects were fed with either low-fat wood stanol ester (2.34 g/day) or vegetable oil stanol ester (2.20 g/day). Decreases in $\alpha + \beta$-carotene concentrations were significantly greater in both experimental groups than in the control group, although change in α-, β or ($\alpha + \beta$)-carotene/cholesterol ratio did not differ significantly among the groups. No significant changes were found in serum lycopene or lycopene/total cholesterol ratios in both experimental groups. The authors concluded that low-fat stanol ester margarine appeared to have little effect on serum concentrations of α- , β- or α+ β-carotene, or lycopene. Gylling *et al.* (1999) investigated whether sitostanol ester margarine affects the serum levels of vitamin D, retinol, α-tocopherol and α- and β-carotenes during 1-year treatment in subjects and controls with hypercholesterolaemia. Vitamin D and retinol concentrations and the ratio of α-tocopherol to cholesterol were unchanged by sitostanol ester. Serum β-carotene and α-carotene concentrations, but not their proportion, were

Table 11.2 Overview of studies on the effect of plant sterols and plant stanols on fat-soluble nutrients

Reference	Dosage	Parameters reported to be changed	Reported % change
Hendriks *et al.*, 2003	1.6 g/d sterol ester	Lipid-adjusted (alpha + beta) carotene	−15% to −25%
Amundsen *et al.*, 2002	1.6 g/d sterol ester	Lipid-adjusted lycopene	−8.1%
Judd *et al.*, 2002	3.6 g/d sterol ester	Alpha-carotene	−12.8%
		Beta-carotene	−12.7%
		Lycopene	−20%
Mensink *et al.*, 2002	3 g/d plant stanol ester	Lipid-adjusted beta carotene	−12.9%
Raeini-Sarjaz *et al.*, 2002	1.92 g/d sterol ester	No changes in carotenoids	
	1.76 g/d stanol ester	and fat-soluble vitamins	
Christiansen *et al.*, 2001	1.5 g/d free sterols	No changes in carotenoids	
	3.0 g/d free sterols	and fat-soluble vitamins	
Plat and Mensink, 2001	3.8 g/d stanol ester	Beta-carotene	−26.1%
		Delta-tocopherol	−10.7%
		Alpha-tocopherol	−7.8%
		Lipid-adjusted carotenoids were not significantly lower	
Gylling *et al.*, 1999	3 g/d stanol ester	Lipid-adjusted beta carotene	−25%
Hendriks *et al.*, 1999	0.83 g/d sterol ester	Lipid-adjusted (alpha + beta)	−8%
	1.61 g/d sterol ester	carotene	no change
	3.42 g/d sterol ester		−15%
Sierksma *et al.*, 1999	0.8 g/d free sterols	Lipid-adjusted lycopene	−9.5%
Weststrate and Meijer, 1998	3 g/d sterol ester	Lipid-adjusted (alpha + beta) carotene	−19%

significantly reduced in the sitostanol group from baseline and in relation to controls. The authors concluded that sitostanol ester did not affect vitamin D and retinol concentrations and the α-tocopherol/cholesterol proportion, but reduced serum β-carotene levels (25 per cent). Hendriks *et al*. (2003) showed that consumption of a plant sterol esters enriched spread during 1 year reduced lipid adjusted α and β-carotene concentrations by 15–25 per cent compared with the control. Furthermore, lipid-adjusted fat-soluble vitamin concentrations were not affected by plant sterol intake.

In the meta-analysis of Katan *et al*. (2003), 18 trials testing doses of 1.5 g/day or more reported plasma concentrations of fat soluble vitamins. Mean reductions across the trials were 9 per cent for α-carotene, 28 per cent for β-carotene, and 7 per cent for lycopene. When adjusted for the change in cholesterol only β-carotene was significantly reduced by plant stanols and plant sterols by 12.1 per cent (6.8–17.4 per cent). The reduction in α-tocopherol was explained by the reduction in cholesterol (Katan *et al*., 2003). The meta-analysis of Katan *et al*. (2003) did not indicate that consumption of plant sterols and plant stanols would affect vitamin A, D or K status.

Carotenoids are not essential nutrients, but both α-carotene and β-carotene have provitamin A activity. They may be of importance in situations where vitamin A requirements are greater than normal, as in pregnancy, lactation or infancy. The consequences of a persistent decrease of blood concentrations of carotenoids, specifically β-carotene, as observed after consumption of plant sterols and plant stanols on human health are unknown. Dietary advice to consume an additional daily serving of a high-carotenoid vegetable or fruit when consuming spreads containing sterol or stanol esters may be effective to maintain plasma carotenoid concentrations (Noakes *et al*., 2002).

11.5 Using plant sterols and stanols as functional foods

Products enriched with plant sterols and plant stanols can be considered as functional foods because functional foods are defined as foods with additional benefits on top of the nutritive value. It has been shown that consumption of plant sterols and plant stanols is effective in lowering total and LDL-cholesterol concentrations. As shown in the present chapter, reductions in total and LDL-cholesterol varying from 0.3 to 6 per cent can be obtained depending on the dosage. The most common dosage given in different trials is about 1.5–2.5 g/day, resulting in a mean decrease of LDL-cholesterol of about 10 per cent. Such a decrease may, on a population basis, substantially contribute to the prevention of coronary heart disease (Law *et al*., 1994). Analysis of cohort studies by Law *et al*. (1994) indicated that the longer-term risk reduction would be about 20 per cent. Plant sterols and stanols were not only effective in healthy volunteers, but also in people with hypercholesterolaemia receiving statin therapy. Besides the cholesterol-lowering potential, animal studies have shown that plant sterols and plant stanols may reduce development of atherosclerotic lesions (de Jong *et al*.,

2003). The potency of reduction of development and progression of atherosclerotic lesions ideally should be confirmed in very large intervention trials. Smaller and shorter-term trials with surrogate endpoints, such as flow-mediated dilatation, pulse wave velocity or intima media thickness, might help in proving the efficacy of plant sterols and stanols in reducing cardiovascular disease. In addition to the effects on CHD risk, effects of plant sterols and plant stanols on risk on colon cancer and prostate cancer are suggested.

Compared with the general population, Seventh-day Adventists have lower rates of cancer at many sites, including colorectal cancer, and higher dietary intakes of plant sterols (Nair *et al*., 1984). Since bile acids are reported to be tumour promoters in colon cancer (Cohen and Raicht, 1981), a proposed mechanism is that plant sterol consumption reduce bile acid excretion and thus the risk on colon cancer. On the other hand, increased consumption of plant sterols and plant stanols results in increased presence of cholesterol in the large intestine, which in turn can be converted into mutagenic metabolites (4-cholesten-3-one). Wolfreys and Hepburn (2002) investigated the mutagenic potential of plants sterols and plant sterol esters as well as 4-cholesten-3-one in a bacterial mutation assay and an *in vitro* chromosome aberration assay. None of the components showed any evidence of mutagenic activity in any of these assays. Moreover, studies with mice and rats showed a decreased mucosal cell proliferation after consumption of different dosages of β-sitosterol (Janezic and Rao, 1992; Awad *et al*., 1997). However, results of a prospective cohort study by Normén *et al*. (2001) indicated that high dietary intake of plant sterols was not associated with a lower risk of colon and rectal cancers in the Netherlands Cohort Study on Diet and Cancer.

It has also been suggested that plant sterols may beneficially affect prostate cancer. Dietary supplementation of sitosterol has been shown to improve the clinical symptoms of prostatic hyperplasia in humans (Berges *et al*., 1995). Awad *et al*. (1998) showed that, among other things, plant sterol feeding in rats reduced serum testosterone concentrations. They concluded that dietary plant sterols may reduce risk of prostate cancer by stimulating the activities of the enzymes of testosterone metabolism. However, Hendriks *et al*. (2003) could not demonstrate an effect of 1-year consumption of spread enriched with plant sterol esters on serum testosterone concentrations in healthy men.

11.5.1 Risks of overconsumption and monitoring of long-term exposure

The introduction of new products containing plant sterols and plant stanols is proceeding. Margarines containing either plant stanol or plant sterol esters have been marketed in the United Stated and several countries in Europe for more than 2 years and in Finland for over 5 years. More recently other formulations, including yoghurt, cream cheese spreads and cereal bars have been introduced in some countries, and cereals containing free (unesterified) plant sterols and stanols are being marketed in the United States. With the growing number of products enriched with plant sterols and stanols, some consumers may reach

high intake levels when consuming different enriched products. Safety of plant sterols and plant stanols has been extensively tested. Several GRAS (generally regarded as safe) notification dossiers are present. The Scientific Committee on Food concluded that the use of plant sterols in yellow fat spread at a maximum level corresponding to 8 per cent free plant sterols is safe for human use (SCF, 2000). More recently the Scientific Committee on Food (SCF, 2002) concluded that 'the available data do not provide a basis for setting a numerical upper level of daily intake of phytosterols'. However, the committee indicated that 'it is prudent to avoid plant sterol intakes exceeding a range of 1–3 g/day'. Based on results of animal studies discussed in this chapter this still leaves a large margin of safety.

With the growing number of enriched foods the Scientific Committee on Foods suggests that 'additional management measures may be needed to avoid excessive intakes' (SCF, 2002). Although there is no major concern on adverse health effects, the lack of very long-term effects leaves a possibility of unforeseen effects. Therefore follow-up of samples from the general population eating these foods by post-marketing surveillance techniques is important to monitor both the beneficial as well as the adverse effects on the very long term. This is underscored by the Scientific Committee on Food (SCF, 2003) in its most recent opinion in which 'the committee encourages the Commission to initiate a programme monitoring the total intake of phytosterols-enriched products'.

11.6 Conclusion and future trends

Products enriched with plant sterols and plant stanols either in its esterified or unesterified form have demonstrated to be effective in cholesterol lowering. Dosages of 1.5–2.5 g per day reduce total cholesterol up to 10 per cent and LDL-cholesterol up to 15 per cent. The mean reduction in LDL-cholesterol is about 10 per cent which on a population basis may result in a 20 per cent risk reduction for coronary heart disease. Sterols and stanols seem to be equally effective. Plant sterols and stanols are not only effective in people with normal and mild hypercholesterolaemia, but also in people with hypercholesterolaemia receiving cholesterol-lowering therapies. No effects are observed on HDL-cholesterol and triacylglycerol level. Plant sterols and stanols are poorly absorbed and plasma concentrations are low, except in subjects with a rare inherited disorder phytosterolaemia. Safety of plant sterols and stanols has been extensively tested and no adverse effects seem to be present, although there is a lack of chronic exposure data. Consumption of plant sterols and stanols results in a substantial reduction of carotenoid levels. The implication of this reduction for human health in the long term is not clear. With the growing number of enriched products entering the market the consumption of plant sterols and stanols should be monitored. An intake not exceeding 1–3 g per day is advised. A follow-up of samples from the general population eating plant sterol and stanol enriched

foods by post-marketing surveillance techniques is important to evaluate both the beneficial as well as the adverse effects in the very long term.

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12

Garlic and cardiovascular disease

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12.1 Introduction

The official wording from the Food and Nutrition Board of the National Academy, USA, defines functional foods as any food or ingredient that may provide a health benefit beyond the traditional nutrients it contains (Thomas and Earl, 1994). The European view states that a food can be said to be functional if it contains a component (whether or not a nutrient) that benefits one of a limited number of functions in the body in a way that is relevant to either the state of well-being and health or the reduction of the risk of a disease or it has a physiological effect (Clydesdale, 1997; Bellisle *et al*., 1998). Garlic clearly falls into the functional food category since it is normally eaten in small amounts either raw, cooked or as a salt. It is also sold as a supplement in the form of powdered tablets, capsules, steam-distilled oil or plant extract, all of which can be taken on a daily basis and guaranteed not to produce the strong smell associated with consumption of the raw garlic.

While the garlic may be taken as a pharmaceutical now, it has been used as a flavouring and food additive for many centuries, as described in detail by Rivlin (2001). The earliest reference is from 2600–2100 BC on Sumerian clay tablets. Its subsequent use was reported by the Egyptians and the Greeks to improve stamina generally, but it was the Chinese who prescribed it for specific illnesses, and in this case it was as an aid to digestion and respiration and more particularly for diarrhoea and worm infestation. In India early texts described its use in the treatment of heart disorders. In Europe it was of value in the treatment of a wide variety of ailments, which ranged from digestive disorders to worm infestation, kidney problems, toothache, constipation, dropsy and plaque (Rivlin, 2001). Fresh garlic is still prescribed for the

treatment of many diseases, including the prevention of heart and circulatory problems (Grieve, 1998).

Garlic has received renewed prominence recently because of the popularity of the Mediterranean diet, which includes the extensive use of fresh and cooked garlic. The Mediterranean diet is thought to reduce the risk of cardiovascular disease through a reduction in cholesterol and its consequent reduction in blood pressure. There is extensive Web page documentation at for example http://www.fair-flow.com and http://www.deliciousitaly.com/mediterraneancom1.htm of the benefits of the diet and in supporting clinical trials (de Lorgeril *et al*., 1996; Laino, 2003), but the exact cardioprotective mechanism of the diet is unknown. Since the Mediterranean diet includes a wide array of vegetables, fruit, fish and oil, the protective effect is likely to be multifactorial. Garlic is an essential component of the diet and is thought to make an important contribution to its medical benefits.

The current enthusiasm for herbalists' remedies and healthy diets has led to detailed studies of the chemical composition of garlic and the medical importance of the chemicals it contains. The commercial direction is now away from the fresh plant and into a variety of garlic extracts. The long-term aim of the supplement industry is either to identify an active single compound in the extract that can then be used as a new pharmaceutical or to use the complete plant extract in concentrated form as a food supplement. It is assumed that since garlic has been prescribed for many centuries, there is no or less need for the supplements to undergo the rigorous (and very expensive) testing that is required for the introduction and acceptance of a new pharmaceutical. Garlic supplements and extracts are advertised extensively on the Web where the accompanying text claims that regular consumption of the supplement could delay the onset of cardiovascular disease. However, the supplement industry is now under increasing scrutiny from external regulators to justify their claims, and is looking towards the scientists to provide medical evidence that garlic can in fact prevent the early onset of specific diseases. There have been a large number of studies recently in which the effect of garlic on specific stages of cardiovascular disease has been explored. As an indication of the interest in the subject there has also been a number of reviews (Ernst, 1987; Orekhov and Grünwald, 1997; Bannerjee and Maulik, 2002; Rahman, 2001, 2003) describing the relationship between garlic and cardiovascular disease.

12.2 Chemical composition of raw and cooked garlic

Raw garlic is eaten in small amounts largely as a flavouring, and although popular especially in the Mediterranean diet, it does lead to a strong odour on the breath. The flavour and pungency is a result of a high concentration of sulphur-related compounds in the cloves which amount to 1.0 per cent of their dry weight. In the undamaged clove, over 70 per cent of the sulphur compounds exists as (+)-*S*-allyl-L-cysteine sulphoxide or alliin, (+)-*S*-*trans*-1-propenyl-L-

cysteine sulphoxide or isoalliin, and *S*-methylcysteine sulphoxide or methiin and γ glutamyl peptides, such as γ-glutamyl-*S*-allylcysteine and γ-glutamyl-*S*-*trans*-1-propenylcysteine. The sulphur is divided approximately 50 per cent between the cysteine sulphoxides and the γ-glutamyl peptides (Mütsch-Eckner *et al*., 1992). In the plant the function of the cysteine sulphoxides is probably one of protection since their breakdown products appear to have antibiotic and fungicidal properties (which also contributes to their medicinal value), while the γ-glutamyl peptides appear to have a storage function for N and S. The major alkylcysteine sulphoxide in garlic is alliin (85 per cent), with isoalliin (5 per cent) and methiin (10 per cent) occupying much more minor roles (Lawson, 1996).

Garlic cloves have a slight smell when intact but on cutting or maceration a strong smell is released rapidly. The volatile is only released on damage because there are different parts to the flavour mechanism: a degradative enzyme, alliinase, and its substrates, the alkylcysteine sulphoxides, which are stored in separate compartments in the plant. Unlike onion where there is an intracellular separation of the alliinase and the alkylcysteine sulphoxides (Lancaster and Collin, 1981), in garlic there is a spatial separation based on location within different tissues of the clove (Ellmore and Feldberg, 1994; Wen *et al*., 1995). The amounts of alliinase in the garlic are unusually large, amounting to at least 10 per cent of the total protein in the clove and would explain why there is such a rapid release of flavour and complete breakdown of the flavour precursors upon cutting the tissue.

The chemistry of the *Allium*s is interesting, since from a small number of original flavour precursors a large number of compounds are produced, many of which have medicinal properties (Block, 1985; Lawson, 1996). In the alliinase-catalysed reaction, the first products of the reaction are the sulphenic acids, which have a very short half life before being condensed to form thiosulphinates. From the three original sulphoxides, eight or nine possible thiosulphinates are formed. The major one derived from alliin is diallyl thiosulphinate, or allicin, which represents about 70 per cent of the total thiosulphinates. Isoalliin does not form the lachrymatory compound found in onion but is rapidly converted into allyl 1-propenyl thiosulphinate and the methiin finally degrades to largely allyl methane thiosulphinate. The thiosulphinates are colourless liquids with a pungent smell reminiscent of fresh cut garlic, of which allicin is the main contributor to the odour. The allicin yield is important since it is used as a measure of garlic quality in commercial preparations. It appears to be relatively stable especially in water, but in homogenates it is less stable, which is a major consideration when assessing the effectiveness of different garlic preparations.

The thiosulphinates are described as unstable and reactive and convert readily to more stable compounds, which contain the thioallyl (*S*-allyl) or thiomethyl group. In crushed garlic at room temperature the reactive thiosulphinates are transformed to diallyl sulphide, diallyl disulphide and allyl methyl trisulphide, which is also the reaction that occurs in the mouth. *In vitro* studies of the thiosulphinates, allicin, and methyl and propyl thiosulphinates show that they

are capable of combining with the sulphydryl group of the amino acid cysteine to form *S*-allyl mercaptocysteine, *S*-methyl mercaptocysteine and *S*-propyl mercaptocysteine, thereby inhibiting the activity of enzymes which contain cysteine as the active site. This ability to react with the sulphydryl group of acetyl-CoA SH, the building block of cholesterol and triglyceride synthesis, may help to explain its biological effect (Wills, 1956). These reactions may have some significance in the intestinal tract during digestion as they may determine the form in which the breakdown products of garlic enter the bloodstream. There are no conclusive data that clearly identify the main metabolites in the bloodstream after garlic consumption (Amagase *et al*., 2001). Evidence has shown that allicin, which is an unstable product of alliin breakdown and could be present in the digestive system after consumption of garlic or garlic supplements, is rapidly converted to diallyl disulphide in the blood (Freeman and Kodera, 1995). This compound was found in micromolar concentrations in the rat plasma and liver tissues after oral administration of diallyl disulphide implicating it as a major garlic-derived metabolite in the body (Germain *et al*., 2002) but it does not seem to remain in the body for long after consumption of raw garlic. The water-soluble compound *S*-allylcysteine, itself a breakdown product of garlic, does seem to be a stable residue in the blood following oral consumption (Nagae *et al*., 1994).

Garlic is normally cooked, so the composition will differ from the raw garlic. Heat inactivates the alliinase and therefore inhibits the formation of allicin and other thiosulphinates, but some breakdown of the alliin occurs leading to the accumulation of small amounts of diallyl trisulphide and di- and tetrasulphides. In crushed garlic the cysteine sulphoxides are largely converted to the thiosulphinates (Lawson, 1996). After boiling in a closed container there was complete conversion of the thiosulphinates to sulphides, whereas in an open container over 90 per cent of the sulphides were lost. After cooking in hot fat, most of the allicin was lost but a much higher proportion of the sulphides remained. In an analysis of the volatiles released after standard periods of cooking such as oil frying, baking and microwaving, the dominant volatile was diallyl disulphide (Yu *et al*., 1993).

12.3 Commercial forms of garlic supplement

The commercial forms of garlic that are sold as a supplements include powdered dried garlic cloves, oils produced upon treating chopped garlic with steam, vegetable oil or ether, and aged extracts of chopped garlic in ethanol or water, as well as garlic cloves pickled in vinegar. An important aspect of the supplements is that the composition of the product will vary according to variety, the year of harvest and on the processing method. The preparation and composition of the major commercial products are described below.

12.3.1 Garlic powder

Garlic powder such as Kwai is prepared by peeling the cloves, oven drying at 50–60 ºC then grinding to a powder. Some conversion of the flavour precursors by alliinase does occur on cutting but the loss is minimised by reducing the amount of cutting before drying. Alliinase retains its activity since the powder is very stable and will only lose about 10 per cent of its allicin yield after 5 years of storage. However it is important that the powder is stored at no more than 4-6 per cent water content to prevent the alliinase being activated. The powder can be stored as tablets when again the allicin potential is also very stable but with an average loss of 36 per cent allicin over 5 years is more variable than the powder. Garlic salt products generally have a lower yield of allicin than quality garlic, which probably reflects the greater degree of chopping prior to dehydration (Lawson and Hughes, 1992).

In order to standardise the various dried products, the quality is measured by the level of allicin. The values of some brands are 4 mg/g which compares favourably with the levels in fresh garlic. An alternative measure is the ability to form allicin from alliin (called the allicin potential), which depends on the presence of active alliinase in the tablet. Alliinase is inactivated by the acid conditions of the stomach. In order to provide the body with the pharmacologically active forms in the garlic, the activity of the alliinase must be maintained until the pills reach the non-acid environment of the intestine. The pills are protected from the stomach acid by being coated with cellulose esters which require the presence of intestinal enzymes for the coating to be removed. This has given rise to another measure, the effective allicin yield, which is obtained by using simulated gastrointestinal conditions, i.e. 1 hour in simulated gastric conditions and 2 hours in simulated intestinal conditions (Lawson and Hughes, 1992). Those brands with an effective enteric coating were those with the largest release after 1 hour. Other factors determining the amount of allicin released will depend on whether the tablets are consumed with or without food. A meal with a high protein content will see the pH of the stomach rise from 1.5 to 4.0, which will be less inhibitory to any alliinase activity. It is recommended that the tablets are taken with or just after a meal when the pH is higher and the alliinase-inactivating ability is lowered (Blania and Spangenberg, 1991).

The other factor determining the release of allicin is the ability of the alliinase to convert all the alliin to allicin. This is measured by the ratio of allicin to alliin. With a 100 per cent conversion this should appear as 0.46, whereas the range normally measures from 0.32 to 0.42 because allyl methyl and allyl 1-propenyl thiosulphinates are also formed. Ratios below this figure indicate a damaged alliinase. The usual reason for a reduced figure is due to excessive drying of the cloves at a high temperature which inactivates the alliinase. Significant amounts of γ-glutamylcysteines are also present in the powders and tablets (Block, 1996).

12.3.2 Oil of steam-distilled garlic

Steam-distilled garlic contains exclusively about 98 per cent allylmethyl and 1-propenyl mono- and polysulphides. The commercial product is diluted by vegetable oil so that the final composition of sulphides represents about the same amount of allicin and other thiosulphinates present in a similar weight of crushed garlic. This dilution stabilises the polysulphides and decreases the extremely strong odour of the undiluted oil. The major compounds are the diallyl di-, tri- and tetrasulphides and the allyl methyl di-, tri- and tetrasulphides. The total content of different brands does vary as a result of the different levels of dilution whereas the percentage composition does not. The composition is also relatively stable over time as a 5-year study showed virtually no change in the amount of the main allyl sulphides (Lawson, 1996).

12.3.3 Oil of macerated garlic

Garlic is macerated in a vegetable oil, and the oil is then filtered or the crushed garlic is left suspended in the oil. The product contains compounds not found in the fresh or powdered garlic, i.e. the ring-structured vinyldithiins and the oxygenated ajoenes. The composition seems not to vary but the total amount of sulphur compounds is dependent on the quality of the original garlic and the amount of dilution by the oil. If the garlic bulb is homogenised with an equal weight of oil, the highest amount of transformation products is about 3–5 mg/g. The yields rarely reach this value but can be increased by crushing the cloves first to produce the thiosulphinates more efficiently before addition of the oil. The oil-based compounds appear to be stable since vinyldithiins were stable over a 5-year period but the ajoenes much less so, even when enclosed in a gelatine capsule. It is recommended that oil-macerated products should be kept refrigerated to retain their ajoene concentration for more than 18 months (Iberl *et al*., 1990).

12.3.4 Garlic aged in dilute ethanol

Chopped garlic is incubated in 15–20 per cent ethanol for up to 20 months at ambient temperatures as in aged garlic extract (AGE) (Lawson and Wang, 1995). The incubation medium is then filtered and evaporated to dryness and sold in dry form as tablets, powder or liquid forms. Analysis showed that allicin and other thiosulphinates were nearly depleted by 90 days, having been transformed into diallyl and allyl methyl tri-, di- and tetrasulphides, most of which was then lost to the atmosphere. There are considerable amounts of alliin in the extract, which had diffused out, whereas the alliinase had remained in the cells. Under these conditions the γ-glutamyl-*S*-allylcysteine and the γ-glutamyl-*S*-1-propenylcysteine were converted to *S*-allylcysteine and *S*-1-propenyl cysteine under the action of an enzyme γ-glutamyl peptidase. The quantity of these products will depend on the content of the original γ-glutamyl cysteine sulphoxides. The range in different sources varied from 1.6 to 6.8 mg/g fresh

weight, which in turn produces an amount of *S*-allylcysteine content of 2.7–11.3 mg/g dry weight which represents the approximate amount that should be found in commercial products. The amount of *S*-allylcysteine that is found in commercial products is much smaller than this figure. The alliin content of commercial aged extracts varies from 0.02–0.32 mg/g dry or fresh weight and only trace levels of the allyl sulphides have been found in commercial extracts. The total sulphur content of the liquid form of the commercial aged extract such as AGE was found to be 0.091 per cent in contrast to the 0.35 per cent typically found in garlic cloves.

12.4 The influence of garlic compounds on cardiovascular disease

Cardiovascular disease includes atherosclerosis, hypertension and myocardial infarction or heart attacks, and as such forms the major cause of death in industrial societies. Atherosclerosis is a disease of the arteries where the inner layer becomes thickened by fatty deposits and fibrous tissue, leading to a condition known as hardening of the arteries. Fatty streaks, which are the earliest indication of atherosclerosis, are areas of yellow discoloration on the inner surface of the artery, but do not protrude into the lumen or disturb the blood flow. The streaks are characterised by the subendothelial accumulation of large foam cells filled with intracellular lipid. The foam cells, which are derived from macrophages, and smooth muscle cells are the likely precursors of fibrous plaques, structures that form pale grey elevated lesions, which may project into the arterial wall and reduce the blood flow through the vessel. Calcification of the fibrous plaque leads to rigidity of the artery and hypertension while rupture of the plaque releases material into the bloodstream, causing a thrombus to form. Occlusion of the vessel locally or following transport to distant sites can lead to myocardial infarctions or strokes (Bhattacharyya and Libby, 1998).

Atherosclerosis has a genetic component, which is difficult to overcome, and an environmental and diet-related component, which is susceptible to modification. Regular consumption of garlic as in the so-called Mediterranean diet or the use of garlic supplements is thought to prevent early onset of atherosclerosis and consequently delay the possibility of hypertension, stroke and heart attack. There are a number of stages during the development of atherosclerosis at which garlic consumption has a delaying effect. These stages are the synthesis of cholesterol, oxidation of cholesterol, platelet aggregation, modification of the arterial cell walls and hypertension. Investigations of the role of garlic on these stages have been based on an *in vitro* approach using the response of blood cells or arterial cell cultures to garlic supplements and an *in vivo* approach involving animal and human trials where the supplement has been consumed regularly. The humans trials are difficult to undertake since there is always the problem of achieving a satisfactory level of replication. Decisions have to be made about the number of volunteers, their age, sex, level of

atherosclerosis, duration of trial and type of garlic supplement. The next part of the review provides examples of the *in vitro* and *in vivo* approach used to examine the effect of garlic on specific stages of the disease.

12.4.1 *In vitro* and animal studies of cholesterol synthesis

The level of cholesterol in the blood is an important factor in the development of atherosclerosis. When fats are ingested as part of the diet, cholesterol and triglycerides are absorbed in the intestine and finally transferred to the venous circulation. These large molecules are hydrolysed by the enzyme lipoprotein lipase, which releases fatty acids into peripheral tissues while the metabolic remnants composed largely of cholesterol remain in the circulation. The liver in an endogenous cycle of cholesterol production and metabolism releases very low-density lipoprotein (VLDL) into the circulation. Lipoprotein lipase acts on VLDL at muscle cells and adipose tissue to release free fatty acids into the cells as before and the residue, intermediate-density lipoprotein (IDL), which contains esterified cholesterol remains in circulation. Further processing results in cholesterol-rich low-density lipoprotein (LDL) which is largely taken up by the liver. Cholesterol released back into circulation is transported by high-density lipoprotein (HDL) which returns the cholesterol to the liver via IDL and LDL for recycling into lipoproteins or excretion in the bile. The HDL appears to act in a protective role, while elevated levels of LDL correlates with a high incidence of atherosclerosis.

The level of cholesterol, particularly LDL, is critical (Bhattacharyya and Libby, 1998) The control of cholesterol synthesis is determined by the enzyme hydroxylmethyl glutaryl CoA reductase (HMG CoA reductase) in the liver and other enzymes in the blood. Inhibition of these enzymes, possibly by the presence of garlic-related compounds provides an important route for slowing down development of atherosclerosis. The consumption of garlic may inhibit the HMG CoA reductase for instance by interfering with the signal transduction pathway such as the AMP-dependent kinase pathway, or adenosine-induced signalling (Gebhardt and Beck, 1996). Much of the approach has been based on establishing an atherosclerotic effect in the blood of whole garlic extracts or separate components of the extracts using *in vitro* and whole organisms studies.

In vitro studies have concentrated on the ability of blood cells to accumulate cholesterol, as this provides the vehicle for the movement of cholesterol about the body. The response of rats to a high-cholesterol diet with and without the garlic supplement AGE showed that plasma concentrations of total cholesterol and triacylglycerol of the AGE supplemented rats were 15 and 30 per cent lower respectively than those of the non-supplemented rats (Yeh and Liu, 2001). Subsequently, rat hepatocytes were used to determine the role of garlic in cholesterol biosynthesis *in vitro* by measuring the incorporation of C^{14} acetate into cholesterol in the presence of different crude fractions from raw garlic (Yeh and Yeh, 1994). These were the water extractable fraction (WEF), methanol-extractable fraction (MEF) and petroleum ether-extractable fraction (PEF),

Kyolic, a liquid form of AGE and SAC (*S*-allylcysteine). The rates of C^{14} acetate incorporation into cholesterol were depressed 44, 56, and 64 per cent by MEF, PEF and WEF respectively, suggesting that water- and lipid-soluble components were effective in inhibiting cholesterol production.

The results showed that water-soluble compounds such as SAC were more effective than the sulphides but maximum activity was exerted by the complete extract, Kyolic. The effect of the individual compounds, i.e. SAC, SEC (*S*-ethylcysteine), SPC (*S*-propylcysteine), SMC (*S*-methylcysteine), GSAC (γ-glutamyl *S*-allylcysteine), GSMC (γ-glutamyl *S*-methylcysteine), GSPC (γ-glutamyl *S*-propylcysteine), SAAC (*S*-allylacetylcysteine), SASA (*S*-allyl sulphonylalanine), SAMC (*S*-allyl mercaptocysteine) and alliin were assessed. Lipid-soluble compounds were diallyl sulphide (DAS), diallyl disulphide (DADS), diallyl trisulphide (DATS), dipropyl sulphide (DPS), dipropyl disulphide (DPDS) and methyl allyl sulphide (MAS). The cells were treated with C^{14} acetate in the presence or absence of the test compound at 0.05–4.0 mmol/L for measurement of cholesterol synthesis as before. Among the water soluble compounds, SAC, SEC and SPC exhibited dose-dependent inhibition on the rate of cholesterol synthesis with maximal 40–60 per cent inhibition achieved at 2.0–4.0 mmol/L. Glutamate derivates, GSAC, GSMC and GSPC, depressed the synthesis by 20–35 per cent. Alliin SAAC and SASA had no inhibitory effect whereas the sulphides, DADS, DATS and DPDS diminished the rate of acetate incorporation into cholesterol at 1.0, 2.0 and 4.0 mmol/L (Yeh and Liu, 2001). On the basis of these results, SAC was regarded as a major factor responsible for the cholesterol-lowering effect seen in the human intervention and animals studies.

12.4.2 Human trials of cholesterol synthesis

A typical human trial to show the cholesterol-lowering effect of garlic supplements involved a double-blind randomised, placebo-controlled intervention study of free-living hypercholesterolemic men (34; 48.2 ± 0.8-years-old). The subjects were divided into two groups to receive garlic extract or placebo as dietary supplement for 5 months. The garlic group consumed nine capsules a day, each containing 800 mg of AGE, whereas the placebo subjects took nine capsules, each containing 800 mg of a common food ingredient. Lipids were assessed after 2, 4, and 5 months (Yeh and Liu, 2001). Plasma concentrations remained unchanged 2 months after the supplements. After 5-months supplementation reduced the mean plasma LDL-cholesterol concentration by 10 per cent from its baseline value. Plasma concentrations of HDL-cholesterol and triacylglycerol remained constant. Examination of food intake and weight of subjects indicated that the changes were not due to a change in diet or life style and were therefore due to the garlic supplement.

In a further trial using a diffent source (Kwai), garlic appeared to have a short-term and a long-term effect (Orekhov *et al*., 1996). Two and four hours after a single dose of Kwai (one tablet containing 300 mg garlic powder), the

atherogenicity of sera as measured by cholesterol levels taken from patients with coronary atherosclerosis was decreased. After 3–4 weeks of long-term Kwai therapy blood serum atherogenicity was significantly lower compared with the initial level. In the control group on a placebo the atherogenicity of sera obtained from patients on a placebo was unchanged. The atherogenicity was considerably reduced after 4 weeks, indicating an initial rapid reduction and a long-term decline. From this evidence, garlic does appear to have an effect on lowering the cholesterol level in the blood.

12.4.3 *In vitro* and animal studies of oxidised LDL

The risk factor in atherosclerosis, such as high LDL or low HDL concentrations can lead to excess cholesterol available being taken up by the intimal layer, which is the inner layer lining the lumen of the arteries. High LDL predisposes the arteries to endothelial dysfunction by making them more permeable to the transport of LDL. Once within the intima, LDL accumulates in the subendothelial space by binding to components of the extracellular matrix. This trapping increases the residence time of LDL within the vessel wall where the lipoprotein may undergo chemical modifications. The LDL becomes oxidised by local free radicals and as oxidised LDL it attracts circulating monocytes to the vessel wall. The modified or oxidised LDL can be ingested by macrophages contributing to the development of foam cells. Following oxidation of the LDL, the next stage is the attraction of leucocytes, primary monocytes and T lymphocytes. After the monocytes have adhered to the luminal surface, they may penetrate the subendothelial space by slipping between the junctions. Once localised beneath the endothelium, monocytes differentiate into macrophages, the phagocytic cells that are able to ingest oxidised LDL. The macrophages then become lipid-laden foam cells – the primary constituent of the fatty streak. More recently, oxidised LDL has been recognised as playing a more important role in vascular dysfunction leading to atherosclerosis than native LDL (Bhattacharyya and Libby, 1998). Garlic seems to play a key role in this stage of the disease.

The approach has been to use an *in vitro* system to show whether garlic supplement can prevent or reduce the oxidation of LDL. In the *in vitro* cell-free system, $CuSO_4$ was used to oxidise LDL and the product, thiobarbituric acid (TBARS), measured after 24 hours incubation in the presence and absence of the garlic supplement, AGE (Lau, 2001). The supplement exerted a concentration-dependent inhibition of Cu^{2+} induced oxidation of LDL. All four water-soluble compounds derived from garlic, N-acetyl-*S* allylcysteine, *S*-allylcysteine, alliin and allyl mercaptocysteine showed significant inhibition of LDL oxidation. In a further *in vitro* test vascular endothelial cells, exposed to Ox-LDL showed signs of cell membrane damage by a significant increase of lactic acid dehydrogenase (LDH) release and a decrease of methylthiazol tetrazolium (MTT) absorbance, indicating mitochondrial injury. Pretreatment of vascular cells with AGE and SAC minimised these Ox-LDL induced parameters of cellular injury. These

garlic compounds also inhibited Ox-LDL induced lipid peroxidation implicating lipids as the principal target in Ox-LDL mediated cellular injury.

12.4.4 Human trials of oxidised LDL

In a small-scale study (a double-blind placebo-controlled crossover study involving eight subjects, four men and four women, mean age 68), four participants took 1.2 g AGE three times a day for 2 weeks, then 2 weeks of no garlic (washout period) followed by 2 weeks of placebo. The remaining four subjects took a placebo for the first 2 weeks, followed by 2 weeks washout and 2 weeks of 1.2 g AGE three times a day (Lau, 2001). Blood was drawn at the beginning of the experiment, at 2, 4 and 6 weeks and when the experiment was completed. Plasma LDL was isolated and the $CuSO_4$ test repeated. The use of garlic supplements was found to significantly increase the resistance of LDL to oxidation.

12.4.5 *In vitro* and human studies of platelet aggregation

One of the consequences of the formation of foam cells and subsequently the fibrous plaque is rupture of the plaque-releasing material into the bloodstream, causing a thrombus to form and platelet aggregation to occur. Aggregation is a consequence of exposure of fibrinogen receptors on the surface of cells. These receptors bind fibrinogen in the presence of extracellular Ca^{2+} and crosslink the platelets to form aggregates. The fibrinogen is a heterodimer of the membrane glycoproteins (GP) IIb and IIIa and although unstimulated platelets express the GPII-III complex at their surface, this complex is unable to bind fibrinogen until platelets are activated for example by ADP. The GPIIb-IIIa receptor has a high content of –SH groups and binding of fibrinogen is inhibited by the organosulphur compound ajoene, which is a component of some garlic extracts. A reduction in aggregation would decrease the risk of thrombosis. The approach has been to test the blood of trial volunteers for the ability to aggregate in response to a number of chemical compounds known to stimulate this process.

In contrast to earlier trials referred to, the importance of aggregation was investigated in 34 normolipodaemic individuals (Steiner and Li, 2001). These volunteers were recruited for a 44-week-long double-blind crossover study. No supplements were given for the first 6 weeks, then participants were selected to receive either AGE or a placebo in a dosage of three capsules/day (each 800 mg) for a period of 6 weeks. The dose was then raised to six capsules/day for 6 weeks and finally to nine capsules for another 6 weeks. The first intervention period was followed by a 2-week washout period. The subjects were then switched to the supplement they had not received during the first arm of the study. A final 2-week washout period concluded the study. Blood was sampled every 2 weeks and processed for platelet aggregation and adhesion studies. Aggregation was tested with the following agonists which would normally encourage aggregation: arachidonic acid, ADP, collagen and epinephrine. Each platelet

stimulant was used in a range of concentrations to determine the threshold levels required to induce complete aggregation. Aggregation studies using ADP as a stimulant showed minimal increases. Collagen and epinephrine-induced aggregation was significantly inhibited by AGE. All individuals consuming AGE showed an increase in SAC in the blood, which dropped when AGE ceased to be consumed.

In a contrasting investigation on platelet aggregation in humans, 23 healthy and normolipodaemic participants (12 men and 11 women) consumed 5 ml AGE daily for 13 weeks, blood samples were taken before and after the last dose. Platelet aggregation was then measured (Rahman and Billington, 2000). The extent and rate of aggregation in response to ADP were reduced after dietary supplementation with 5 ml AGE. Serum lipid concentrations were not affected. It is thought that the effect of AGE was to inhibit the ADP-induced rise in cytosolic Cu^{2+} concentrations. Both trials suggested that regular consumption of garlic supplements would reduce the level of aggregation in the blood and therefore the risk of thrombosis.

12.4.6 *In vitro* and animal studies of the arterial cell wall

The most important consequence of plaque formation is that it leads to a restricted blood flow and hardening of the arteries. Resistance in the arterial circulation is increased and there is a risk that the plaques might break, releasing material into the bloodstream and causing a thrombus to form and strokes to follow. Garlic consumption appears to have a direct effect on the artery wall by reversing or reducing wall hardening. The approach has been to examine the response of cell cultures of arterial cells to compounds such as LDL, then the arteries of animals fed a high-cholesterol diet for lesion development.

Free cholesterol and cholesterol esters are present in arterial cells and contribute to the eventual formation of plaques. Uptake and metabolism of cholesterol by arterial cells are critical to the final stages of atherosclerosis. The control of this process has been examined *in vitro* by using the response of cultured smooth muscle cells to cholesterol. Cultures of such cells derived from a fibrous plaque of an atherosclerotic human aorta loaded with cholesterol were incubated for 24 hours with an aqueous extract of garlic powder. Free cholesterol decreased by 30 per cent, cholesterol esters by 30–40 per cent and triglycerides by 20 per cent (Orekhov *et al*., 1995; Orekhov and Tertov, 1997). This change is achieved because garlic appears to suppress lipid synthesis in the cells by inhibiting the activity of acetyl-CoA:cholesterol acyltransferase (ACAT), the enzyme involved in the formation of cholesteryl esters, the main component of the excessive fat accumulated by cells. In the untreated atherosclerotic cells, ACAT activity was three-fold higher than in normal cells. The aqueous extract of garlic cells decreased this enzyme activity to normal levels. Garlic extract also stimulated cholesterol ester hydrolase, an enzyme that degrades and therefore reduces cholesterol esters in atherosclerotic cells (Orekhov and Tertov, 1997).

In addition to a drop in cholesterol concentration and retardation of atherosclerotic lesion formation, garlic enhanced the fibrinolytic activity of the blood plasma, which is lowered as a result of cholesterol feeding. A reduction in fibrinolytic activity may accelerate atherosclerosis by exposing the vascular walls surfaces to recurrent thrombi and clot association mitogens. The effect of garlic may result from an increased fibrinolytic activity (Bordia *et al*., 1975). In a further correlation it was found that in cultured cells there was a strong correlation between atherogenicity of LDL (measured by the lipoprotein's capacity to induce lipid accumulation in cultured arterial cells and stimulate cell proliferation) and sialic acid content of LDL. Desialylated LDL caused cholesterol accumulation in arterial cells via increased uptake, through interaction with both the B,E-receptor and an unregulated scavenger receptor, whereas non-desialylated LDL is taken up through the B,E-receptors only. Desialylated LDL may be responsible for the onset and further development of atherosclerotic lesions since atherogenic LDL isolated from patients' blood had a lower content of sialic acid compared with native LDL of healthy subjects. Long-term therapy with garlic tablets increased the LDL sialic content up to normal (Orekhov *et al*., 1992).

In an animal study atherosclerosis was induced in rabbits by feeding them a cholesterol-rich diet for 2–4 months when lesions were found to have formed in the aorta of a sample of the animals. Cholesterol feeding was withdrawn, then the rabbits given essential oil of garlic for 8–9 months or garlic homogenate for 3 months. After cholesterol feeding was discontinued, serum cholesterol concentration decreased. In the garlic-fed animals the fact that the area of atherosclerotic lesions had decreased more than 50 per cent and the lipid content of the artery wall decreased by 69 per cent indicated a direct effect of garlic on the arterial wall and a regression of the disease (Bordia and Verma, 1980). Garlic supplementation also partially suppressed biosynthesis and accumulation of collagen in the aorta of the cholesterol-fed rabbits.

When the aorta of rabbits fed a standard diet was examined directly there were no fatty lesions on the intimal surface in the presence or absence of Kyolic. In the cholesterol-fed rabbits 70 per cent of the luminal aortic surface was covered by lipid-filled lesions and the neointima was greatly increased with a concentrated thickening of fibro-fatty plaques. The Kyolic-treated group had only 25 per cent coverage of the aortic surface by lesions, the accumulated cholesterol in the aorta was significantly reduced and the neointima was only half the size (Campbell *et al*., 2001). The function of smooth muscle cells is to contract but during atherosclerosis there is a change to a cell that can accumulate lipid, migrate, proliferate and synthesise appreciable extracellular matrix. This change to smooth muscle cells also occurs in culture but it was inhibited by the presence of Kyolic supplement in the culture medium. Inhibition of the conversion of the smooth muscle cell to this altered phenotype may represent a mechanism by which garlic achieves its protective effect against atherosclerosis under *in vivo* conditions.

12.4.7 *In vitro* studies of hypertension

Hypertension is a very common condition and one that results from atherosclerosis. One result of the changes during the development of atherosclerosis is that the arterial wall tissue hardens and loses its ability to relax and contract. In order to test the effect of garlic on the contractility of aorta, rats were fed a high-cholesterol diet with and without garlic extract, then killed after 16 weeks of this treatment. The arteries were cut into rings. These were suspended between two hooks, so the contractile response could be measured. Aortic rings were contracted with noradrenalline and exposed to acetylcholine or sodium nitroprusside. When contractions reached a plateau, the endothelium-dependent relaxation and independent relaxation were tested. Contractions were higher in those rings treated with garlic (Slowing *et al*., 2001), suggesting that garlic consumption can offset the hardening effect of atherosclerosis.

Chronic hypertension is a major contributor to the development of myocardial ischaemia infarction and strokes, but hypertension can be induced by factors other than age-related changes. One of the causes of induced hypertension is stress, with its accompanying depressive effect on the immune system and reduced control of oxidative stress. Activation of the transcription factor NF−κB, which is associated with the regulation of numerous genes encoding proteins in immune function, is an important part of this stress response (Grimme and Baeuerle, 1993). Oxidised LDL is a product of high blood cholesterol, which can act as a second messenger in the activation of NF−κB. In *in vitro* studies using cultured arterial endothelial cells, activation of NF−κB by oxidised LDL led to the expression of cell adhesion factors, VCAM-1 and ICAM-1, formation of which can accelerate the development of atherogenic lesions and cell death. Anti oxidants such as garlic derived compounds inhibited oxidant-induced NF−κB (Geng *et al*., 1997). The transcription factor NF−κB can also be activated by the tumour necrosis factor (TNF)-α. When endothelial cells from the human umbilical vein (HUVEC) were incubated with TNF-α, the cells showed activation of NF−κB expression, whereas preincubation of HUVEC with *S*-allylcysteine, a major products of alliin breakdown in fresh garlic and a component of some supplements, inhibited the activation (Ide and Lau, 2001).

Antihypertensive medications are the standard means to lower chronically elevated blood pressure. These are diuretics, whose side effects include elevation of cholesterol and triglyceride levels, beta-blockers, through a reduction in heart rate and a mild decrease in contractility with side effects that may include a rise in serum triglycerides, and a reduction of good cholesterol (HDL), and Ca^{2+} channel blockers, which have fewer side effects but are costly (Deshmukh *et al*., 1998). Although the influence of garlic on the risk factors of atherosclerosis and ultimately hypertension is weaker than that of synthetic drugs, the broad spectrum of garlic is an important advantage. Of equal importance is safety, and here garlic has the advantage of having being taken as a supplement for many centuries with no obvious ill effects except bad breath and the occasional gastric complaint.

12.5 Future trends: developing new functional foods

An obvious method of increasing the uptake of active organosulphur compounds into the body is simply to eat more garlic or consume more supplements. Another strategy is to optimise the levels of the precursor compounds such as alliin in the existing garlic. This both improves the amounts of precursor compounds and breakdown products in the raw and cooked garlic and ensures the highest possible concentration in the garlic supplements. Increases in levels of organic sulphur compounds in garlic can be achieved in a number of ways. These are by the addition of sulphur fertiliser to the soil, by classical breeding techniques of selection and crossing and by genetic manipulation.

In an example of the first approach when sulphur fertiliser was added to the soil as 3.4×10^{-2} kg.m^2 $CaSO_4$, it increased the total sulphur in the fresh garlic from 96.4 mmol/kg to 190 mmol/kg. In the dried garlic powder from these bulbs, there was an increase in concentration of organosulphur compounds, alliin, allicin, γ-glutamyl propylcysteine and γ-glutamyl-phenylalanine. The effect of increasing soil sulphur led indirectly to a change in levels of NF$-\kappa$B in blood cells The nuclear factor, NF$-\kappa$B, is an important regulator of the immune system and is involved in atherosclerosis through activation of adhesion factors. Lipopolysaccharide are able to induce the inflammatory cytokines interleukin (IL)-1β and TNF-α in human blood which can activate production of NF-*K*B (Li and Verma, 2002). Pretreatment of the blood cells with garlic powder extract reduced the lipopolysaccharide production of cytokines and in turn reduced the production of the nuclear factor NF$-\kappa$B. The effect of garlic was to reduce the induction of the nuclear factor by 25 per cent, whereas samples treated with sulphur-fertilised garlic lowered the NF$-\kappa$B by 41 per cent (Keiss *et al*., 2003). The effect of the garlic was probably through diallyl disulphide, a breakdown product of alliin, since Keiss *et al*. (2003) found this compound reduced the lipopolysaccharide activation of interleukin (IL)-1β and TNF-α levels in blood.

A classical and genetic engineering approach to plant breeding to enhance the yields of organosulphur compounds in garlic has been undertaken by participants of the Garlic and Health grant, an EU-financed project (http://www.plant.wageningen-ur.nl). Garlic is vegetatively propagated and none of the current varieties shows any seed formation, which makes it difficult to attempt varietal improvement by standard selection and breeding techniques. The approach undertaken was to return to the country of origin of the garlic and obtain plant varieties that showed seed formation, in combination with high yields of alliin in the bulb. It should now be possible to select high-yielding individuals, which can be put through a standard breeding programme involving crossing and seed production. In a genetic engineering approach an attempt was made to alter biosynthesis of the flavour precursors by genetic manipulation. This approach required identification of the pathway intermediates and the controlling enzymes. The mechanism of alliin biosynthesis is largely speculative but it is thought that there are two possible routes: one via serine and an allyl source and the other via glutathione and an allyl source (Fig. 12.1) (Lawson,

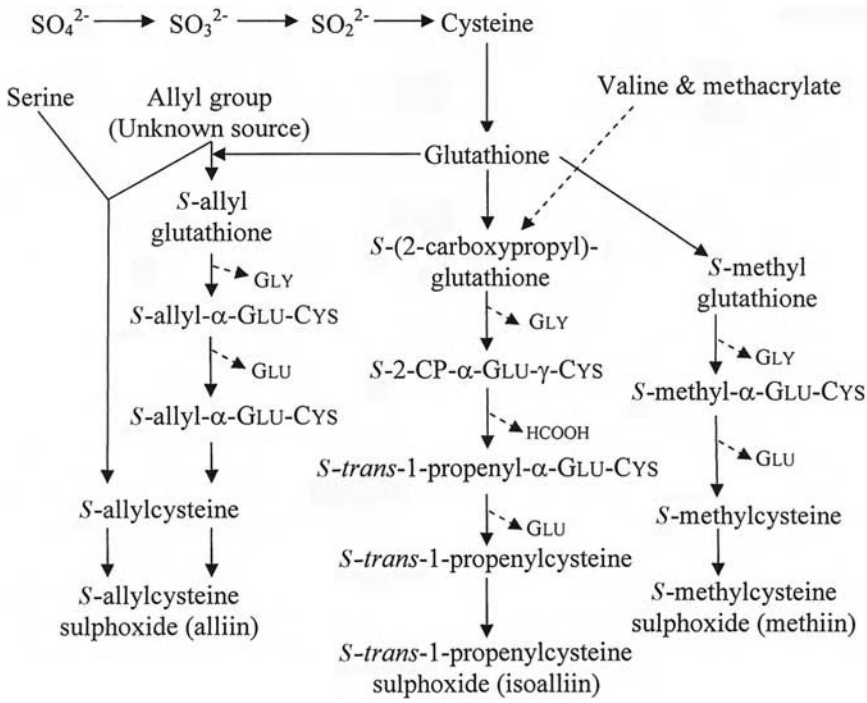

Fig. 12.1 Proposed pathways for the synthesis of alliin, isoalliin and methiin in garlic (modified from Lawson, 1996).

1996). These two stages represent the beginning of the secondary pathways and probably where the control of the secondary pathways occurs. Genetic manipulation of the control enzymes would require identification of the enzymes and the genes responsible, followed by transformation of the garlic and overexpression of the control enzymes to overcome any metabolic limitations on the pathway.

12.6 Sources of further information and advice

While the large array of different garlic supplements available to the customer can be confusing, the Web is now a valuable reference for both products and medical advice. Many of the statements, however, have not been evaluated by regulatory bodies such as the American Food and Drug Aministration, and the European Union (Thomas and Earl, 1994; Clydesdale, 1997; Bellisle *et al*., 1998), so the garlic cannot be seen as an official cure, or treatment or for the prevention of cardiovascular disease. The evidence only suggests that garlic may have a beneficial effect on cardiovascular disease. An example of a helpful website is http://illness.altmedangel.com/heart.htm which is a non-profit site offering educational information and broad-based research on various health

conditions, medications, supplements and therapies. It advocates the use of garlic supplement to regulate blood pressure and to thin the blood. It claims that consumption of the garlic will also preserve the elasticity of the arteries and delay their stiffening. In another site, http://www2.vitaminconnection.com, garlic as fresh garlic (up to one clove daily) and 400 mg tablet (standardised to 1 per cent total allicin potential) was shown to cause a lowering of cholesterol. There were also beneficial effects on blood pressure, fibrinogen and LDL. In the more commercially orientated sites the descriptions of the effects of garlic consumption are sometimes vague, using such language as cleansing cholesterol and normalising blood pressure, helping to reduce harmful LDL-cholesterol and triglycerides, reducing blood clotting and strengthening blood vessels.

Another site (http://www.americanheart.org/presenter.jtml) was more critical since it made an objective analysis of a number of clinical trials on the effect of garlic consumption on cardiovascular disease. The analysis showed that there were a number of modest short-term benefits of garlic supplements. It maintained that the main problem with making a comparison between the trials was the variation in the composition of the different commercial preparations. In a larger meta-analysis the effect of garlic on blood pressure reduction and lipid lowering was examined by Silagy and Neil (1994a,b). The conclusions from analysis of 11 randomised controlled trials for blood pressure and 25 randomised controlled trials for serum cholesterol was that garlic therapy may reduce both blood pressure and serum lipids. Silagy and Neil (1994a,b) suggested that this result should be treated with caution because of shortcomings in methodology of some of the trials examined. In a later meta-analysis (Stevinson *et al*., 2000), the effect of garlic on total cholesterol in persons with an elevated level of cholesterol of at least 200 mg/mL was analysed in a number of randomised, double-blind, placebo-controlled trials. The results showed that compared with the placebo, garlic reduces the total cholesterol level in those persons whose levels were elevated. The findings confirm the results of earlier studies but the effect may be smaller than originally thought with a reduction of 4–6 per cent of cholesterol due to garlic. The meta-analysis included only 13 trials since it was only these that satisfied the strict requirements of the test and of these there were serious variations in the methodology used. One of the most significant was the unblinding of the test because of the difficulty of disguising the garlic odour. Empirical research suggested that trials that are not wholly double-blind tend to exaggerate the treatment effects. The other major problem in trying to establish a significant effect was the variation in the garlic source. The test used Kwai, garlic oil and spray-dried garlic powder. Of these there was known variation in the allicin content of the samples in addition to the known variation between the different sources of garlic. According to Stevinson *et al*. (2000), what is required is to have well-designed placebo-controlled trials comparing the efficiency of the different types of garlic.

The trials took essentially a short-term view of the effect of garlic consumption, i.e. the increase in cholesterol was measured, whereas it is more important to take a long-term view in terms of clinical effects, such as heart

disease. Many of the other protective effects of garlic such as reduced blood pressure, platelet inhibition and stimulation to blood flow have been assessed *in vitro* and *in vivo*, but the clinical effects have been implied but not measured. The results of the various meta-analyses suggested that garlic is superior to a placebo in reducing elevated cholesterol levels but the size of the effect is moderate. Compared with the use of pharmaceutical drugs such as the modern statins, which can cause a reduction of 17–32 per cent, the reduction in cholesterol from garlic is small. Even dietary modifications can cause a 5.3 per cent reduction over a 6-month period. The implication for clinical practice is that garlic use is not an efficient way to decrease serum cholesterol level. Large-scale and long-term trials are required to establish the association between garlic consumption and the clinical outcomes. The one test that attempted this involved 152 patients over a 4-year period when it was shown that there was deceleration of the development of atherosclerosis in those who included garlic in the diet (Koscielny *et al*., 1999). The other more long-term test for cardiovascular health is perhaps the Mediterranean diet, which contains a significant amount of garlic and which appears to have a protective effect on the cardiovascular system.

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Part III

Controlling dietary fat

13

Diet, oxidative stress and cardiovascular disease

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13.1 Introduction: oxidative stress and cardiovascular disease

Interest in the role of uncontrolled oxidative processes in response to oxidative stress in the onset and progression of disease, and as contributing factors in organ and system dysfunctions related to ageing, is underlined by the volume of research work and reviews devoted to this area since 1990. These studies cover diverse fields, from the chemistry of oxidative reactions of susceptible substrates in a test-tube, to the occurrence and detection of oxidative processes in living systems, and the relevance of these events in pathophysiology. Although these processes are considered factors in altering or modulating biological functions, it is difficult to reach reliable quantitative estimates of their contribution to multifactorial events such as diseases. This review discusses the links between oxidative stress, with special relevance to lipid oxidation, and cardiovascular functions and disease.

13.1.1 Oxygen–substrate interactions: non-enzymatic and enzymatic processes

Oxygen is a key factor in energy metabolism in the animal kingdom since it participates in the major energy-releasing reactions, such as substrate utilization and formation of high-energy compounds. Oxygen is also directly involved in oxidative reactions catalysed by several enzymes, such as the oxygenases, resulting in the formation of a large number of bioactive products from lipids (e.g. the eicosanoids), or the hydroxylating enzymes involved in the formation of neurotransmitters from amino acids (e.g. the catecholamines), or in the metabolism of xenobiotics (e.g. hydroxylations or other types of oxygen-dependent reactions).

Under aerobic conditions, in addition to the participation of oxygen to redox reactions, highly reactive unstable and short-lived chemical entities, the so-called reactive oxygen species (ROS) are produced. ROS can be considered as the outcome of oxidative stress, a condition that, in addition to resulting from endogenous processes, can also be induced by a wide range of environmental factors, including UV exposure, pathogen invasion (hypersensitive reactions) and tissue reperfusion after oxygen deprivation. The term ROS, generally referring to the superoxide anion radical ($O_2^{\bullet}$), H_2O_2, and the hydroxylradical ($OH^{\bullet -}$), includes also hypochlorous acid (HOCl), chloramines, singlet oxygen and peroxyradicals. ROS are highly reactive and interact with major biomolecules: they bind to proteins, break DNA strands, react with vital cellular components, and alter structural lipids in biomembranes by attacking double bonds of polyunsaturated fatty acids (PUFA) in membrane phospholipids. The oxidative modification of proteins and lipids is commonly defined as protein oxidation and lipid peroxidation, respectively. Free iron (Fe^{3+}) and, in general, bivalent metal ions accelerate the decomposition of lipid hydroperoxydes ($LOOH^{-}$) into compounds such as alkoxyl and peroxyl radicals, 4-hydroxynonenal (4-HNE) and malonylaldehyde (MDA). These compounds, in addition to being end products of peroxidative decomposition of polyenoic fatty acids in the lipid peroxidation process, are also reactive compounds that in turn can continue, amplify and extend the process beyond the initial oxidative event by oxidizing cellular thiol groups.

13.1.2 Lipid peroxidation and biomembranes

Lipid peroxidation can spread throughout the cell, and disrupt lipid membranes, even at sites not immediately associated with those where ROS have originated. Lipid peroxidation may be considered a major alteration in ROS-induced cellular derangements for various reasons. Interactions between ROS and cellular structures mainly take place at interfaces between water and cells. Cell membranes, the most obvious site for these interactions, are largely composed of structural lipids. The route oxygen takes from the atmosphere to animal cells and tissues involves a sequence of highly expanded cellular membranes and of membrane-bound particles: red blood cells (highly enriched in oxygen), other types of circulating cells, lipoproteins of different size and composition and endothelial cells. The area of the surface of red blood cell membranes is in the order of about 0.5 m^2/mL blood and that the global surface of the lipoprotein particles is over 1 m^2/mL blood, an enormous value that suggests a high surface of exposure to lipid peroxidation. Of particular relevance in this respect is the role of erythrocytes, loaded with ‘quanta’ of oxygen, and endowed with highly expanded membranes (the peculiar shape of these cells results in a very high surface/volume ratio) enriched in PUFA in structural lipids. Erythrocytes in the arteries continually ‘bombard’ endothelial cells with ‘quanta’ of oxygen, and create a persisting highly oxygenated ‘background’ condition for the major components of vessel walls. In addition, the endothelium interacts with

subpopulations of polymorpho-nuclear neutrophils (PMN) and leuckocytes that, upon activation, release ROS.

13.1.3 Lipid peroxidation in oils and fats

In addition to occurring in biological systems, lipid peroxidation may also occur in separated fats (e.g. oils) and in lipids present in edible materials (foods), especially when inadequately stored (oils) or during cooking. Some general distinction should be made between oxidation in foods and in separated fats/oils. Natural foods are not just a mixture of chemicals, but generally constitute parts of other organisms, plants or animals (organs, tissues and cells). Fats (as triglycerides) and lipids (polar lipids and cholesterol) in living systems are present as micelles or as cell constituents, i.e. in conditions characterized by a high surface/volume ratio (HSV). HSV ratios are characterized by highly structured and functionally developed interactive sites, where amphiphilic dispersants, e.g. phospholipids, create a very polar display in a polar medium (Porter, 1993), and by quantitatively balanced proportions between substrates of oxidative processes, enzymes and antioxidant systems or compounds. Separated fats and oils, on the other hand, represent a condition that does not occur in nature and that is a product of human manipulation: a very low surface/volume ratio (LSV). In separated fats, lipid oxidation processes and substrate/antioxidant interactions take place under very different conditions. The different behaviour of oxidative processes and especially the different actions of antioxidants in LSV and in biological systems (HSV) can in fact be considered paradoxical, since some compounds that are very effective in the former condition are rather ineffective in the latter (Porter, 1993).

The processes involved in the oxidation of lipids, mainly PUFA and their esters, by molecular oxygen have been described in detail in the literature as autoxidation processes (Niki, 1987). Briefly, after an *initiation* process, a lipid radical is formed through free radical chain mechanisms generated by an attack of radicals, light, UV, heat, irradiation or metal. The lipid radical reacts with oxygen to give a lipid peroxy radical, which attacks another lipid molecule, giving a lipid hydroperoxide, while at the same time a new lipid radical starts the sequence again (*propagation*). The propagation cycle is broken by *termination* reactions.

13.2 Antioxidants in foods and their effects

An antioxidant (AO) can be defined as any substance that, when present at a concentration lower than that of an oxidizable substrate, significantly slows down or inhibits the oxidation of the substrate itself (Halliwell and Gutteridge, 1999). It is clear that the definition is exclusively functional, and that this class of compounds includes substances with highly diverse structures. In addition, while a classical distinction between AO considers only water-soluble and lipid-

Table 13.1 Actions of antioxidants

- Removal of oxygen
- Removal of ions with catalytic activities
- Removal of key intermediates in the oxidation process
- Trapping of initiating radicals
- Chain-breakers

soluble compounds, a wide range of compounds with potent AO properties in various systems, but also with additional effects on various cellular functions (e.g. interactions with enzymes), is characterized by amphiphilic features. This type of characteristic is not generally considered in describing AO compounds.

The main actions of antioxidants are listed in Table 13.1. The contexts in which antioxidants operate may differ as follows.

13.2.1 Food products

The past decades have brought about major changes, both in quantitative and qualitative terms, in our way of eating. Before the Second World War, food, mainly as unprocessed natural food items, was purchased in relatively small quantities, to be consumed quickly, stored for short times, in the presence of unsophisticated refrigeration systems, and cooked by few experienced persons in a family. In recent times, instead, foods are purchased and stored in bulk, as LSV (oils or solid fats, artefacts introduced by humans), which tend to oxidize at the surface, or, frequently, as fast foods, preprocessed mechanically or by heat or freezing. The antioxidant actions in LSV systems are based mainly on the presence and activities of a few natural antioxidants, such as tocopherols, carotenoids and, in special conditions, glutathion and ascorbic acid. Some antioxidant compound from natural sources, however, have been shown to be antioxidant in bulk lipids, but do not function as antioxidant in tissues. The main natural antioxidants acting in LSV systems are hydrophilic phenols, such as the tocopherols. They are true membrane, organelle and adipocyte, or oil droplet antioxidants, since this is the way lipids are displayed in natural tissues. Supporting the tocopherols is the reductive glutathione–ascorbic acid cascade. The tocopherols show some paradoxical behaviour: α-tocopherol is almost ineffective in vegetable oils, modestly effective by itself in animal fats, but more effective, even, than the synthetic antioxidants buthyl hydrox anisole (BHA) and buthyl hydroxy tyrosol (BHT), in HSV situations (Porter, 1993). In bulk oils the effectiveness of tocopherols in the descending order is δ, γ, β and α, the opposite of the order predicted from common indexes (e.g. reduction potential). The natural antioxidants present in food are listed in Table 13.2.

A general feature of antioxidants in natural foods, especially from plant sources, is that most of them (e.g. phenolics) are produced as protective compounds against several stressful conditions (oxidative and other), and are

Table 13.2 Antioxidants present in food

Antioxidant	Function	Sources
Vitamin C	Diverse antioxidant functions	Fruits (especially citrus fruits) and vegetable (tomatoes, potatoes, peppers) including spinach
Vitamin E	Radical chain-breaking	Vegetable oils (soy, corn and sunflower) and derivatives (margarines), grains, seeds, nuts vegetables
Beta-carotene	singlet oxygen quencher	Yellowish-orange fruit (apricots) and vegetables (carrots) and green leafy vegetables
Lycopene	Singlet oxygen quencher	Tomatoes and derived products
Ubiquinol-10	Radical scavenger	
Phenols, flavonoids	Plant antioxidants with several functions	Widely distributed in foods of plant origin
Glutathion	Diverse antioxidant functions	

present as complex mixtures, with somewhat diversified functional features (redundance) and in given quantitative proportions. Some of the latter properties are transferred to animals through the food chain.

13.2.2 Biological systems

The generation of oxygen-derived radicals in biological systems, through cell-independent and cell-mediated processes, results in the production of a variety of oxidation products, generated from lipids, proteins, nucleic acids and sugars. Owing to the complexity of the processes leading to substrate oxidation, it is relevant that the antioxidant defence strategies in biological systems are generally highly evolved. In fact, although several complex biological molecules (e.g. lipoproteins) are quite susceptible to oxidation *in vitro*, i.e. after isolation from biological systems, they are instead rather resistant to oxidative stress in the physiological medium (plasma). Also, cells *in vivo* appear to behave rather differently, with respect to susceptibility to ROS, from cultured cells, frequently used for several types of studies. Cells in culture are often exposed to unphysiological states of oxidative stress, while being depleted of AO (Visioli *et al*., 2000), and the consequence is that effects are produced that are largely artefactual owing to an abnormal generation of ROS. Several studies on the effects of antioxidants may therefore have been affected by these artefacts (Halliwell, 2003). The antioxidant defence system is summarized in Table 13.3.

Table 13.3 Antioxidant defence

System		
Non-enzymic	Enzymic (direct)	Enzymic (ancillary)
Food antioxidant (Table 13.2) Urate Bilirubin Plasma proteins Food addictives, drugs	Superoxide dysmutase (CuZn-, Mn-, Fe-enzyme) GSH peroxidase (GPx, PHGHPx) Catalase (haemprotein, peroxisomes)	Conjugating enzymes (glutathione-S-transferases) UDP-glucuronosyl-transferases NADHP-quinone oxidoreductose (two electron reduction) GSSG reductase NADPH supply (NADPH for GSSG reductase) Transport systems Repair systems (DNA repair, oxidized protein turnover, oxidized phospholipids turnover)

13.3 Biomarkers of oxidative stress

A general problem in studing oxidative stress in biological systems and in the evaluation of the effects of AO *in vivo*, i.e. in patients, concerns the strategies for reliable measurements of oxidative parameters. Several markers and methods have been used for the assessment of the generation of oxidation products (markers of oxidation) of various biomolecules *in vitro*, in *ex vivo* systems and *in vivo* (Table 13.4). The *in vitro* measurements, although quite effective in the assessment of the antioxidant potential of a given compound in a controlled system, are not greatly predictive of the possible activities *in vivo*. It should also be added that since different antioxidants act through different mechanisms and different oxidative substrates may yield different types of products, assays should be aimed at measuring various oxidative products using different substrates (Halliwell, 1995). *Ex vivo* measurements are often also used in connection with the evaluation of oxidative processes in pathological states, but again in some cases some artefactual modification may occur during the collection of the samples (e.g. cells, plasma preparation). The *in vivo* assays are made directly on samples collected without any manipulation, e.g. urines, but although they reflect processes occurring in the organism, they do not imitate the site(s) of these events. Measurement of isoprostanes, non-enzymatically produced oxidative metabolites of arachidonic acid, is considered, with the above-mentioned limitations, a valid indicator (biomarker) of lipid peroxidation. Increments of this marker have been observed in conditions in which enhanced lipid peroxidation may be predicted (in people who smoke, or have diabetes or hyper-cholesterolemia) (Pratico *et al*., 2001).

Concerning specifically the measurements of lipid peroxidation markers, ideal assays should have the following features (Halliwell, 1999):

Table 13.4 Markers of oxidation

In vitro (susceptibility of substrates to oxidation under controlled conditions)
Substrates/markers:

A. Substrates = lipids: fats, oils, lipids in membranes and lipoproteins
   Markers: TBARS, conjugated dienes, lipid peroxides, oxygen uptake, fall of PUFA and vitamin E, isoprostanes
B. Substrates = Proteins: –SH groups, amino-acid residues, etc.
   Markers: electrophoretic mobility. adduct formation, carbonyl content, etc.
A. Substrates = Nucleic acids: DNA bases, deooxyguanosine
   Markers: mass spectrometry (MS) of high performance liquid chromatography (HPLC) of modified bases, electrophoresis of damaged 5′-GG-3′ doublets, 'comet assay' for DNA bases.
A. Substrates = sugars: ribose and deoxyribose in DNA
   Markers: oxidation products

Ex vivo (evaluations on samples, e.g. blood, or cells, obtained from animals/humans without further treatments, except those made *in vivo*)
Antioxidant/oxidant status, antioxidant capacity, antioxidant levels and activities of AO enzymes, levels of negatively charged LDL (a fraction with different chromatographic behaviour in HPLC systems), antibodies against modified LDL, *ex vivo* assays of DNA oxidation, *ex vivo* assays of protein oxidation

In vivo (determinations in biological samples collected non-invasively)
Lipids/lipoprotein oxidation): urinary levels of isoprostanes, hydrocarbons in expired air
DNA damage: urinary levels of modified DNA bases

- Quantitation of major products of the peroxidation process.
- Low coefficients of variation of analyses.
- No interference by other biomolecules.
- Methods: Chemically reliable (e.g. mass spectroscopy, MS or high performance liquid chromatography, HPLC) or validated.
- Possibly not confounded by oxidized lipids ingested with the diet.
- Assess steady-state levels of peroxidation products and total rates of ongoing lipid peroxidation.
- Parameters measured should be stable on storage and not produced artefactually.

Measurement of valid biomarkers of oxidative processes should be promoted before conducting studies on the effects of antioxidants in human studies (Mayne, 2003).

13.4 The role of lipid oxidation in cardiovascular disease

A vast literature over the past two decades has been produced, devoted to the possible involvement of oxidative stress and of ROS-derived products in various

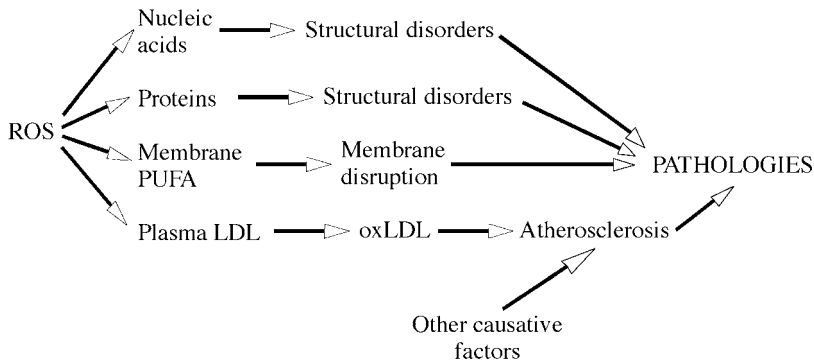
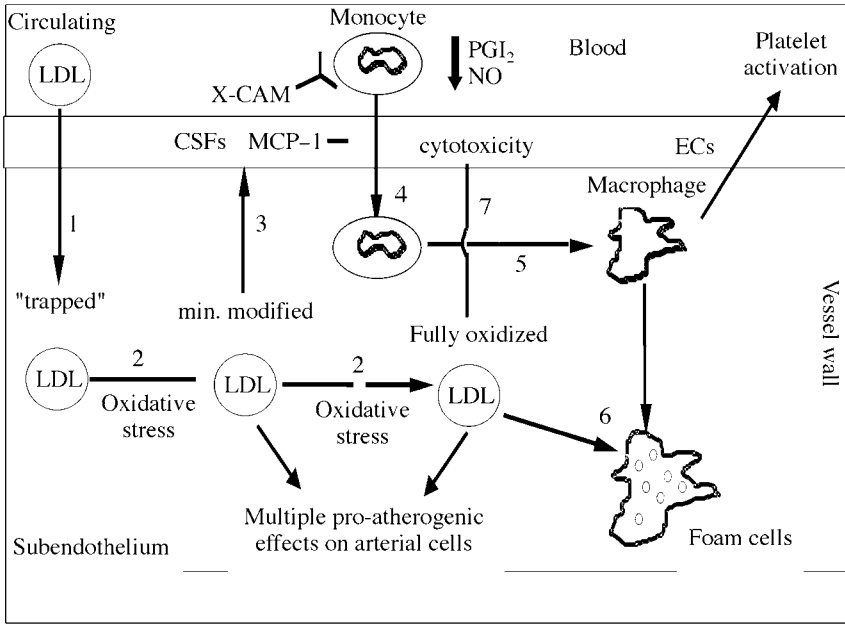


Fig. 13.1 Oxygen radicals and pathologies.

pathological states. To some extent the published information is speculative, owing to major conceptual and analytical difficulties in the assessment of oxidative processes *in vivo* and in the evaluation of their real contribution to pathologies. Uncontrolled free radical production has indeed been advocated as a factor in a number of diseases: atherosclerosis, arthritis, diabetes, pulmonary diseases, cancers, Alzheimer's disease, lateral amyothrophic sclerosis, neuritis, hepatitis and senile cataracts, but most of the attention has been devoted to the possible involvement of lipid/lipoprotein oxidation in atherogenesis and in cardiovascular disease, CVD (Steinberg, 1997, Berliner and Heinecke, 1996). The proposed mechanisms for the involvement of ROS in diseases are described in Fig. 13.1.

As to the issue of oxidative stress and atherosclerotic CVD, certainly rather convincing evidence has been produced in *in vitro* studies, showing that LDL that have been exposed to oxidative stress (oxLDL) through various mechanisms (exposure to chemicals, to physical factors or to cellular processes) are highly atherogenic. Atherogenesis induced by oxLDL has been shown to activate a sequence of events, involving several types of circulating cells (monocytes, platelets) and cellular components (e.g. smooth muscle cells, SMC) and present within the vessel walls (macrophages). The major steps in the whole sequence are presented in Fig. 13.2.

There are, however, still several issues to be defined. First, LDL are rather etherogenous molecular complexes, with significant individual differences in macro- and micro-components, including a number of lipophilic compounds that are associated to them, and it is difficult to identify and quantify all the products generated after exposure to oxidative stress, which may contribute to atherogenesis. Second, *in vitro* LDL oxidation is generally carried out in conditions that maximize the oxidative process, e.g. removal or depletion of hydrophilic and amphiphilic antioxidant compounds that are normally present in plasma, exposure to strong pro-oxidant factors that are difficult to compare quantitatively with *in vivo* free radical generating systems. Therefore the final products, i.e. oxidized LDL, cannot be easily compared with oxLDL possibly



1. LDL crosses the endothelium and becomes trapped
2. In the subendothelium (oxidizing environment) the trapped LDL becomes oxidized
3. Products in min. ox. LDL enhance the expression of monocyte binding proteins (X-CAM), monocyte chemoattractant protein (MCP-1), and colony stimulating factors (CSFs), in endothelial cells (Ecs)
4. Monocytes are recruited
5. Monocytes differentiate into macrophages
6. Further oxidation in Apo B leads to internalization of LDL by macrophages, progenitors of foam cells
7. LDL become cytotoxic leading to further endothelial injury with reduced production of prostsacyclin or prostaglandin I_2 (PGI_2) and NO

Fig. 13.2 Sequence of events involved in activating atherogenesis by inducing oxLDL.

generated *in vivo*. *In vitro* studies have also convincingly shown that several types of antioxidants are able to prevent LDL oxidation induced by various agents, but the use of AO, mainly in the form of supplements, in clinical studies has not shown significant protection against CVD. Although some of these issues are considered in detail in other chapters of the book, it is worth underlining some the strong and the weak points in the overall relationships between oxidative stress and CVD.

There is evidence that lipoproteins (LP) with some of the general features of oxLP produced *in vitro*, evaluated with the use of the typical markers of oxidation (see further), are present in atherosclerotic plaques. On the other side, it is not completely clear whether oxLDL are generated within the vessel wall exposed to high oxygen fluxes, from previously accumulated particles, or whether they are deposited in the vessel walls after being produced in the

circulation, i.e. whether the presence of oxLDL is a secondary or an associated process, rather than a causative event.

For monocytes, again, the accumulated reactive material could be produced in a secondary process. In addition, the recognition by antibodies has several limitations: poor characterization of the oxLDL used as antigens for the preparation of the antibody, and eventual (epitope) differences between the artificially produced oxLDL and those generated *in vivo*. In addition there may be some lack of specificity and poor quantitative responses in the reaction.

Some of the previously mentioned limitations may apply to the presence of autoantibodies against oxLDL in sera of atherosclerotic patients. There is also some evidence that antioxidant consumption may slow the progression of the disease. This, however, is a rather controversial aspect. In essence, the difficulties in the evaluation of the outcome of the studies concern the form and doses of administration of the AO and in the selection of the people to be treated.

In addition to the role of oxidized LDL in the atherogenetic process, a number of studies have been devoted to assess the involvement of oxidative stress in several CV conditions and functions, as discussed in the following reviews: endothelial functions (Cai and Harrison, 2000; Lum and Roebuck, 2001; Matsuoka, 2001; Terada, 2002), neutrophil activation (Kaminski *et al*., 2002), macrophage involvement (Jessup *et al*., 2002), smooth muscle cell function (Bomzon and Ljubuncic, 2001), vascular ageing (Yu and Chung, 2001), congestive heart failure (Mak and Newton, 2001), arterial hypertension (Zalba *et al*., 2001) and diabetes (Bayraktutan, 2002). However, as already discussed, most of the evidence is derived from *in vitro* models, animal studies or *ex vivo* situations, i.e. in somewhat artefactual conditions where some of the processes may be amplified. It is therefore rather problematic to assess and quantify the actual role and relevance of oxidative stress in CVD.

Based on all the direct and indirect evidence in support of the hypothesis that free radical-mediated processes and specific products arising from them may play a role in CVD, great interest has been devoted to the possible protective effects of AO in the diet, or as pure compounds, on biomarkers and on clinical endpoints in population studies.

A vast number of studies have been carried out since 1990 on various aspects of the issue of AO protection (see Table 13.5): they range from epidemiological investigations to controlled trials and have involved a great number of participants. In reality, early observations on the relationships between dietary antioxidant vitamins and disease date back to the 1930s (Seventh-Day Adventists) and the 1950s (Mormons) (reported by Enstrom *et al.*, 1992), and the whole area has been recently reviewed systematically (Asplund, 2002). This review is based on the following inclusion criteria: human studies only, published after 1989, reporting only original data, obtained in case-control, cohort or randomized controlled trials; related to AO vitamins only; mainly reporting on morbidity and mortality of clinically meaningful manifestations of ischaemic heart disease or stroke. The following contexts have been considered: primary prevention of various endpoints (ischaemic heart disease, stroke or combined cardiovascular events), the effects on

Table 13.5 Cohort studies (CS) and randomized control trials (RCT) on the effects of AO on CV risk

Study	Odds ratio
CS – high vs. low intake	
Beta-carotene (8 studies)	No difference
Vitamin E (9 studies)	0.74
Vitamin C (11 studies)	No difference
CS – high vs, low plasma/serum/tissue levels	
Beta-carotene (4 studies)	0.46
Vitamin E (9 studies)	1.61 (unexpected and contrary to the finding for food intake of vitamin E in the same study)
Vitamin C (11 studies)	0.58
Randomized controlled trials of food supplements	
Beta-carotene (6 studies)	No difference
Vitamin E (9 studies)	No difference
Vitamin C (11 studies)	No difference

intermediary endpoints (e.g. blood lipids and blood pressure), studies on secondary prevention in patients with manifest CV disease.

The main conclusions are: in observational studies (case-control or cohort design) people with high intake of AO vitamins by regular diet or as food supplements generally have a lower risk of myocardial infarction and stroke than low consumers. In randomized controlled trials, however, AO vitamins as food supplements have no beneficial effects in the primary prevention of myocardial infarction and stroke, with some report also of adverse events. In addition, in contrast with the initial favourable reports on AO in the secondary prevention of CVD, recent reports apparently failed to show beneficial effects. Some of the negative findings on the effects of AO vitamins, however, may be attributed to pitfalls in the design of the experiments: inadequate characterization of subjects under investigation in terms of ongoing oxidative stress, inappropriate formulations and dosages, especially in comparison with the situation in natural sources: single compounds rather than mixtures, concentrations too high (possibly pro-oxidant) or too low (ineffective), administered as a bolus (capsules or tablets) rather than in the context of foods (better absorption, protection *vs*. oxidation of dietary components, balance between various ingredients with maintenance of natural structural and functional relationships).

In summary, some relationship exists between intakes/plasma levels of some risk factor for vitamin C (reduction of cholesterol and blood pressure with high intakes/levels), for vitamin E (reduced platelet adhesiveness with high intakes) and for multivitamin supplementation (reduced platelet aggregation), but correlations are generally weak and the area has not been investigated in detail. For case-control studies there is some support for low plasma concentrations of

Table 13.6 Summary of results of randomized controlled trials of dietary supplements of AO in the prevention of CVD

- Primary prevention in healthy subjects: 1 out of 8 studies has shown protective effects with beta-carotene *vs*. retinol on a limited number (1203) of subjects. 1 study with beta-carotene show enhanced risk of lung cancer in smokers
- Secondary prevention of CVD in patients with manifestations of the disease
- Out of 14 studies
  - In 5, reduction of CV events
  - In 9, no effect
  - In 1 increase of CV events (beta-carotene).

beta-carotene, and possibly of vitamin E, being linked to increased risk of myocardial infarction. The same does not apply to vitamin C. Altogether, owing to rapid changes in plasma AO vitamins during CV events, the data must be interpreted with caution. Concerning cohort studies, people with high intakes of AO vitamins (regular food or food supplements) have a modest reduction of risk for CV events. Plasma levels of carotene and vitamin C are stronger predictors of future CV events than dietary intakes.

The effects of dietary supplements of AO in the primary and secondary preventions of CVD in randomized controlled trials are summarized in Table 13.6. The general conclusions from these studies are as follows:

- People affected by ischaemic heart disease and stroke, and populations with high occurrence of CVD often have low intakes/plasma levels of AO vitamins (causal or unfavourable lifestyle factors?).
- In case-control or cohort studies, people with high intakes of AO vitamins (food or supplements) have a low risk of myocardial infarction and stroke.
- In randomized controlled trials, AO vitamins as supplements have no beneficial effect on risk for MI or stroke (not recommendable for prevention).
- Some support from observational studies that low intakes of fresh fruits/vegetables may confer a high risk for CVD.

Diets, however, especially those rich in fruits and vegetables, contain several factors or mechanisms other than AO or AO other than vitamins, exerting protective effects on various systems (Halliwell, 1999). The issue of the effects of bioactive compounds in foods and their role in the prevention of CV disease is therefore quite complex, since a large number of potentially health beneficial substances have been described (Kris-Etherton *et al*., 2002). These are summarized in Table 13.7.

Flavonoids in particular have been investigated in relation to possible health benefits (Ross and Kasum, 2002), owing to their potential antioxidant and free-radical scavenging activities observed *in vitro*. Human feeding studies have shown that their absorption and bioavailability are higher than originally believed, but their overall function *in vivo* has yet to be clarified, whether antioxidant, anti-inflammatory, enzyme inhibitor, enzyme inducer, inhibitor of cell division, or some other function (Rice-Evans, 2001). Epidemiological

Table 13.7 Selected bioactive compounds with potentially beneficial effects on the CV system

Compounds	Examples	Sources
Flavonoids		
Flavones	Apigenin, luteolin	Parsley, thyme, celery
Flavonols	Quercetin, myricetin	Onions, broccoli, apples, cherries, berries, tea
Flavanones	Naringenin, hesperedin	Cirtus foods, prunes
Catechins	Epicatechin, gallocatechin	Tea, apples, cocoa
Anthocyanidins	Pelargonin, malvadin	Cherries, grapes
Isoflavones	Genistein, daidzein	Soya beans, legumes
Phytoestrogens		
Lignans, coumestran	Enterolatone, coumestrolk	Flaxseed oil, clover
Resveratrol		Grapes, red wine, peanuts
Lycopene		Tomatoes, tomato products
Organosulphur compounds	Allicin, diallyl sulphide	Garlic, onion, leek
Isothiocyanates	Phenethyl benzyl, sulphoranes	Cruciferous vegetables
Monoterpenes	d-Limonene, perillic acid	Essential oils of citrus fruit, rice bran oil, cherries, mint
Plant sterols	Sitostanol, stigmasterol	Tall oil, soybean oil, rice bran oil
Olive oil	Hydroxytyrosol, oleuropein	Olives, virgin olive oil

studies exploring the role of flavonoids in human health have been inconclusive: some studies support a protective effect of their consumption on CVD and cancer, other studies demonstrate no effect and a few studies suggest potential harm (Ross and Kasum, 2002). Additional selected classes of bioactive compounds with antioxidant and other types of potentially healthful activities are the large groups of phenolics that are present in edible fluids – obtained from fruits of plants exposed to stressful conditions, such as grapes and olives – which, since the beginning of recorded history, have been part of the diet of populations living in certain areas, such as the Mediterranean basin, i.e. wine and olive oil. A vast literature is available on the properties of these compounds (German and Walzem, 2000; Visioli *et al*., 2002), although the impact of their consumption on health through the diet has not yet been fully assessed.

13.5 Dietary fat consumption and lipid oxidation

Human fat consumption has certainly changed drastically from the hunter-gatherer conditions, through the beginning of agriculture to modern times. Changes concerned both the amounts and the quality (Simopoulos, 1999), from the low amounts of fats, especially of vegetable origin, with relative abundance of long-chain polyunsaturated fatty acids (LC-PUFA), components of structural

lipids in lean meat of wild animals and fish, in prehistoric conditions, to the progressive increment in the consumption of fats from farmed animals and cultivated vegetables.

The introduction and development of agriculture have changed fat intake markedly, although for a long time changes concerned mainly the continuity of fat supply after agriculture development as opposed to the sporadic intake in hunter-gatherers. Following the progressive depletion of food obtained from small mammals, fish, fowls and gathered plants, associated with the increase in human population numbers, cereal grains became the dominant caloric and protein source of most early cultures.

Drastic changes in fat intakes have occurred however, especially in recent times, i.e. in the period after the Second World War, for a number of reasons: fats represented in the past the most expensive part of the diet, since fat/oil productions in developing countries were limited by climatic and economic reasons, and importation from fat-producing countries was expensive. Fat consumption was therefore strictly correlated with national per capita incomes (FAO, 1977). With the introduction of extensive cereal grains and seed oil-raising crops, the availability of fats for human consumption and animal feeding increased dramatically. Fats became recently rather inexpensive, even used as fuel, and available on a global scale to most populations, which in several situations appear to be exposed to hypercaloric and yet deficient (in several essential micronutrients) dietary conditions. In addition, increments in seed oil consumption brought about marked increments in the consumption of PUFA, especially of the omega-6 series (i.e. linoleic acid, 18:2 omega-6). However, differences in fat intakes among populations are still present, with generally lower intakes (7–15 energy per cent) in countries from the East and Far East, e.g. Bangladesh, Korea, China, India, Philippines, and from Africa, e.g. Tanzania, Nigeria, Ethiopia (FAO, 1994), *vs*. around 32–38 energy per cent (en per cent) in several countries on Western diets. A relatively recent study carried out in Tanzania dealt with populations on diets with 8–13 en per cent from fats (Pauletto *et al*., 1996), i.e. still much lower than the levels in Western countries. High fat intakes are generally associated with high saturated fatty acids (SFA), and also relatively high intakes of PUFA, especially of the omega-6 series.

There are also still appreciable differences in fat intakes among Western populations as indicated by a cross-evaluation in 14 European countries (Hulshof *et al*., 1999). Variations concerned both the absolute intakes with values ranging from around 31 en per cent, in Finland, Italy, Norway and Portugal, up to over 40 en per cent in Germany, Iceland, Spain and Belgium. As to the qualitative differences, SFA range between around 10 en per cent, in most Mediterranean Countries to about 19 per cent, monounsaturated fatty acids (MUFA) contribute to about 9–12 en per cent, with higher values in Greece and the southern parts of Italy and Spain (high olive oil intake), and PUFA ranging between 3 and 7 en per cent. *Trans* FA range between 0.5 en per cent in Greece up to round 2 en per cent in Iceland, and are therefore not considered to be a major problem. As to the trends in nutrient intakes over time, it is of interest that

a study carried out in 10-year-old children over two decades (1973–94) in Louisiana, revealed that total energy intake remained unchanged during that time period (although it declined as Kcal/body weight), but there was a trend toward weight gain. There was a significant increase in percentage energy from proteins and carbohydrates and a decrease in percentage energy from fat (mainly SFA and MUFA). In general, although more children met the recommendations for total fat, SFA and dietary cholesterol, the vast majority continued to exceed prudent diet recommendations.

Recently, it has been proposed that the role of the diet, and particularly of dietary fats in vascular disease and in its protection, have been vastly underestimated, owing to failure to understand the importance of postprandial events (Spencer, 2002). The compounds that repeatedly enter the circulation every day during our lifespan certainly result in the exposure of vessel walls to a large variety of nutrients, and also of potentially stressful factors. These include postprandial oxidative stress, consequent to the consumption of meals containing oxidized and oxidizable lipids. This results in the postprandial elevation of plasma lipid peroxides (Ursini and Sevanian, 2002), while on the other hand, AO in meals may minimize postprandial oxidative stress.

13.5.1 Dietary fat consumption: raw *vs*. cooked fats

Based on the above considerations, it appears that the consumption of heated/fried fats may be a contributing factor in the impact of dietary fat on health. Alterations of fats and oils and of the lipid components of meals are induced by various factors (heat, light, irradiation, pH, oxygen, moisture, pro-oxidizing agents, storage at room temperature) through several processes. Refining of vegetable fats and oils has no deleterious effects upon their composition as far as deliming, neutralization or bleaching are concerned, but during deodorization or physical refining, small amounts of dimeric triglycerides and of *trans* fatty acids are formed depending upon temperature and duration (Billek, 1992). In general, boiling and baking have no effects, and short-term shallow frying shows only minor changes in quality. The situation is different for deep-fat frying, which can cause serious alterations, especially if the oil is used for too long. The chemical reactions involved are predominantly isomerizations, polymerization and oxidation processes. There are certainly differences related not only to the cooking/frying conditions, e.g. conventional cooking methods *vs*. microwave cooking (Regulska-Ilow and Ilow, 2002), but also to the type of fat, the oils containing more unsaturated fatty acids, e.g. several seed oils rather than olive oil, being more susceptible to oxidative changes. In addition to alterations in the chemistry of fats, changes can also occur in antioxidant levels and antioxidant activity of the oils (Warner, 1999). The addition of antioxidants to the oils may protect the fatty acids from oxidation, and during heating/frying the loss of lipid-soluble vitamins (e.g. the tocopherols) from the oil precedes that of more polar compounds, e.g. phenolics in the case of olive oil (Gomez-Alonso *et al*., 2003), suggesting that they may act as protecting agents against AO vitamins. Fried

foods are generally considered detrimental to our health especially in relation to lipid oxidation, but not all food components are equally affected, since there is, for example, little or no effect on the protein or mineral content of fried food. In addition, it should be considered that when fat/oils are used in frying food, e.g. potatoes, the temperature reached at the surfaces between food and oils is markedly lower than the temperature of the boiling oily phase, owing to the extensive evaporation of the water in the food, and that the formation of a 'crusty' surface prevents a significant penetration of the oxidized products into the food.

Among the oxidative products generated from lipids in foods, attention has been expressly paid to cholesterol oxides. Several cholesterol oxides are commonly found in foods with high cholesterol contents, such as meat, egg yolk and egg-based products (cakes, sweet biscuits, mayonnaise) if fresh materials are not used in their manufacture, and in full-fat dairy products (Savage *et al*., 2002). Fresh foods generally contain very low levels of cholesterol oxides, while their levels are increased by storage, cooking and processing. Dietary cholesterol oxides appear to be well absorbed, and to influence postprandial lipoprotein particle size and composition. These changes may have effects on the clearance of chylomicrons from plasma, arterial delivery of oxysterols and possible deposition in arterial lesions (Vine *et al*., 1997). In general, lipid peroxides from the diet may contribute significantly to the whole process of lipid peroxidation, especially during the postprandial phase, in addition to the peroxides produced through endogenous processes.

A number of studies have been devoted to investigate the health effects of thermoxidized oils and fats (Billek, 2000). While early studies using extremely overheated fats showed toxic effects in animals, the administration of fats and oils heated in equipment for deep-fat frying under the conditions of good commercial practice did not show detrimental effects on classical parameters (e.g. growth, toxicity tests) even when fed in high amounts for long time periods. However, human studies specifically related to the postprandial effects of unheated or heated oils showed increments of markers of lipid oxidation in serum related to the type of oil (e.g. safflower had greater effects than olive oil, both when uncooked and especially when cooked) (Sutherland *et al*., 2002). The effects of the administration of both cooked oils on major functional parameters, e.g. endothelium-dependent dilatation were, however, minimal (Williams *et al*., 2001). Other parameters not directly related to lipid peroxidation and to changes in plasma antioxidants have also been shown in animal studies after the administration of thermally oxidized fats: an increase in plasma thyroxine concentrations irrespective of the vitamin E and selenium status (Eder *et al*., 2002). In general, this type of study needs to be substantially extended and applied to more practically relevant conditions. The experimental design is crucial in this respect since the type of oxidized fats to be administered and their actual chemical composition, the context of the other components (macro- and micro-nutrients) of the diet, the doses and duration of the experiments and selection of subjects are major determinants in the outcomes.

13.6 Sources of further information and advice

Several health organizations over the last few years have provided recommendations on fat intake with the aim to improve our health status especially with respect of CV disease and cancer. The Scientific Conference on Dietary Fatty Acids and Cardiovascular Health (AHA, 2001) and the Executive Summary of the NCEP Expert Panel (NCEP, 2001) have provided the following recommendations: total fat 25–35 en per cent, SFA < 7 en per cent, MUFA up to 20 en per cent and PUFA up to 10 en per cent. On the other side, since evidence has been accumulating on the differential and somewhat contrasting biological roles of the omega-6 and omega-3 fatty acids, it has also been proposed by the board of the International Society for the Study of Fatty Acids and Lipids (ISSFAL) (NIH Workshop 7–9 April 1999) that individual PUFA should be considered separately and that, in addition to a value not exceeding 7 en per cent for total PUFA, LA, the major omega-6, should not exceed 4-5 en per cent, while the omega-3 ALA (alpha linolenic acid) should be at least 1 en per cent and EPA + DHA in a range of at least 0.3 g up to 1 g/day. The omega-6/omega-3 ratio is also considered an important parameter and a ratio of about 4 or 5/1 has been recommended, i.e. a ratio in the range of that apparently present in the diet before the explosion of modern agriculture, and lower than the ratio greater than 10/1 in our diets. Practical approaches to the definition of a diet with an adequate FA composition and can be based on the use of food composition data, such as those in the web site of the USDA (http://www.usda.gov). Although information from databases may not be totally adequate with reference to some minor FA components, e.g. some omega-3 FA, their use is valuable.

As to the recommendations concerning the consumption/intake of AO, although randomized controlled trials of AO vitamins as supplements have shown that they have no beneficial effect on risk for myocardial infarction or stroke, increments in the consumption of vegetables and fruits should be highly recommended. As an example, the list of 10 foods recommended as very healthy by *Time* magazine (2002), on the basis of generally accepted scientific evidence, and selected also for the content in AO in addition to other bioactive compounds, include the following vegetables and fruits: tomatoes (rich in the carotenoid lycopene and vitamin C), spinach (rich in the AO phytochemicals lutein and zeaxanthine, in addition to providing iron and folate), broccoli (rich in beta-carotene and vitamin C, in addition to some phytochemicals, e.g. indole-3-carbinol with detoxifying activity), nuts (rich in vitamin E, as well as in the omega-3 FA alpha-linolenic acid, and in ellagic acid, with potential anticancer activities), red wine (polyphenolic AO derived from the skin of the grapes), oats (rich in tocotrienols, AO with vitamin E-like activities, and in fibres, e.g. beta-glucan), (green) tea (rich in the AO phenols, the catechins), blueberries, very rich in several types of AO (especially the antocyanins). Dietary AO, in addition to providing precious protective and health-promoting agents, may play a special role by acting at the gastrointestinal tract, possibly a major site of production of toxic oxidized products (Halliwell *et al*., 2000).

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14

Dietary fat, pregnancy and the prevention of heart disease

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14.1 Introduction: pregnancy and foetal growth

In his book, first printed in 1992,[1] Barker made a statement of 'foetal' or 'metabolic' programming. Based on studies using medical records in Britain and other countries,[2–5] the basic hypothesis is that impaired development *in utero*, leading to babies of low birthweight, is a strong predictor of heart disease, arterial disease, hypertension or type 2 diabetes mellitus in later life.[6–8] The statement could now be extended to include slow growth in the first year of life.[9,10] It is the intention of this chapter to consider whether changes in the content of fat, or the composition of that fat, in the maternal diet may, by improving the development of the foetus or by some other mechanism, help prevent such problems.

It is well known that the developing foetal brain has a definite requirement for the long-chain polyunsaturated fatty acid, docosahexaenoic acid (DHA), but other fatty acids are required for structural purposes (membrane synthesis), as a source of precursors (e.g. for eicosanoids, a group of compounds including prostaglandins and thromboxanes involved in cell-to-cell communication) or as the substrate for fat stores to be used after birth as a source of energy. Furthermore, the use of fats, as a source of energy for the mother, means that glucose is available for use by the foetus.

In this chapter, we first examine the mechanisms underlying the materno-foetal relationship in terms of lipid metabolism so that the roles of the maternal diet during stages of pregnancy and of maternal dietary history may be understood. We also examine the complex interactions between the dietary fatty acids, their synthesis *in vivo* and other complicating factors, such as susceptibility to oxidative stress. We finish by summarizing the complexity of the situation and by suggesting some avenues for future research.

14.1.1 Changes occurring in the mother during pregnancy that help to sustain foetal growth under normal conditions

Foetal development depends upon the continuous supply of metabolites, derived from the maternal circulation, across the placenta. Quantitatively, the most abundant nutrient crossing the placenta is glucose, followed by amino acids.[11–15] Placental transfer of lipid components is limited in comparison,[16,17] but the lipid components also play a major role in foetal development. Changes in the availability of lipid components, such as those produced by changes in dietary fat composition, are known to have implications for foetal and postnatal development.[18] In addition, the adaptations of maternal lipid metabolism during gestation also have major implications for foetal growth; for instance, it is known that deviations from normal maternal plasma lipid status, such as hypercholesterolaemia, even when temporary and limited to pregnancy, can trigger pathogenic events in the foetal aorta and may influence atherosclerosis later in life.[19–21]

From the metabolic point of view, there are two distinct stages of pregnancy. During the first two-thirds, foetal growth is small and the mother stores a large proportion of the nutrients she eats, which, in combination with her hyperphagic state, causes accumulation of fat stores.[22,23] This condition is facilitated by hyperinsulinaemia and normal, or even enhanced, insulin sensitivity.[24–26] During the last third of gestation foetal growth is very rapid, being sustained by an enhanced transfer of nutrients through the placenta. Hence the mother switches from the previous anabolic condition to a catabolic one. This change is seen most clearly in terms of an enhanced breakdown of lipid stores by lipolysis in adipose tissue,[27–29] and is facilitated by the development of an overt insulin-resistant condition.[30–32]

14.2 Carbohydrate, amino acid and maternal lipid metabolism in gestation

14.2.1 Carbohydrate and amino acid metabolism

During late pregnancy the mother tends to develop hypoglycaemia, which is especially evident during fasting.[33,34] Indirect studies in women[35] and direct experiments in rats[34,36] have shown that the rate of gluconeogenesis is enhanced during pregnancy under fasting conditions. Of the common gluconeogenic substrates, glycerol was converted into glucose even more rapidly than others such as pyruvate and alanine.[37] Thus, gestational hypoglycaemia must be a consequence of increased utilization of glucose: this is despite the decreased consumption of glucose by the insulin-resistant tissues and results from a rate of placental transfer much higher than for other metabolites, even amino acids.[11,38] The placental transfer of glucose is carried out by facilitated diffusion according to concentration-dependent kinetics[14,39] and is therefore dependent on the positive materno-foetal glucose gradient.[12] The gradient is maintained by the low concentration of glucose in the foetal circulation and, on occasion, by active maternal gluconeogenesis.

In contrast, the concentration of amino acids in foetal plasma is even higher than in the mother,[12,40,41] because placental transfer of amino acids is carried out by an energy-dependent process, using selective transporters.[12,14,42–44] This ensures the availability of these essential precursors in appropriate quantities to the foetus and can result in a tendency to maternal hypoaminoacidaemia.[11]

14.2.2 Maternal lipid metabolism

Two consistent manifestations of altered maternal lipid metabolism during normal gestation are the accumulation of lipids in early-pregnant maternal tissues[22,45] as a result of major changes in adipose tissue metabolism and the later development of maternal hyperlipidaemia.[46,47]

Adipose tissue metabolism: accumulation of body fat

Fat accumulation is a characteristic feature of pregnancy, occurring in both women[22,45,48] and experimental animals.[23,49,50] The accumulation of maternal fat in maternal depots takes place during the first two-thirds of gestation but stops or even declines during the last third,[45,49,51,53] as a consequence of enhanced adipose tissue lipolytic activity.

Body fat accumulation during early pregnancy seems to be the result of both hyperphagia and increased lipid synthesis. Hyperphagia during pregnancy occurs in women[54,55] and rats.[50,56] Both fatty acid synthesis and the conversion of glucose to form the 'glycerol backbone' of fat molecules have been found to increase progressively in rat adipose tissue until day 20 of gestation and then to decline sharply on day 21, just before parturition.[38,57]

Changes in adipose tissue lipoprotein lipase (LPL) activity could be a means by which fat accumulation is controlled during early pregnancy. This enzyme, present in the capillary endothelium of extra-hepatic tissues, hydrolyses triacylglycerols circulating in plasma in the form of triacylglycerol-rich lipoproteins,[58] and the hydrolytic products, fatty acids and glycerol, are mostly taken up by the subjacent tissue.[59] In this way, LPL activity is a prerequisite for the uptake of circulating fat by adipose tissue. Some reports suggest that there is an increase in the activity of LPL in rat adipose tissue by day 12 of gestation,[60,61] but the change is small and not always reproduced.[29] Furthermore, no significant change has been found in the postheparin LPL activity in pregnant women at mid-gestation.[46] During late pregnancy, however, LPL activity in rat adipose tissue has consistently been found to be decreased.[52,62–64] Postheparin LPL activity has also been found to decrease in pregnant women during the third trimester of gestation.[46]

Thus, it is proposed that fat uptake by adipose tissue decreases during late pregnancy and that this change, together with the enhanced lipolytic activity (see below), results in the net accelerated breakdown of fat depots during the last trimester of pregnancy, which coincides with the phase of maximal foetal growth.[52,65]

Adipose tissue metabolism: lipolytic activity

Increased lipolysis of adipose tissue fat stores occurs both in women and rats during the last third of gestation.[28, 66–69] At the same time, increased activity of hormone-sensitive lipase (HSL, the key enzyme of adipose tissue lipolysis), and increased concentrations of the mRNA that codes for it, are observed in pregnant rats.[29]

The majority of the products of adipose tissue lipolysis, fatty acids (often called non-esterified fatty acids or NEFA) and glycerol, are released into the circulation. Since the placental transfer of these products is quantitatively low,[17] their main destination is the maternal liver[70] where, after conversion into active forms acyl-CoA and glycerol-3-phosphate respectively, they are re-esterified for the synthesis of triacylglycerols that are released into the circulation as part of very low-density lipoproteins (VLDLs). Since insulin inhibits both adipose tissue lipolytic activity[71, 72] and hepatic VLDL secretion[73] but increases LPL activity,[74] the insulin-resistant condition of late pregnancy contributes to both the increased lipolysis of fat stores[75] and the increased VLDL production, although for the latter the enhanced oestrogen concentration at late pregnancy seems to be its major activator.[47]

During late pregnancy, the lipolytic activity of maternal adipose tissue increases markedly under (experimental) fasting conditions.[27, 28, 69, 76] In addition to the use of the lipolytic products in the resynthesis of triacylglycerols described above, glycerol may be used for glucose synthesis (required for brain function) and NEFA for β-oxidation to acetyl-CoA, leading to energy production and synthesis of ketone bodies; these pathways also increase markedly under fasting conditions in late pregnancy.[34, 36, 77, 78]

The preferential use of glycerol for gluconeogenesis and the efficient placental transfer of the newly formed glucose may be of major importance to the foetus under such fasting conditions, where the availability of other essential substrates such as amino acids is reduced.[36, 61] The enhanced maternal ketogenesis during fasting also benefits the foetus in two ways: ketone bodies are used by maternal tissues, thus sparing glucose for essential functions and delivery to the foetus; placental transfer of ketone bodies is very efficient,[79] attaining the same concentration in foetal plasma as is found in the maternal circulation.[80] Consequently, ketone bodies may be used by the foetus as oxidative fuels[81] as well as substrates for brain lipid synthesis.[82]

Maternal hyperlipidaemia

The catabolic condition of maternal adipose tissue during late gestation is associated with hyperlipidaemia, mainly corresponding to rises in triacylglycerols, with smaller rises in phospholipids and cholesterol in the circulation.[47] Although the greatest increase in plasma triacylglycerols corresponds to VLDL, there is also an enrichment of triacylglycerols in other lipoprotein fractions that normally do not transport them, such as low-density lipoproteins (LDL) and high-density lipoproteins (HDL).[46] This increase in plasma VLDL triacylglycerols during gestation results from enhanced production by the

liver[83, 84] and decreased removal from the circulation as a consequence of reduced adipose tissue LPL activity.[29, 46]

The abundance of VLDL triacylglycerols in the presence of an increase in cholesteryl ester transfer protein (CETP) activity taking place at mid-gestation[46, 85] contributes to the accumulation of triacylglycerols in the lipoprotein fractions of higher density, LDL and HDL.[46, 86] Another factor contributing to the same effect is the decrease in the hepatic lipase activity, which also occurs during late pregnancy.[46] The decrease in this enzyme's activity decreases the conversion of buoyant HDL_2 triacylglycerol-rich particles into smaller, denser, triacylglycerol-poor HDL_3 particles, allowing a proportional accumulation of the former.[46]

The hormonal factors responsible for the metabolic changes, which result in the development of maternal hypertriacylglycerolaemia, are the insulin-resistant condition and the increase in plasma oestrogen concentrations, both occurring during late pregnancy. The insulin-resistant condition contributes to the enhanced adipose tissue lipolytic activity, which, as described above, speeds the transport of glycerol and NEFA to the liver, to their subsequent conversion into circulating VLDL-triacylglycerols,[75] and to decreased LPL activity.[74] The increase in plasma oestrogen concentrations during gestation[87, 88] also contributes to maternal hypertriacylglycerolaemia since it enhances hepatic production of VLDL[89, 90] and decreases the expression and activity of hepatic lipase.[91, 92]

14.3 Placental transfer of lipid metabolites

14.3.1 Availability of essential fatty acids to the foetus

Essential fatty acids (EFA) are fatty acids containing double bonds either six or three (or both) carbons from their methyl end, the so-called n-6 and n-3 positions (often referred to as ω-6 and ω-3) respectively. As animals are incapable of inserting such double bonds themselves, EFA can be obtained only from the diet and it follows that the foetus can obtain them only from the maternal circulation via the placenta. The simplest n-6 polyunsaturated fatty acid (PUFA) is linoleic acid with 18 carbons and two double bonds (18:2 in shorthand), which is a precursor of arachidonic acid (20:4 or AA). The simplest n-3 PUFA is α-linolenic acid (18:3), a precursor of docosahexaenoic acid (22:6 or DHA).

Triacylglycerols circulating in plasma lipoproteins do not directly cross the placental barrier,[17] but EFA from maternal diet, which are transported as triacylglycerols in triacylglycerol-rich lipoproteins in maternal plasma,[93] must be made available to the foetus. The presence of both the VLDL/apo-E receptor and the LDL receptor-related proteins in placental trophoblast cells[94–99] allows these lipoproteins to be taken up by the placenta. In addition, the trophoblasts also express at least three different lipolytic activities including LPL,[100–102] phospholipase A_2[103, 104] and an intracellular lipase.[105–107] Thus, maternal triacylglycerols in plasma lipoproteins are either taken up intact by the placenta

by receptors or, after hydrolysis, their constituent fatty acids are taken up by the placenta, where the fatty acids are re-esterified to synthesize glycerolipids to provide a reservoir of fatty acids.[108] Subsequent intracellular hydrolysis of the glycerolipids releases fatty acids to diffuse to foetal plasma, where they bind to the alpha-foeto protein.[109, 110] In this way, they are transported to the foetal liver, where they are re-esterified and released back into the foetal circulation in the form of lipoprotein-triacylglycerols.

Thus, maternal hyperlipoproteinaemia seems to play a key role in the availability of EFA to the foetus, and reductions in maternal hypertriacylglycerolaemia, such as that caused by treatment with hypolipidaemic drugs, have detrimental effects on foetal development.[111, 112]

Transport of non-esterified fatty acids

There are important differences among mammalian species in the net flux of fatty acids across the placenta. In species with placentas that comprise both maternal and foetal layers, such as sheep, pig and cat, the maternal foetal fatty acid transfer is small,[113–116] whereas species where the placenta is formed by layers of foetal origin, such as the rabbit,[117] guinea pig,[118] primates[119] and rat,[120, 121] the amount of fatty acid crossing the placenta exceeds even that needed to provide an adipose store of lipids sufficient to support postnatal growth and development.[122] In humans, although small in proportion to lipoprotein triacylglycerols, maternal plasma NEFA are an important source of PUFA to the foetus.[123, 124]

In human placenta there is a membrane fatty acid-binding protein ($FABP_{pm}$)[125, 126] which is responsible for the preferential uptake of long-chain polyunsaturated fatty acids (LC-PUFA) and allows the preferential transfer of certain LC-PUFA: docosahexaenoic > α-linolenic > linoleic > oleic > arachidonic acid.[127] In the case of arachidonic acid, its uptake by syncytiotrophoblast membranes has been shown to occur by an ATP-dependent active process.[128] This selective transport of certain fatty acids may contribute to the efficacy of the overall placental transfer process and may contribute to a degree of selective metabolism such as the conversion of a proportion of 20-carbon fatty acids to prostaglandins and other eicosanoids,[124] the relative proportions of 2-series and 3-series prostaglandins being formed,[129, 130] the incorporation of some fatty acids into membrane phospholipids,[131] placental fatty acid oxidation[132] and placental fatty acid synthesis.[133] Thus, the combination of all these processes determines the actual rate of placental fatty acid transfer and its selectivity, resulting in the proportional enrichment of certain LC-PUFA, such as arachidonic acid and docosahexaenoic acid in the foetal compartment compared to maternal compartment.[134]

An interesting point here is that that thromboxane A_3, a prostaglandin-like messenger derived from n-3 fatty acids, has been reported to be a less effective vasoconstrictor than thromboxane A_2, derived from n-6 fatty acids, and that this could provide a link between diet and hypertension.[135]

Cholesterol

The remaining major lipid, cholesterol, is an essential component of cell membranes, where it affects the fluidity and passive permeability.[136] It is the precursor of bile acids, used in the digestion of dietary lipids, of steroid hormones, required for cell proliferation[137, 138] and development of the growing body (e.g. sexual differentiation),[139, 140] cell differentiation and cell-to-cell communication,[141] and of oxysterols, which regulate certain metabolic processes.[142] Consequently, the demand for cholesterol in the embryo and the foetus is relatively high.

Placental transfer of maternal cholesterol has been shown to be effective in different species, such as the rat,[143] guinea pig[144] and rhesus monkey.[145] Cholesterol synthesis in foetal tissues, and especially in foetal brain, has also been shown to be highly active in some species,[146–150] and the expression of the genes for the enzymes involved in cholesterol synthesis, as measured by mRNA contents and by enzyme activities, is elevated in foetal tissues.[151–153]

Cholesterol can be transferred by the placenta and it can be synthesized from a simple two-carbon precursor by a complex biochemical pathway. In the rat, the foetus receives little or no cholesterol from its mother and satisfies its need for cholesterol through endogenous synthesis[149, 150] as illustrated by the following experiment. Feeding late-pregnant rats with cholesterol, sufficient to increase the maternal plasma cholesterol concentration and to reduce maternal cholesterol synthesis, had no effect on these same parameters in the foetus[146, 147, 154, 155] or on foetal development.[156] However, some circumstantial evidence exists for a role for maternal cholesterol during the early stages of gestation. Treatment of pregnant rats during early pregnancy with an inhibitor of the enzyme Δ^6-reductase, AY 9944, resulted in foetal teratogenesis, whereas simultaneous administration of oral cholesterol prevented this effect.[157–159]

In humans, comparison of concentrations of lipoprotein-cholesterol in maternal plasma with umbilical cord blood cholesterol gave positive correlations in some experiments[160, 161] and no correlation in others.[162–165] Gestational age could influence these comparisons, since plasma foetal cholesterol levels are higher in 5-month than in 7-month-old foetuses, and in foetuses younger than 6 months, plasma cholesterol concentration is significantly correlated to the maternal concentration,[19] suggesting that, at these early stages of gestation, maternal cholesterol actively contributes to foetal cholesterol. At term, umbilical venous concentrations of HDL-, LDL- and total cholesterol were higher than in umbilical arterial plasma, indicating the delivery of cholesterol from placenta to the foetus; however, the contribution of such cholesterol to the foetal plasma cholesterol pool is very small.[162]

14.4 Foetal development: the role of dietary fatty acids

Essential fatty acids (EFA) and their LC-PUFA derivatives are required during normal foetal development to support the synthesis of structural lipids, notably

the phospholipids of brain and retinal tissue.[166–169] Although both term and preterm infants seem able to form arachidonic acid (20:4 n-6 or AA) and docosahexaenoic acid (22:6 n-3 or DHA) from their respective EFA precursors, linoleic acid (18:2 n-6) and α-linolenic acid (18:3 n-3) by a process of sequential desaturations and elongations,[170–175] the degree to which the foetus is capable of carrying out these processes is not clear. In fact, it has been shown in the newborn infant during the first week of life that the endogenous synthesis of AA seems to contribute very little to the plasma AA pool,[174] the limiting factor being a low Δ5-desaturation activity, although foetal baboons have been shown to synthesize both AA and DHA from their respective EFA precursors.[176, 177]

In humans, a reduced nutritional status with respect to EFA during gestation has been correlated with reduced neonatal growth[178] and, in untreated healthy women, maternal plasma concentrations of LC-PUFA have been consistently correlated with those in the foetus or newborn.[179–181] Furthermore, supplementation with fish oil during pregnancy increases DHA in both mothers and newborns.[182, 183] These results have led to the issue of advice that maternal diets should be routinely supplemented with fish oil during the last trimester of pregnancy.[182, 183] However, care must be exercised because the competitive inhibition of the Δ6- and Δ5-desaturases (two enzymes that control the conversion of EFA into LC-PUFA by the n-3 and n-6 pathways), by specific fatty acids present in excess, may inhibit the synthesis of other specific LC-PUFA that could be essential for foetal growth.[184] In fact, when fish oil is consumed, low plasma AA levels are found,[184, 185] the effect being caused by the abundance of both eicosapentaenoic acid (20:5 n-3 or EPA) and DHA (22:6 n-3) in this oil, which specifically inhibit the Δ6 desaturase activity – an obligatory step in the conversion of dietary linoleic acid into AA.[186, 187]

During the perinatal period, the inhibitory effects of an excess of certain dietary fatty acids on LC-PUFA synthetic pathways may acquire major relevance, since plasma AA concentrations have been correlated to body weight in preterm infants[188–190] and adverse effects of low AA concentrations on growth during infancy have been reported.[188, 191, 192]

An excess of LC-PUFA may also increase the susceptibility to lipid peroxidation. The susceptibility of LDLs to oxidative modification *in vitro* was reported to increase when the LDL were isolated from animals given diets rich in n-6 PUFA.[193–195] Also, an increase in plasma thiobarbituric acid-reacting substances (TBARS – a measure of lipid peroxidation) was found after periods of dietary enrichment with n-6 PUFA.[196] Whether or not a diet high in n-3 PUFA also increases lipid peroxidation is controversial.[197, 198] Whereas several studies in humans have shown that dietary supplementation with fish oil rich in n-3 PUFA does not increase lipid peroxidation *in vivo*,[199–202] studies in rats and in cell culture have shown that this same treatment reduces the antioxidant capacity[185, 203] and increases susceptibility to oxidative damage.[204–206]

That increased lipid peroxidation is a ‘bad thing’ is not in doubt. Experimental studies in diabetic pregnancy have shown that increased reactive oxygen species and lipid peroxidation result in foetal damage, the effect being

prevented by treatment with the anti-oxidant vitamin E.[207–213] The detrimental effect on offspring of high dietary fish oil intake during pregnancy could be mediated either by the reported consequent decrease in AA[214–216] or by an increased usage of α-tocopherol (a form of vitamin E) to protect the high LC-PUFA content of fish oil. Experiments, in which pregnant and lactating rats were fed diets supplemented with 10 per cent fish oil or olive oil, concluded that low AA, rather than low α-tocopherol, was responsible for the delayed postnatal development seen in the offspring of the rats fed the fish oil diet.[185]

14.4.1 Possible effects of dietary fat on 'foetal programming'

The role of foetal and childhood nutrition in the development of long-term effects on its health has been firmly documented during the past years.[217–227] Although most problems in foetal nutrition may be rapidly corrected after birth, as recently reviewed,[228] there are conditions such as maternal hypercholesterolaemia during the early stages of pregnancy that may promote lesion formation in the foetus, increasing the susceptibility to atherosclerosis later in life. Hypercholesterolaemia is known to be accompanied by increased lipid peroxidation,[229–231] and evidence for a role for oxidative stress in the effects of maternal hypercholesterolaemia has been obtained in a rabbit model,[20] where plasma concentrations of cholesterol in offspring were unchanged but lipid peroxidation end products increased. Thus, it may be hypothesized that conditions enhancing the susceptibility of oxidative stress, such as the exaggerated proportional increase in certain dietary LC-PUFA referred to above, could also increase the susceptibility to atherosclerosis later in life.

Several studies have addressed the question of whether early fat-feeding practices are relevant in the development of atherosclerosis (for a recent review see Viikare *et al.*).[225] Breast milk has a high cholesterol content, and prolonged breastfeeding in infancy was related to impaired arterial distensibility 20 years later.[232] However, other studies have proposed a protective effect of exclusive and prolonged periods of breastfeeding against type 2 diabetes, dyslipidaemia and overweight in adults[233] or in adolescents.[234]

14.5 Dietary recommendations for the avoidance of heart disease later in life

In order to avoid future heart disease it is possible to take two approaches. On the one hand, simply addressing the Barker Hypothesis[1] and aiming to improve (increase) birth weight by better foetal nutrition is predicted to improve the cardiac outcomes. On the other hand, identifying a mechanism by which a specific fat may predispose to, or protect from, cardiac disease could lead to much more precise advice.

Given the complexity of the physiology of fat transfer to the foetus from the maternal circulation, it is difficult to make precise recommendations for dietary

modifications for the pregnant mother. The question may be broken down into three components: 'how much dietary fat?', 'what type of fat?' and 'at what stage of pregnancy should it be taken?'

By its very nature, pregnancy is a time of growth and therefore a time of greater energy requirement. In the first two-thirds of gestation this is seen mostly as fat storage in the mother and in the last third by mobilization of those stores to satisfy the requirements of the now rapidly growing foetus. Greater energy requirements are met most easily by consumption of the most energy-dense nutrient, fat, and it seems sensible that the total proportion of fat in the diet should rise, above that normally recommended for an adult, to meet these requirements. If fat can be used to meet normal energy requirements, maternal hypoglycaemia and ketosis can be avoided and the materno-foetal glucose concentration gradient, enabling glucose transport to the foetus, can be maintained. The more glucose that can be 'spared', the more that can be used directly to fulfil foetal needs.

In terms of foetal growth and development, specific fatty acids – DHA and AA – are required, especially at times of brain and retinal development. As neither of these fatty acids are available by synthesis *de novo*, the maternal diet must be sufficient to ensure adequate supply to the placenta. As a dietary supplement, the n-3 acid, DHA is most readily available as part of the triacylglycerols constituting the oils of oily fish. Oils rich in its precursor, α-linolenic acid, are less common. There is no obvious dietary source of AA; so shorter n-6 acids such as linoleic acid, found in most vegetable oils, or γ-linolenic acid of the fashionable oil of evening primrose or blackcurrant oil can provide precursors for subsequent desaturation and elongation. The fact that, as stated above, other acids of fish oils can interfere with these enzymic processes illustrates just how complex the situation is.

The timing of dietary manipulations, if required, is complicated by the role of adipose tissue in the process. Maternal mobilization of adipose stores in late pregnancy means that the fatty acids available to the placenta, which itself selectively transports those most required, depends not only upon her current diet but also on her diet during the anabolic phase of pregnancy and even on her dietary history as reflected in the fatty acid composition of her stores. This argues for intervention on a woman-by-woman basis rather than the issuing of blanket advice for a whole population. Hence, a woman with low reserves of DHA and AA (or their precursors) may well be advised to supplement their diets appropriately and a woman with low total fat reserves could be advised to increase her fat intake from the time of becoming pregnant.

Although the studies reported above in Section 14.4.1 together indicate that early fat-feeding can have a significant influence on future vascular health, the mechanisms are not yet understood and more studies are required to establish the safety window for an appropriate quantity and quality of fat components before dietary supplements with high intakes of LC-PUFA, with or without supplements of antioxidant vitamins, can be recommended with confidence.

14.6 Future trends

After reviewing the literature above, the questions asked at the outset might be revised. Instead of looking for a 'functional food' which may, at a stroke, improve foetal growth and development, or elicit some protective mechanism, thereby reducing the likelihood of future arterial disease, we perhaps should be looking for particular sets of circumstances for which individual dietary treatments are more or less appropriate.

If future advances in technology make it possible to monitor in detail the fatty acid status of a potential mother prior to and during her pregnancy (without resorting to numerous and painful biopsies) and given a knowledge of how dietary fats affect adipose stores, in early pregnancy, and plasma lipids in later pregnancy and of how placental transfer responds to changes in the available substrates, it may become possible to design regimes that maximize the benefits for the growing foetus and to avoid the distinct syndromes, such as hypercholesterolaemia, which predispose to later problems.

In the more immediate future, research work is likely to concentrate on the unknowns identified above. What are the complex interactions between n-3 and n-6 acids in the diet and how can a diet containing an optimum mix of the two be devised? When and by how much should diets be supplemented with antioxidant vitamins? Experiments with animal models have been useful in examining the effects of a single lipid source on the animals' subsequent physiology but we need to develop those models to give answers about more complex and variable mixtures as consumed by humans.

14.7 Sources of further information and advice

The references cited throughout this chapter provide details that we have sometimes only been able to summarize. Some of the references are to review articles which provide substantial information on the subject. Some of the questions herein analysed, as well as being addressed by several research groups throughout the world, are the subject of a project funded by the European Union called PERILIP, a collaboration of groups in six European countries in which both authors are active partners. In the website of this project, at http://www.perilip.org, besides a detailed account of the background to the project and news of recent developments, there is an extensive list of references in the bibliography section. Under 'Useful links' there is a list of other websites that we judge to be useful and carefully prepared. These sites cover such topics as intrauterine development, placental research, lipid research, nutrition in pregnancy and lactation, and neonatal care. The site is revised and updated at regular intervals.

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15

Developing polyunsaturated fatty acids as functional ingredients

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15.1 Introduction: long-chain polyunsaturated fatty acids and cardiovascular disease

Fat is an essential component of the diet,[1] and the fatty acids have different roles in the human body. In the 1970s, Danish researchers discovered that Greenland Inuits, who consume large amounts of marine lipids as part of their native lifestyle, had a much lower cardiovascular mortality (10–30 per cent) compared with the Danes, who consume much lower levels of these lipids.[2,3] These findings triggered new research on the role of the long-chain polyunsaturated fatty acids (LC PUFA) in the development of cardiovascular disease and on the possibilities of utilising the beneficial effects of n-3 LC PUFA by incorporating marine lipids into foods. This chapter will summarise the latest evidence for the positive effects of n-3 LC PUFA on the prevention of cardiovascular diseases and the proposed mechanisms behind the protective effect of n-3 LC PUFAs. Moreover, the problems associated with using marine oil in foods, especially the problems related to off-flavour formation, will be discussed together with examples of how such problems can be solved.

15.1.1 The n-3 and n-6 PUFA families

There are two distinct families of PUFA that cannot be interconverted. The parent fatty acids of the n-6 (linoleic acid) and n-3 (α-linolenic acid) families are essential fatty acids as they cannot be synthesised by the human body (Fig. 15.1). The body is able to synthesise the LC PUFA from the parent fatty acids. However, linoleic acid and α-linolenic acid are competing for the same

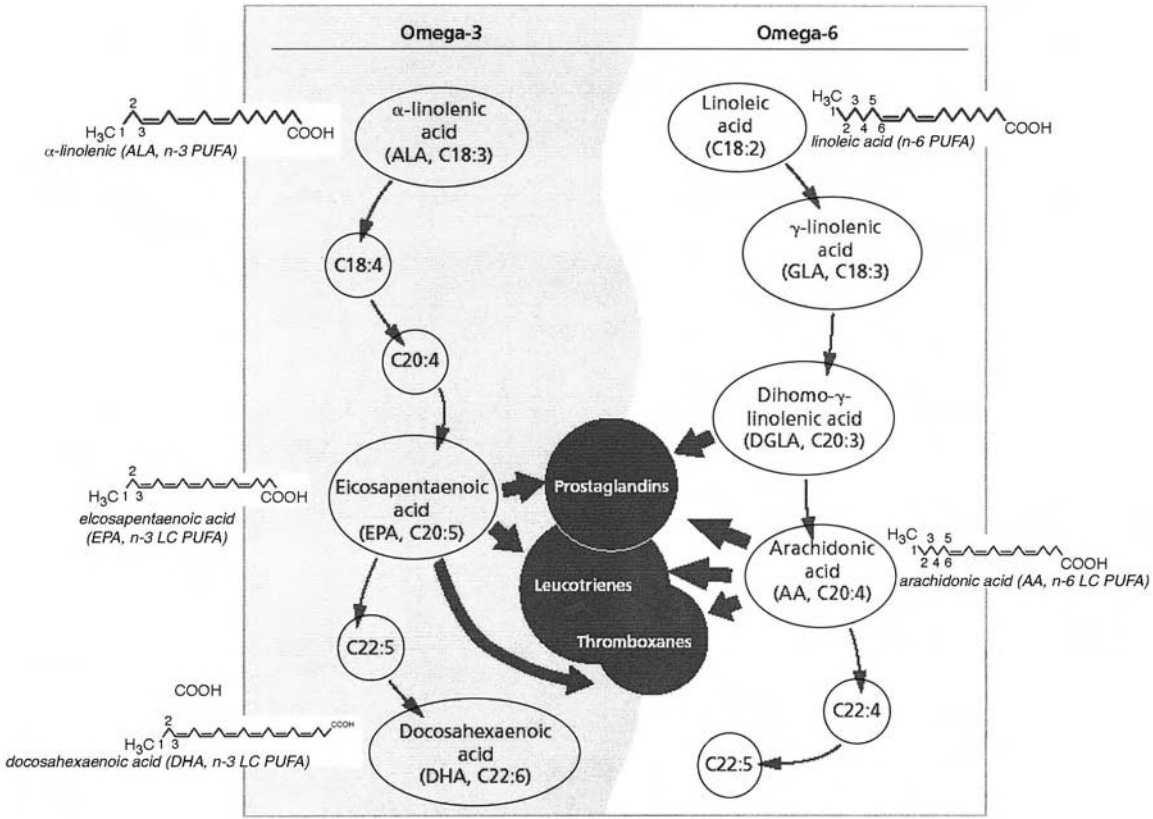

Fig. 15.1 Metabolism of PUFA (adapted from Anselmino and Hornstra[4] http://www.nutrivit.co.uk/professional/PDFs/Omega_3%20book.pdf).

enzyme systems for the synthesis and, therefore, it is important that there is the right balance between the intake of n-6 and n-3 fatty acids.

The n-6 PUFA are found mainly in vegetable products. The parent n-3 fatty acid α-linolenic acid, is also present in some vegetables (rapeseed, soybean and nut oils), but fish and marine animals are the best sources of the n-3 LC PUFA eicosapentanoic acid (EPA) and docosahexanoic acid (DHA). Low levels of n-3 LC PUFA are also found in meat. The current intake of n-3 PUFA in industrialised countries is only 4–10 per cent of the intake of n-6 PUFA, compared with an estimated ratio of 1:1 about 150 years ago.[5] Therefore, several bodies have issued PUFA guidelines to encourage a more balanced ratio of n-6/n-3 fatty acids that would optimise the benefits of both fatty acids (Table 15.1).

15.1.2 n-3 PUFA and cardiovascular disease

Several large-scale epidemiological studies have demonstrated a negative association between fish consumption and cardiovascular and/or overall mortality.[6–11] The cardioprotective effect of fish consumption seems to be more prevalent in high-risk populations.[12] Intervention studies in cardiac patients have shown that fish or fish oil supplementation *vs*. placebo reduced the mortality risk up to 45 per cent.[13–15] Apparently, fish or fish lipids do not reduce the risk of a new cardiovascular incident, but fewer incidents are fatal. At least half the deaths from coronary artery disease are sudden cardiac deaths with fatal arrhythmia caused by ventricular fibrillation.[16] A number of studies have shown that n-3 LC PUFA prevent arrhythmias and this seems to be an important property of these fatty acids.[17–19]

Several mechanisms have been suggested to explain the preventive effect of n-3 LC PUFA on cardiovascular diseases. It is now well established that n-3 LC PUFA reduce triglyceride levels by lowering hepatic triglyceride synthesis and by decreasing the release of triglyceride-rich very low-density lipoproteins (VLDLs) into the blood.[20–23] A high plasma triglyceride level is a cardiovascular risk factor. Hypertension is another important cardiovascular risk factor. High doses of n-3 LC PUFA have been shown to reduce hypertension, probably by influencing membrane fluidity and the balance of the prostanoids that control the constriction and dilation of the small arteries and arterioles.[5]

Numerous studies have shown that n-3 LC PUFA have antiaggregant activity.[24–29] This is probably due to EPA's role in the eicosanoid synthesis and its ability to reduce the levels of arachidonic acid (AA) in the membrane. EPA is a precursor of the 3-series prostanoids TXA3 and PGI3 while AA is a precursor of TXA2 and PGI2. TXA2 and TXA3 are both prothrombotic, but TXA3 is less prothrombotic than TXA2. In contrast, PGI2 and PGI3 are equally antithrombotic. Moreover, it seems that EPA and DHA reduce the gene expression of the enzymes involved in eicosanoid synthesis.[30, 31]

The ability of EPA and especially DHA to prevent arrhythmias may be due to their effect on (i) the ion channel (modulation of the ionic currents in heart

Table 15.1 PUFA recommended dietary allowances

Source	n–3/n–6 ratio	n–3 recommendation	EPA + DHA
Nordic Nutrition Committee		0.5%* (1–2 g/day)	
NATO Workshop, 1989			0.27%* (0.8 g/day)
Scientific Review Committee Canada, 1990	1:5–1.5:6	0.5% (1–2 g/day)	
British Nutrition Task Force, 1992	1:6		0.5%* (1.1 g/day)
Scientific Committee for Food, EU, 1993	1:4.5–1.5:6	0.5%* (1–2 g/day)	
FAO/WHO Expert Committee, 1994	1:5–1:10		
Committee on Medical Aspects of Food Policy, 1991, 1994		0.2%*	0.1–0.2 g/day
National Nutrition Council, Norway, 1996		0.5%* (1–2 g/day)	
NIH Workshop, 1999		1%* (2.22 g ALA)	0.3%* (0.65 g/day)
ANC 2000, France	1:5	2–2.5 g/day	0.12 g/day (DHA)
FDA, 2000			1 g/day
AHA, 2000			0.9 g/day
The Japanese Society of Nutrition and Food Science, RDA for the Japanese, 6th revision 2000	1:4		
Health Council of the Netherlands	1:7.5	0–5 months: 80 mg/kg day above 5 months 1%*	0–5 months: 20 mg/kg day DHA above 5 months 150–200 mg/day

*% energy intake.
Source: Anselmino and Hornstra,[4] http://www.nutrivit.co.uk/professional/PDFs/Omega_3%20book.pdf.

cells),[32,33] (ii) adrenoreceptors (DHA decreases the production of the main β-adrenic messenger, cyclic AMP, which transmits the message from catecholamins to the heart about the rhythm and force of contraction,[34] (iii) prostaglandins (prostaglandins from EPA are less effective in promoting arrhythmias than prostaglandins from AA5), and (iv) energy production (EPA produces energy at a lower oxygen cost than other fatty acids and this is important in ischaemia where the tissue is deprived of oxygen).[35]

15.1.3 Other beneficial effects of n-3 fatty acids

EPA and DHA have inflammatory properties and are similar in action to certain anti-inflammatory agents by inhibiting the production of inflammatory mediators such as prostaglandin E2 and leukotrine B4 derived from leuckocyte and macrophage activation. Because of these properties, n-3 LC PUFA may help to prevent or reduce the symptoms of rheumatoid arthritis[36–38] and Crohn's disease.[39] There is also some evidence that n-3 LC PUFA may prevent certain cancer forms, but more research is necessary to support this hypothesis.

The n-3 LC PUFA have a very important role in the brain, retina and nervous tissue as DHA constitutes up to 50 per cent of the phospholipid fatty acids. Therefore, the brain and retina are dependent on a continuous DHA supply for optimal function.[40] DHA is particularly important during the development of the central nervous system in the foetus in the last trimester of the pregnancy, in pre-term infants and also during childhood. Maternal LC PUFA intake under the present dietary conditions seems to be inadequate to keep up with the increased demand for n-3 LC PUFA during pregnancy. Therefore, it has been suggested that pregnant women should increase their intake of DHA and that infant formulas for both pre-term and term infants should contain DHA. Infant formulas with DHA are now available in several countries.

15.2 Problems in using fish oil in food products: lipid oxidation and off-flavours

The current intake of n-3 LC PUFA is far too low in the industrialised world. The easiest way to increase the intake of these healthy fatty acids would be to increase dietary fish consumption, but it seems to be difficult to change the habits of the Western populations. Therefore, several attempts have been made to substitute part of the vegetable/animal fat in foods by marine lipids in products such as mayonnaise, milk, bread and dressing. Owing to the unsaturated nature of the n-3 LC PUFA, they are more susceptible to lipid oxidation than less unsaturated lipids. Lipid oxidation gives rise to the formation of undesirable off-flavours and unhealthy compounds such as free radicals and reactive aldehydes. The off-flavours formed from n-3 LC PUFA oxidation are particularly nasty. n-3 LC PUFA-enriched mayonnaise, milk drink, fish oil

powder, egg and bread developed off-flavours that were described as fishy, train oil, metallic, painty or simply an unspecific off-flavour.[41–43] Thus, in order to develop and market fish oil-enriched foods, lipid oxidation must be prevented. To do so, a fundamental understanding of the antioxidation and oxidation mechanisms in food systems is required. This section will summarise mechanisms of lipid oxidation and off-flavour formation.

15.2.1 PUFA and lipid oxidation

There are three types of lipid oxidation: autoxidation, photo-oxidation and enzyme-catalysed oxidation. Most emulsified foods do not contain enzymes that can catalyse oxidation and therefore only the first two types of lipid oxidation will be dealt with.

Autoxidation

Autoxidation proceeds via complex free radical chain reactions. This process is characterised by initiation, propagation and termination (Fig. 15.2). In the initiation stage, free radicals are formed when a hydrogen radical is lost from the unsaturated lipid in the presence of initiators (I). Iron and copper are typical examples of initiators. The oxidation process cannot be initiated in the absence of initiators, as the lipid ground state of single multiplicity has an opposite spin direction compared with that of triplet oxygen. Therefore, oxygen in its ground state ($^3O_2$ = triplet oxygen) cannot react directly with unsaturated lipids. After the formation of a free lipid radical, the reaction with oxygen readily takes place to form a peroxyl radical (Fig. 15.2). These radicals propagate the chain reaction by reacting with unsaturated lipids to produce lipid hydroperoxide and a new free radical that is capable of reacting with triplet oxygen. The last stage of

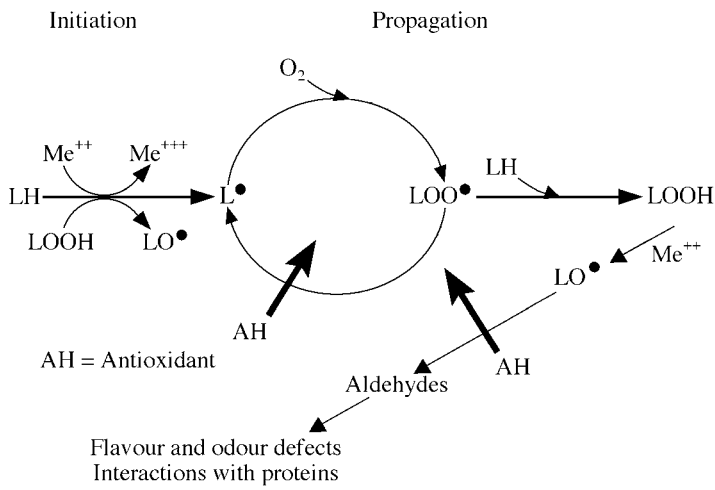

Fig. 15.2 Initiation and propagation stages and prevention of lipid oxidation by free radical chain-breaking antioxidants.

Table 15.2 PUFA oxidisability

PUFA	Symbol	H	Oxidisability
Linoleate	18:2	2	20
Linolenate	18:3	4	41
Arachidonate	20:4	6	55
DHA	22:6	10	102

Source: Adapted from Frankel.[44]

oxidation is the termination phase in which different types of free radicals react with each other to form non-radical end products (not shown in Fig. 15.2).

The oxidisability of lipids increases approximately two-fold for each *bis*-allylic methylene group (double bond) and the oxidisability was thus five times higher for 22:6 than for 18:2 (Table 15.2).[44] The number and complexity of the lipid hydroperoxides increase with the number of double bonds in the lipid substrate. For example EPA resulted in eight peroxides and DHA in 10.[44] The relative amount of the different hydroperoxides is dependent on the temperature and the concentration of the substrate.

The lipid hydroperoxides may be decomposed by homolytic β-scission to alkoxyl radicals as indicated in Fig. 15.2. Subsequently, the alkoxyl radical may undergo further homolytic cleavage. Thereby, secondary volatile oxidation products such as hydrocarbons, furans, alcohols, aldehydes and ketones are formed (Fig. 15.3). Flavour deterioration of fish oil-enriched foods is caused mainly by the presence of these compounds, but their flavour thresholds differ substantially as shown in Table 15.3.

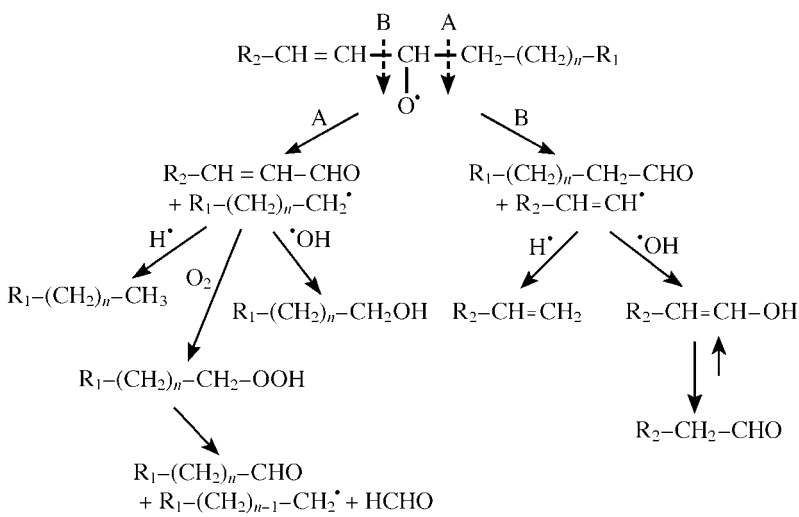

Fig. 15.3 Formation of volatiles by β-scission of monohydroperoxides (source: Franke).[44]

Table 15.3 Flavour thresholds of volatiles in oils

Type of compound	Flavour threshold in oil (ppm)
Hydrocarbons	90–2150
Substituted furans	2–27
Vinyl alcohols	0.5–0.3
1-Alkenes	0.02–9
2-Alkenals	0.04–2.5
Alkanals	0.04–1.0
E,*E*-2,4-alkadienals	0.04–0.3
Isolated alkadienals	0.0003–0.1
Isolated *Z*-alkenals	0.0003–0.1
E-3-*Z*-4-alkadienals	0.002–0.006
Vinyl ketones	0.000 02–0.007

Source: Adapted from Frankel.[44]

Several volatile compounds have been identified in different fish species,[45–47] bulk fish oils,[48–50] microencapsulated fish oil powder[51] and in foods tainted with fishy off-flavours such as butterfat[52] and soybeans.[53] Recently, Macfarlane *et al.* reported that 4-*Z*-heptenal, 2, 6,-*E*, *Z* nonadienal and 3, 6-*E*, *E*-nonadienal were important contributors to the fishy off-flavour in fish oil and a mathematical relationship between the concentration of these compounds and the fishy taste was proposed.[54] However, information about the volatile profiles and their exact sensory impact in fish oil-based emulsions and real foods is very limited. To the author's knowledge, it is only in mayonnaise and milk drink, among fish oil-enriched food emulsion systems, the profile of volatiles has been established.[55–60] From these studies it seems that compounds like 1-penten-3-one, 2, 6-nonadienal, 2, 4-heptadienal, 2, 4-decadienal, 1-octen-3-one, 1-octen-3-ol, 2-*E*-octenal, ethylfuran and 4-*Z*-heptenal may contribute to the fishy, rancid and metallic off-flavours in these emulsified foods. However, none of these compounds exhibited fishy off-flavours when analysed by gas chromatography (GC)-sniffing. Therefore, a single compound is probably not responsible for the fishy off-flavour. Rather, a combination of different compounds are required before the fishy off-flavour occurs.

Photo-oxidation

In the presence of light, oxygen and a photosensitiser, which are pigments such as chlorophyll, haemproteins and riboflavin present in foods, the so-called photo-oxidation reaction will take place. The photosensitising pigments can absorb visible or near-UV light whereby they become electronically excited. Two types of sensitisers exist: the first type reacts in its triplet state directly with the unsaturated lipid to form a lipid radical. Subsequently, the lipid radical can react with oxygen as in autoxidation (Fig. 15.2) whereby the same peroxides as those from free radical autoxidation are formed. In contrast to free radical autoxidation, chain-breaking antioxidants have, however, no effect on this

reaction. The second type of sensitiser transfers its energy to triplet oxygen whereby singlet oxygen is formed. Subsequently, singlet oxygen reacts directly with the lipid substrate. Thereby, lipid hydroperoxides that are different from those formed from free radical reactions are produced. Light is a very powerful catalyst of oxidation, which is illustrated by the fact that the oxidative stability of sunflower oil at room temperature under sunlight is approximately equal to that when the oil is oxidised at 80 °C in the dark.[61]

15.3 Factors affecting lipid oxidation in complex food systems

When n-3 LC PUFA are incorporated into food systems the oxidation mechanisms and thereby the oxidation rates may change dramatically. This is due to the fact the oxidisability depends on the physical structure and form of the lipid. Moreover, food systems contain a wide range of different ingredients that may influence lipid oxidation, as will be discussed in the following.

15.3.1 Metal catalysis

Metals can catalyse oxidation by two different mechanisms:

1 by electron transfer:

$$M^{(n+1)+} + RH \longrightarrow M^{n+} + R^{\bullet} + H^{+} \qquad 15.1$$

2 by catalysing the decomposition of hydroperoxides:

$$M^{n} + ROOH \longrightarrow M^{n+1} + RO^{\bullet} + OH^{-} \qquad 15.2$$

$$M^{n+1} + ROOH \longrightarrow M^{n} + ROO^{\bullet} + H^{+} \qquad 15.3$$

Fe and Cu are the most active metals, but Mn, Zn, Co and Ni can also act as pro-oxidants. Ferrous iron (Fe^{2+}) is more effective in decomposing peroxides than the ferric ion (Fe^{3+}) and both ferrous and ferric iron are more effective than copper.[62]

Trace metals are present in most foods. Even after refining and deodorisation most oils will contain trace levels of lipid hydroperoxides. Therefore, metal-catalysed decomposition of lipid hydroperoxides is probably the reaction responsible for the initiation of lipid oxidation in most foods. The reactions in equations 15.2 and 15.3 not only generate free radicals, which may initiate further oxidation reactions, but will also give rise to the formation of secondary volatile oxidation compounds as previously described. In fish oil-enriched mayonnaise, the iron present in the egg yolk, which is used as an emulsifier, was suggested to be the most important catalyst of oxidation.[63, 64] The mechanism by which iron promotes lipid oxidation in mayonnaise was suggested to be as follows: the low pH in mayonnaise (pH < 4.2) is responsible for releasing small amounts of iron ions from the oil–water interface where iron is bound to the egg

yolk protein phosvitin. Subsequently, the released iron promotes the decomposition of pre-existing lipid hydroperoxides located at the oil–water interface and perhaps also in the aqueous phase.[63–65] The effect of pH on iron release will be discussed further below. Recent results have indicated that metals from certain milk proteins are also important oxidation catalysts in fish oil-enriched milk.[42]

15.3.2 Oxygen

Oxygen is required for oxidation to occur. Therefore, lipid oxidation may be reduced by reflushing the food product with nitrogen as observed in fish oil-enriched mayonnaise[66] and by packaging in an air-tight container. The total amount of oxygen in the headspace above the product and the oxygen dissolved in the product will limit the extent of oxidation. However, usually oxidation can proceed for a relatively long time, because plenty of oxygen will be available even in a closed container, unless oxygen has been completely removed. Importantly, the oxygen is not required for the decomposition of lipid hydroperoxides. Therefore, off-flavour products may be formed even after all oxygen is consumed.[67]

15.3.3 Droplet size/interfacial area

The large interfacial area in emulsions increases the potential contact area between the oil droplet and trace metals in the continuous, aqueous phase. The interfacial area is governed by the size of the droplets in emulsions. In the literature, contradicting reports are available on the effect of the droplet size on oxidation. In fish oil-enriched mayonnaise, lipid oxidation was faster in mayonnaises with small droplet sizes in the initial part of the storage period, whereas no effect of droplet size was observed in the later part of the storage period.[58] The following mechanism was suggested to explain these findings: in the initial phase of the oxidation period a small droplet size, i.e. a large interfacial area, would increase the contact area between iron located in the aqueous phase and lipid hydroperoxides located at the interface and this would increase oxidation. In the later stage, oxidation proceeds inside the oil droplet and therefore the droplet size is less important. Further studies are required to elucidate this matter.

15.3.4 Moisture

Lipid oxidation is significantly affected by the water activity in foods, especially in powders. Water may act as a solvent for metal ions, and metal salt hydrates may be formed. These hydrates are less lipid soluble and less active than the metal ions themselves. Lipid oxidation may decrease, owing to the formation of hydrogen bonds between water and lipid hydroperoxides, which prevent their decomposition into initiating free radicals.[44,68] Water may facilitate the

breakdown of alkoxyl radicals formed by the decomposition of hydroperoxides.[44] Moreover, a decrease in water activity will also decrease the so-called glass transition temperature, which is the temperature at which the food matrix changes from the glassy state to the rubbery state. It has been suggested that lipids are more susceptible to oxidation in the rubbery state, because they can react more readily with oxygen in this state. In the glassy state, the lipids are encapsulated because there is less free volume that is not taken by the macromolecules and therefore diffusion of oxygen is also limited. Thus, bringing the powder into the glassy state by optimising the recipe or by decreasing the storage temperature/water activity may reduce oxidation. It is important to take these phenomena into consideration in relation to the incorporation of fish oil into powders such as infant formula.

15.3.5 Temperature

Temperature affects oxidation rates in an exponential manner. The mechanism of oxidation changes with temperature, especially above 60 ºC, and the lipid hydroperoxides from different fatty acids decompose into secondary volatile oxidation products at different temperatures.[44] Therefore, it is difficult to mathematically predict the effect of temperature on shelf-life and sensory properties of foods. Nevertheless, Presa-Owens *et al.*[69] attempted to predict the shelf-life of fish oil-enriched infant formula using an accelerated stability test (Rancimat). They reported that shelf-life predicted by long-term studies at 25 ºC and 60 ºC based on peroxide values and sensory evaluation were in accordance with the shelf-life predicted by repeated Rancimat measurements at temperatures ranging from 60 to 130 ºC.

15.3.6 Salt

Mei *et al.*[70,71] showed that the effect of NaCl on oxidation depended on the charge of the emulsifier, the concentration of NaCl and the concentration of ferrous in corn and salmon oil emulsions. In traditional mayonnaise, salt was shown to increase anisidine values and the effect of salt was shown to depend on the salt concentration and salt type.[72] In fish oil-enriched mayonnaise, NaCl did not, however, promote free radical formation, which indicated that NaCl was not a pro-oxidant.[65] Taken together, these data suggest that the effect of NaCl on lipid oxidation should be investigated in each individual food system.

15.3.7 pH

Metal ions are generally more soluble at low pH than at high pH.[44] This may explain why lipid oxidation generally is slowest at high pH values. Furthermore, pH influences the emulsifier charge and this may significantly affect oxidation as will be discussed later. In fish oil-enriched mayonnaise, lipid oxidation increased with decreasing pH.[63,64] The following hypothesis was suggested to

explain this phenomenon: the egg yolk used as an emulsifier in mayonnaise contains large amounts of iron, which is bound to phosvitin. At the natural pH of egg yolk (pH 6.0) the iron also forms iron bridges between phosvitin and other components in egg yolk, namely LDL and lipovitellin. These components are located at the oil–water interface in mayonnaise. When pH is decreased to 4.0, which is the pH in mayonnaise, the iron bridges between the egg yolk components are broken and iron becomes dissociated from LDL and lipovitellin. Thereby, iron becomes more active as a catalyst of oxidation.[63, 64]

15.3.8 Emulsifiers (proteins and surfactants)

Proteins are commonly used as emulsifiers in foods to facilitate the formation and enhance the stability of oil-in-water emulsions. During homogenisation they are absorbed to the oil droplet surface where they lower surface tension and prevent coalescence of droplets by forming protective membranes around the droplets. Proteins also have a stabilising effect on the emulsion by providing the emulsion droplets with a positive or negative electrical charge at pH values below or above the p*I* of the proteins. It has been suggested that the electrical charge of the interfacial layer around the oil droplet significantly influences oxidation in emulsions in the presence of metal ions.[70,71] Compared with a non-ionic emulsifier, an anionic emulsifier was shown to increase oxidation in corn oil model oil-in-water emulsions whereas a cationic emulsifier decreased oxidation. These results were explained by the ability of the emulsifier to attract and repel metal ions to the oil–water interface, respectively. Therefore, the charge and thereby the type of emulsifier may affect the oxidative stability of the emulsion.

pH will affect the charge of the emulsifier and this may in turn affect the oxidative stability of emulsions. Recently, it was reported that oxidation increased with increasing pH in salmon oil-in-water emulsions stabilised by whey proteins.[73] More specifically, lipid oxidation rates were significantly lower at pH values below the p*I* of the whey protein isolate. This was suggested to be due the fact that the proteins would be positively charged at pH values below p*I* and therefore they would repel metal ions near the oil–water interface. However, the surface charge of the emulsifier does not seem to be the only factor influencing lipid oxidation. Thus, Hu *et al.*[73] also reported that the order of lipid oxidation rates in salmon oil-in-water emulsions stabilised by either whey protein isolate, sweet whey or two of the proteins present in whey protein, namely α-lactalbumin or β-lactoglobulin, did not equal the order of the positive charge of the emulsion droplets. In another study, in corn oil-in-water emulsions it was observed that casein resulted in lower oxidation rates than whey protein isolate and soy protein isolate, even though all emulsions were cationic at low pH.[74] Based on these findings it was proposed that other factors responsible for the differences in oxidative stability of protein stabilised oil-in-water emulsions could be differences in how the proteins influences the thickness or packing of the emulsion droplet interface.[73, 74] Increasing the thickness of the interfacial

layer could make it more difficult for aqueous iron to interact with lipid hydroperoxides located near the interface. Another factor affecting the antioxidative effectiveness of proteins could be their amino acid composition. The sulphydryl group of cysteine has thus been reported to have antioxidant activity because of its ability to scavenge free radicals.[75] Antioxidative effects of tyrosine, phenylalanine, tryptophan, proline, methionine, lysine and histidine have previously been reported in the literature.[74]

Surfactants are small lipophilic and hydrophilic molecules that are used to form emulsions. Normally, surfactants will be present in excess in emulsions and surfactants not associated with the emulsion droplets will form micelles in the continuous phase. It has been suggested that surfactant micelles are able to reduce lipid oxidation by altering the physical location of lipid hydroperoxides and/or iron in emulsions.[76, 77]

More research is required to completely understand the role of emulsifiers in the lipid oxidation in complex food systems.

15.3.9 Carbohydrates/viscosity

Previous studies have indicated that some carbohydrates in high concentrations are capable of scavenging free radicals and thereby act as antioxidants.[78] Sucrose addition has been suggested to be able to decrease oxidation by decreasing the concentration of oxygen in the aqueous phase, and sucrose may also decrease the diffusion coefficient of oxygen via its increasing effect on the viscosity of the emulsion.[79] Apart from reducing the diffusion of oxygen, a high viscosity of the emulsion may also reduce the diffusion of metals and other reactants and reaction products, and this may slow down oxidation rates.

15.3.10 Antioxidants

Addition of antioxidants to foods may delay the onset of oxidation or slow down the rate at which it proceeds. Antioxidants are usually classified as either primary or secondary antioxidants. The former are also referred to as free radical scavengers as they are chain-breaking antioxidants that delay or inhibit the propagation stage by donating a hydrogen atom to the lipid radical, the peroxyl radical or the alkoxyl radical as shown in Fig. 15.2. Primary antioxidants are often phenolic compounds such as the synthetic antioxidants BHA, BHT, propyl gallate or as naturally occurring compounds, such as tocopherol, and plant polyphenols, such as carnosic acid. The secondary antioxidants act by a number of different mechanisms such as metal chelation, oxygen scavenging and replenishing hydrogen to primary antioxidants. The secondary antioxidants often exert synergistic effects together with primary antioxidants. EDTA, lactoferrin and citric acid are examples of metal chelators that have been shown to reduce lipid oxidation in fish oil emulsions and fish oil.[71, 80, 81] Ascorbic acid and the glucose oxidase–catalase enzyme system are examples of oxygen scavengers.[82] Ascorbic acid is also able to regenerate tocopherol by replenishing hydrogen.[83]

The possibility of controlling lipid oxidation in fish oil-enriched foods will be discussed in Section 15.4.

15.3.11 Other problems in using fish oil as a food ingredient

During the last ten years, there has been an increasing focus on the content of pollutants such as dioxin and PCBs (polychlorinated biphenyls) in our foods. Fish from certain areas that have been particularly exposed to pollution, e.g. air polluted with dioxin from incineration plants, may have elevated levels of dioxin and PCBs. These components are lipid-soluble and therefore they will be extracted together with the fish oil during the fish oil manufacturing process. In July 2002, a new regulation was imposed in the EU where the limit for dioxin in fish oil was set to 6 ng WHO-TEQ*/kg. It is also expected that a similar regulation will be imposed for the PCBs. Because of the strict rules, new technologies have been developed to remove dioxin from fish oil. The most common method is to remove the dioxin by activated carbon, but new deodorisation techniques are also under development. Such new technologies will be required to remove PCBs as they cannot be removed by activated carbon.

15.4 The successful use of fish oil in food products: improving shelf-life and sensory properties

The application of fish oil in a wide range of different products such as bread, mayonnaise, salad dressing, ice cream spread, milk drink and infant formula has been attempted. [42, 56–59, 84–87] Attempts have also been made to increase n-3 fatty acid contents in animal products such as milk fat, pork meat and hen eggs by adding fish oil to the animal feed.[88–90] Several n-3 LC PUFA-enriched food products have entered the marketplace, as illustrated in Table 15.4.[91] Some of these products have been withdrawn from the market again owing to poor market performance, but other products are still on the market. The reasons for the poor market performance for some of the products are not known, but it may be speculated that some of the reasons could be either that the products had

- poorer sensory properties than traditional products;
- higher prices;
- poorer shelf-life; or
- the fact that health claims on the beneficial effects of n-3 LC PUFA are not yet allowed in many countries.

No matter the reason, the fact is that the industry is still searching for better methods to improve the sensory properties and shelf-life of n-3 enriched foods.

* A TEQ is a dioxin Toxic EQuivalent – calculated by looking at all toxic dioxins and furans, and measuring them in terms of the most toxic form of dioxin (2, 3, 7, 8-TCDD). Some dioxins/furans might only count as half a TEQ – that is half as toxic as 2, 3, 7, 8-TCDD.

Table 15.4 Commercial products enriched with n-3 LC PUFA

Bread and bakery products	
Germany	Omega-3 bread and omega-3 rolls (VK Mühlen, Hamburg), Diamant Vital omega-3 Kruste (Diamant Mühle), Ruf Wellness Aktifit-Brot (Ruf Lebensmittelwerke)
Sweden	Leva omega-3 bread (Pågen)
Japan	DHA bread and DHA table rolls (Yamazaki Baking)
Australia	Tip Top sliced bread (Tip Top bakeries), Hi Q DHA bread (Bunge Defiance)
South Africa	Richbake Omega Brown bread mix (Credin Bakery)
Margarine and spreads	
Scandinavia	Gaio spread (Arla Foods)
Chile	Dos Alamos margarine (Grasco)
UK	Vitaquell Omega-3 (Vitaquell), Heartwatch omega-reduced fat spreads
Brazil	Vigor Omega Vitta (Vigor)
Eggs and egg products	
Germany	Eiplus eggs (Eifrisch)
Netherlands	Columbus eggs (Belovo)
Italy	Ovo3 eggs (Maia Agrolimentare), Oro omega-3 eggs (Unione Cascine Valpadana)
USA	Gold Circle Farms eggs (OmegaTech & NutraSweet Kelco, USA), Eggs Plus (Pilgrim's Pride, USA)
Finland	Minicol omega pasteurised eggs (Wammala Food)
Pasta	
Switzerland	Val Farella pasta (Morga)
UK	Heartwatch Omega Pasta spirals and Omega Pasta crests (Functional Nutrition)
Culinary products	
USA	Millina's Healthy Kitchen Omega-3 Pasta Sauces (Organic Food Products)
Milk and dairy products	
Italy	Plus omega-3 milk and omega-3 yoghurt (Parmalat, I)
Spain	Puleva omega-3 milk (Puleva), Lauki omega-3 skim milk (Candia)
Portugal	Especial omega-3 milk (Mimosa)
Brazil	Omega-3 milk (Parmalat, USA)
Soy and tofu products	
Japan	Ajiwai Tofu (Asahi Shokuhin Sangyo Corp., Japan)
USA	Gold Circle Farms soy milk in different flavours (OmegaTech, Boulder, CO, USA)
Juice and soft drinks	
USA	Tidal Wave Superfood juice (Naked Juice, CA)
UK	Bertrams omega functional juices (Bertrams)
Germany	My Way Wellness drink (Designer Foods)
Japan	Cola-like soft drinks
Meat and poultry products	
Spain	Omega-3 Jamon Cocido cooked ham, Terra I Mar turkey breast (Carnicas Serrano)
Israel	Mega Off frozen chicken pieces enriched (Off Tene)

Source: Adapted from Trautwein.[91]

The literature on the oxidative stability of real foods enriched with n-3 PUFA is scarce. Most studies have either been carried out in bulk fish oil or in simple fish oil-in-water emulsion systems. In the following, the major findings will be summarised.

15.4.1 Mayonnaise

Jafar *et al.*[92] reported that mayonnaise containing 70 per cent fish oil prepared without antioxidants had a shelf-life of 1 day when stored at room temperature and when evaluated by sensory analysis (only the odour was evaluated). Addition of citric acid or sodium citrate and propyl gallate in the oil phase and EDTA and ascorbic acid in the aqueous phase increased the shelf-life to 49 days. The shelf-life could be further increased to 89 days by refrigeration and to 132 days by the addition of the glucose oxidase–catalase oxygen scavenging system.

Li Hsieh and Regenstein[93] reported that after 14 weeks of storage a sensory panel was not able to distinguish between a traditional soy bean oil mayonnaise and a mayonnaise containing fish oil provided that EDTA (0.075 per cent) and TBHQ (0.02 per cent) were used, the storage temperature was low (2 ºC) and that oxygen was excluded.

In Europe, TBHQ is not allowed and therefore this antioxidant cannot be used. Jacobsen *et al.*[41,56–59] evaluated the antioxidative effect of EDTA, propyl gallate, gallic acid, tocopherol, ascorbic acid or a mixture of ascorbic acid, lecithin and tocopherol (the so-called A/L/T system) by sensory profiling, measurements of lipid hydroperoxides and volatiles and in some cases also by electron spin response (ESR) determination of free radical formation. The volatiles were measured by dynamic headspace gas chromatography mass (GC-MS). Weak pro-oxidative effects of propyl gallate and gallic acid were observed. Tocopherol was inactive as an antioxidant and ascorbic acid and the A/L/T were strong pro-oxidants, probably because ascorbic acid promoted the release of iron from the egg yolk located at the oil–water interface. In contrast, EDTA (0.0075 per cent) was observed to be a strong antioxidant that totally inhibited oxidative flavour deterioration during storage at 20 ºC, as illustrated in Table 15.5. The strong antioxidative effect of EDTA was proposed to be due to the metal-chelating properties of this antioxidant. In our laboratory, we have subsequently shown that lower concentrations (i.e. 10 ppm) of EDTA is sufficient to retard lipid oxidation in mayonnaise (unpublished data).

Table 15.5 Sensory scores during storage at 20 ºC for fishy off-flavour in fish oil enriched mayonnaise with and without 75 ppm EDTA. Sensory scale from 0 to 9

	0 weeks	1 week	2 weeks	3 weeks	4 weeks
Mayonnaise without antioxidant	0.2 ± 0.4	2.1 ± 1.5	2.4 ± 1.6	2.8 ± 1.3	3.4 ± 1.8
Mayonnaise with 75 ppm EDTA	0.2 ± 0.3	0.2 ± 0.4	0.1 ± 0.3	0.3 ± 0.5	0.2 ± 0.3

Source: Adapted from Jacobsen *et al.*[59]

15.4.2 Margarine/spreads

Young[94] developed low-calorie (40 per cent fat) spreads of commercially acceptable quality. The spreads had 20 per cent of the fat replaced by fish oil and the oxidative stability was optimised by adding EDTA (150 ppm). Moreover, the fish oil contained 300 ppm of Grindsted 117 (ascorbyl palmitate, propyl gallate and citric acid) and 1000 ppm Toco 50 (50 per cent tocopherol in vegetable oil). In another margarine study, tocopherol (0.2 per cent) in combination with ascorbyl palmitate (0.01 per cent) and propyl gallate (0.01 per cent) was found to be the most effective in retarding lipid oxidation as evaluated by peroxide, carbonyl and anisidine values. No sensory evaluation was performed. TBHQ also retarded lipid oxidation, but it was less efficient than the combination of the above three antioxidants and it gave rise to discoloration. The effect of EDTA was not evaluated in this study. Kolanowski *et al.*[84] suggested that spreads could only be enriched with up to 1.5 per cent fish oil (i.e. 0.5 per cent EPA and DHA) without deteriorating the sensory quality of the product. No antioxidants were added to this product. In another study, Kolanowski *et al.*[95] concluded that low-calorie spreadable fats (soft margarine and mix of butter and vegetable oil) could be enriched with up to 1 per cent EPA and DHA without significantly affecting the sensory quality. The margarine spread may be stored up to 6 weeks and the spread based on butter and vegetable up to 3 weeks without significant decrease of quality. These spreads contained 55 per cent fat and no antioxidants were added.

15.4.3 Milk products

In a study by Kolanowski *et al.*,[84] it was not possible to incorporate even low levels of EPA and DHA (0.15 per cent fish oil which equals 0.05 per cent EPA and DHA) into milk without significantly reducing the palatability of the milk. In contrast, the same authors found that enrichment of flavoured yoghurt with up to 0.3 per cent fish oil (i.e. 0.1 per cent EPA and DHA) resulted in a product with acceptable sensory characteristics. In our laboratory, we have shown that the oxidative deterioration of fish oil-enriched milk strongly depends on the quality of the fish oil. Cod liver oil with a relatively low peroxide value (PV) (1.5 meq/kg) was found to give rise to the development of fishy off-flavours when added to milk in a concentration of 1.5 per cent. In contrast, milk with a more unsaturated tuna oil (20.1 per cent in cod liver oil *vs* 30.2 per cent EPA + DHA in tuna oil) with a lower PV (0.1 meq/kg) oxidised much slower and off-flavours were not detected.[42] These findings indicate that the presence of both lipid hydroperoxides and trace metals in relatively low levels will lead to oxidative flavour deterioration in fish oil-enriched milk because of the mechanism described in equations 15.2 or 15.3 in Section 15.3.1. On the other hand, it seems to be possible to enrich milk with n-3 LC PUFA and obtain a product of satisfactory sensory quality provided that the fish oil is of a high quality. Moreover, preliminary results in our laboratory also suggest that the process for homogenising the fish oil together with the milk must be optimised in order to reduce lipid oxidation in this product.

15.4.4 Beverages

Kolonowski and co-workers[84] found that addition of up to 0.3 per cent fish oil (0.15 per cent EPA and DHA) to orange juice resulted in an acceptable product, but after 10 days of storage a strong fishy off-flavour had developed. The effect of antioxidant addition was not evaluated in this study.

15.4.5 Formula concentrates

The sensory quality of formula concentrates consisting of an instant flavoured powder milk-based protein-carbohydrate formulae enriched with up to 6 per cent microencapsulated fish oil (0.3 per cent EPA and 0.6 per cent DHA) was acceptable after 6 months of storage.[84]

In a study by Satué-Gracia *et al.*[80] on traditional infant formula without n-3 LC PUFA, lactoferrin was able to reduce lipid oxidation. The antioxidative effect of lactoferrin was suggested to be due to its ability to chelate metal ions. These findings indicate that lactoferrin could be an important efficient antioxidant in infant formula enriched with n-3 LC PUFA.

15.4.6 Bread

Becker and Kyle[86] evaluated the sensory stability of bread baked with either a regular pharmaceutical grade fish oil made from sand eel, a specialty tuna oil or an algal oil. The taste panel indicated that sensory off-flavours were less likely to be detected in the DHA bread made with algal oils compared with those made with fish oils. Based on these data they suggested that the algal source had a better stability than the fish oils. This proposition was later invalidated by Frankel *et al.*[96] They showed that the high oxidative stability of the commercial DHA-rich algal oil was lost when the triglycerides were purified to remove tocopherols and other antioxidants. Moreover, an oil-in-water emulsion with the same algal oil had a lower oxidative stability than corresponding fish oils.

15.4.7 Encapsulation of n-3 LC PUFA for food products

n-3 LC PUFA for foods are commercially available both as neat oils (fish oil or algal oil) or as microencapsulated fat powders. The latter are essentially dried, homogenised emulsions of an oil or fat where proteins, modified starches or hydrocolloids are used as emulsifying materials. A non-emulsifying, water-soluble material such as sugar or hydrolysed starch is also used as filler.[43] Different drying techniques, i.e. spray drying or freeze drying, may be used to produce the powders. The advantage of microencapsulation for fish oil is that the shelf-life of the fish oil may be extended by protecting the fish oil from contact with atmospheric oxygen. Keogh *et al.*[43] studied the effect of emulsifier type (three different casein types), free fat, surface fat and the air content of the spray-dried fish oil powder on the shelf-life as monitored by sensory evaluation. They concluded that fish oil powder with a low level of off-flavour can be

produced with a shelf-life of 31 weeks at 4 ºC using dairy ingredients alone as encapsulating ingredients. They also found that the shelf-life increased when the free fat and vacuole volumen of the powder decreased. They did not find any effect of the surface fat. In another study by Heinzelmann *et al.*,[51] fish oil was microencapsulated using freeze-drying techniques. It was shown that the best shelf-life was obtained when the fish oil contained a combination of ascorbic acid, lecithin and tocopherol (i.e. the A/L/T system) and when the freezing rate was slow. No sensory evaluation was performed on these powders. Based on the authors personal communication with the industry producing microencapsulated n-3 LC PUFA, more research seems to be required to optimise the quality of the n-3 powders.

15.4.8 Recommendations

On the basis of the above summary of how lipid oxidation has been retarded in products enriched with n-3 LC PUFA the following strategies to avoid lipid oxidation are suggested:

- Exclude oxygen from the system, for example by packaging under vacuum.
- Store the enriched products at chilled temperatures.
- Ensure that ingredients have a low content of hydroperoxides, transition metals and other pro-oxidants. It seems to be especially important that fish oil has a low PV. Therefore, marine oils should be stored at low temperatures (<0 ºC), in the dark, with reduced oxygen and the fish oils should be used as fast as possible after deodorisation as hydroperoxides will form even at temperatures below 0 ºC.
- The choice of emulsifier may significantly affect lipid oxidation rates. Therefore, when applying n-3 oils in a new food product it may be necessary to reformulate the conventional recipe to include other emulsifier types. In turn, the recipe may also need to be changed with respect to addition of thickening agents in order to obtain the same rheological properties as the traditional product.
- Use metal chelators such as EDTA, citric acid, proteins, polysaccharides and metallic chelating plant polyphenols to prevent lipid hydroperoxide decomposition.
- Addition of free radical chain breaking antioxidants may further reduce lipid oxidation. Select antioxidants that will be located where they are required, i.e. normally near the oil–water interface where the decomposition of lipid hydroperoxides takes place.
- Optimise the processing conditions. In some food systems the particle/droplet size will affect the oxidation rates, in other foods they may not. In addition, the emulsification process may disrupt natural membranes that may protect the fish oil from protein-bound metals. Emulsification processes should be optimised to minimise lipid oxidation.

15.5 Future trends

Public awareness of the beneficial effects of the n-3 LC PUFA seems to have increased since the late 1990s. Likewise, the industrial interest in exploiting health effects of the n-3 oils has apparently also increased. Therefore, it is expected that more new food products enriched with n-3 PUFA will enter the marketplace in the coming years. A promising new area is baby food. As previously mentioned, the infant's requirements for DHA has recently received substantial attention, and several formulas enriched with DHA are now on the market. It is therefore likely that efforts will also be made to develop other types of baby foods enriched with n-3 LC PUFA in the coming years.

Dairy products are the fastest growing product within the functional food area.[97] So far, most dairy products in this category have been 'functional' due to the addition of probiotic bacteria, and consumers already perceive low-fat dairy products as being healthy. Therefore, milk drinks and yoghurts may be a good vehicle for n-3 LC PUFA enrichment and we may see a number of new products in this category in the future.

Ice cream producers are now also targeting healthy consumers. In the recent years, new fat reduced ice cream formulations have entered the market, and recently calcium-enriched ice creams have been marketed. Efforts are currently being made to develop ice cream enriched with n-3 LC PUFA.

This chapter has mainly dealt with EPA and DHA from marine sources. However, with the increased focus on the beneficial effects of n-3 LC PUFA in general, it can be expected that products enriched with the 18:3 PUFA will also receive more attention from the industry. Traditionally, the industry has tried to protect food products from lipid oxidation by the addition of free radical chain-breaking antioxidants. However, this strategy does not seem to be very efficient in preventing lipid oxidation in emulsified food systems, especially when they are enriched with n-3 LC PUFA. With our increased understanding of the important role of trace metals, emulsifiers and processing conditions in the lipid oxidation processes, more efforts will be dedicated to use this knowledge to develop alternative strategies to retard lipid oxidation in real foods with n-3 oils.

The aquaculture industry is the main customer to fish oil, which is almost entirely produced from different types of industrial fish species such as sand eel, menhaden, etc. With the growing aquaculture industry, the demand for fish oil for fish feed production is increasing. This could lead to a situation where the demand for fish oil for human consumption and fish feed exceeds the production. However, sources of n-3 LC PUFA other than the traditional industrial fish species are available. Thus, efforts are being made to use the oil from waste products from fish species used for human consumption. In addition, n-3 LC PUFA can also be produced from algae and commercial products are already available.

15.6 Sources of further information and advice

The Omega-3 Information Network at:
PO Box 24,
Tiverton EX16 4QQ, UK
Tel: (44) (0) 1884-257547, Fax: (44) (0) 1884-242757
E-mail: rayrice@eclipse.co.uk

International Fishmeal & Fish Oil Organisation
2 College Yard, Lower Dagnall Street,
St. Albans AL3 4P4, UK
E-mail: secretariat@iffo.org.uk

Oils and Fats International
DMG World Media (UK) Ltd
Queensway House, 2 Queensway,
Redhill RH1 1QS, UK
Tel: +44 (0) 1737 855068, Fax: +44 (0) 1737 855470
Email: anitarevis @uk.dmgworldmedia.com

NutriVit: http:// www.nutrivit.org/whatsnew/index.htm
The Fish Foundation: http:// www.fish-foundation.org.uk/references.htm

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16

Marine micro-organisms as new sources of n-3 polyunstaurated fatty acids (PUFA)

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16.1 Introduction: PUFA and their health benefits

In recent decades the interest in polyunsaturated fatty acids (PUFA) for nutritional and pharmaceutical applications has increased remarkably. PUFA are long-chain (18–22 carbon atoms) fatty acids containing two or more double carbon bonds. They are classified according to the position of the first double bond as counted from the methyl terminus. A so-called n-3 PUFA has its first double bond at position 3 as counted from the methyl terminus. Other PUFA groups are n-6 where the first double bond is located six carbons from the methyl terminus and n-9 where the first double bond is located nine carbons from the methyl terminus. Instead of n, the symbol ω is often used to classify PUFA. Double bonds in PUFA may also be counted from the carboxylate group and are then represented by the symbol Δ. In Table 16.1 several n-3 PUFA are listed. α-linolenic acid (LNA, 18:3 Δ9,12,15), eicosapentaenoic acid (EPA, 20:5 Δ5,8,11,14,17) and docosahexaenoic acid (DHA, 22:6 Δ4,7,10,13,16,19) are the best studied polyunsaturated fatty acids within this group. The chemical structures of DHA and EPA are shown in Fig. 16.1.

16.1.1 Health aspects of n-3 PUFA

The beneficial effects of n-3 PUFA were first noticed by Dyerberg and collegues in the early 1970s. They reported that the Greenland Eskimo (Inuit) population had a lower incidence of heart disease compared with an equivalent Danish population even though they consumed large amounts of fat. These Greenland

Table 16.1 List of ω-3 PUFA

Common name	Systematic name*	Short name
α-Linolenic acid (LNA)	Δ9, Δ12, Δ15-Octadecatrienoic acid	ω-3 18:3
	Δ6, Δ9, Δ12, Δ15-Octadecatetraenoic acid	ω-3 18:4
	Δ8, Δ11, Δ14, Δ17-Eicosatetraenoic acid	ω-3 20:4
Eicosapentaenoic acid (EPA)	Δ5, Δ8, Δ11, Δ14, Δ17-Eicosapentaenoic acid	ω-3 20:5
	Δ7, Δ10, Δ13, Δ16, Δ19-Docosapentaenoic acid	ω-3 22:5
Docosahexaenoic acid (DHA)	Δ4, Δ7, Δ10, Δ13, Δ16, Δ19-Docosahexaenoic acid	ω-3 22:6
	Δ5, Δ8, Δ11, Δ14, Δ17, Δ20-Tetrasahexaenoic acid	ω3 24:6

* All double bonds are in *cis*-configuration.

Eskimos had a favourable lipid profile with low levels of triglycerides, plasma cholesterol and very low-density lipoproteins (VLDL) and high levels of high-density lipoproteins (HDL) (Dyerberg *et al*. 1975). As the Eskimos consume a large amount of marine mammals and arctic fish in their diet, which are rich in n-3 fatty acids, these PUFA were presumed to play a major role in the health effects of fish oil. Since then several epidemiological and medical studies have been performed to investigate the beneficial effects of n-3 PUFA on humans (Kromhout *et al*. 1985; Daviglus *et al*. 1997; Albert *et al*. 1998).

DHA, for instance, attracted much attention because of its various physiological functions in the human body. DHA reduces or inhibits risk factors involved in various diseases such as cardiovascular diseases (Kromann and Green 1980; Kang and Leaf 1996; Nordøy *et al*. 2001) and has some positive effects on diseases such as hypertension, arthritis, arteriosclerosis and thrombosis (Horrocks and Yeo 1999). Furthermore, DHA is an essential component of cell membranes in some human tissues and, for instance, accounts for over 60 per cent of the total fatty acids in the rod outer segment in the retina (Giusto *et al*. 2000). DHA is regarded as essential for the proper visual and

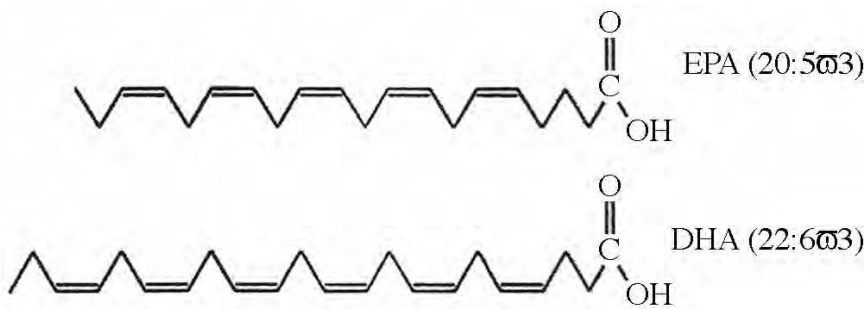

Fig. 16.1 Schematic representation of docasahexaenoic acid (DHA; ω-3 22:6) and eicosapentaenoic acid (EPA; ω-3 20:5).

neurological development of infants because of its roles as structural lipid component (Nettleton 1993; Crawford *et al* 1997; Das and Fams 2003). As pre-term and young infants are unable to synthesise DHA at a fast enough rate to keep up with the demand from the rapidly growing brain (Crawford 1987) they must obtain these compounds from their diet. In general, breastfeeding serves as a good source of PUFA (Huisman *et al*. 1996). However, although it has been recommended that all infant formulas include DHA (FAO/WHA Expert Committee 1994), application of DHA in some infant formulas only started recently. EPA is the precursor of a family of eicosanoids that are widely involved in metabolic regulation (Hwang 2000). Some studies also suggest that EPA is a potential anticachexia and anti-inflammatory agent (Calder 1997; Gill and Valivety 1997; Babcock *et al*. 2000).

With respect to the biological function of PUFA the position of the double bond strongly affects the properties of the fatty acids. For instance, eicosanoids derived from the n-6 polyunsaturated fatty acid arachidonic acid (AA, 20:5 Δ5,8,11,14) have strong inflamatory properties, whereas those produced from EPA are anti-inflammatory (Gill and Valivety 1997).

Although the optimal intake of PUFA has not yet been established, there is some consensus that the PUFA intake should be at least 3 per cent of the total lipid intake (Gill and Valivety 1997). Studies suggest that while total fat levels in the typical Western diet are too high, the intake of long-chain n-3 PUFA is too low (Newton 1998). At present, most consumed PUFA originate from plant oils and belong to the n-6 group. The excess of n-6 fat intake compared with n-3 intake has practical consequences because, as they are very similar except for the position of one double bond, they may compete for the same enzymes that metabolise them. An excess of n-6 over n-3 fatty acids leads to poor metabolism of ingested n-3 fatty acids to the longer n-3 fatty acids EPA and DHA (James *et al*. 2000). In order to improve the balance generally seen as optimal for human health, an increase in n-3 PUFA consumption and a reduction in n-6 PUFA is needed. The British Nutrition Foundation recommended a n-6 to n-3 PUFA ratio between 5:1 and 3:1 (British Nutrition Foundation 1992).

16.2 Sources of n-3 PUFA and microbial production of PUFA

16.2.1 Current sources of n-3 PUFA

Although plant materials such as flaxseed, canola and soybean oil contain the n-3 PUFA α-linolenic acid, this paragraph will focus on the n-3 fatty acids with 20 and 22 carbon atoms. Currently, the main sources of DHA and EPA are fatty fish species such as herring, mackerel, sardine and salmon (Gunstone 1996), as their flesh usually contains a high proportion of fat tissues. The quality of the fish oil, however, is variable and depends on fish species, seasons and location of catching sites. The application of fish oil PUFA in foods, for inclusion in infant formulas, or for pharmaceutical applications may have some disadvantages because of contamination of the fish oil by environmental pollution such as PCBs

(polychlorinated biphenyls) or dioxin-like compounds and problems associated with the typical fishy smell and unpleasant taste. Furthermore, as marine fish oil is a complex mixture of fatty acids with varying lengths and degrees of unsaturation, expensive purification may be required before application.

At present the fish oil production amounts to about 1.1 million tonnes annually (Gunstone 2001), of which 70 per cent is utilised for production of fish feed for farmed fish (Tuominen and Esmark 2003). The demands for n-3 PUFA are rapidly increasing owing to a rapid increase in aquaculture and application in food and pharmacy. It is therefore expected that within 10 years the production of PUFA from current sources will become inadequate for supplying the expanding market. In order to meet the expected rise in demand and to circumvent the detrimental aspects of fish oils, alternative production processes for PUFA are currently being developed. These include the development of refining techniques of fish oils (Yamamura and Shimomura 1997) and the exploitation of microbial PUFA sources (Barclay *et al*. 1994; Kyle 1996; Ratledge 2001; de Swaaf 2003) which may offer a sustainable production of n-3 PUFA.

16.2.2 Microbial production of PUFA

Although marine fish and mammals appear to have some capacity for *de novo* biosynthesis of n-3 PUFA, the majority of the PUFA in their body originates from their diet. Fish consume marine zooplankton that have fed on phytoplankton (Ackman *et al*. 1964) such as bacteria, lower fungi, microalgae and some microalgae-like organisms. These organisms are known as the primary producers in the marine food chain and they are the actual primary synthesisers of PUFA (Yap and Chen 2001).

In human and animal nutrition, lipids have been obtained traditionally from plant and animal sources. However, some valuable lipids are now being produced from micro-organisms. As a source of oil or, in more general terms, lipids, micro-organisms are less well known than plants and animals. Microbial oil or single cell oil (SCO) production is a relatively new concept, first proposed in the twentieth century (Ratledge 2001). Microbial oils may be produced in stirred bioreactors in the dark with an organic carbon source and sufficient amounts of minerals, nitrogen, oxygen and micronutrients by so-called heterotrophic micro-organisms. Alternatively phototrophic species may be cultivated under light in open or closed systems but this process is less well established for SCOs. Upon harvest lipids may be extracted from the dried biomass, formulated and used for their different applications.

As the prices for most bulk plant oils are relatively low, and animal fats are even cheaper, it is likely that processes for the microbial production of oils should focus on high value added products. Although technically feasible, earlier attempts to commercially produce SCOs have failed because of economics (Davies 1992; Nakahara *et al*. 1992; du Preez *et al*. 1995; Ratledge 2001). However, the SCO concept has now yielded several successes with

regard to PUFA and industrial interest is increasing (Barclay 1991; Barclay *et al*. 1994; Kyle 1994, 1996, 1997; Ratledge *et al*. 2001b; de Swaaf 2003).

Based on their percentage of n-3 PUFA, oleaginous marine micro-organisms such as microalgae or marine fungi may be interesting alternatives for fish oils (see Table 16.2). At present the contribution of microbial PUFA to the oil industry is nearly negligible but there are several reasons to increase their use in the near future. In heterotrophic systems microbial oils can be produced all over the year as these processes are usually independent of light, and temperature can be well controlled. Another advantage is that microbial oils are free from contaminants such as PCBs and dioxin-like compounds. Compared with fish oil, microbial oils often contain high levels of the desired fatty acids and, because of their lipid composition, purification of PUFA from microbial oils may be easier or not required.

Micro-organisms capable of producing n-3 PUFA above C20 include lower fungi, bacteria and marine microalgae (Bajpai *et al*. 1991; Kendrick and Ratledge 1992; Gunstone *et al*. 1994; Kyle 1996, 1997; Vazhappily and Chen 1998; Ratledge 2001; de Swaaf 2003). Bacteria, however, are probably not suitable as PUFA producers, as they do not accumulate high amounts of triacylglycerols and may contain unusual fatty acids and lipids not found in other systems (Ratledge 2001).

Oleaginous micro-organisms could provide an economically feasible source of PUFA, provided that most of the PUFA occur in triacylglycerols which is the preferred form to take lipids in the diet (Kendrick and Ratledge 1992). Furthermore, micro-organisms preferably contain one specific PUFA rather than a mixture of various acids. This gives the microbial oils an additional value as compared to fish oils, which contain mixtures of PUFA. The development of a microbial PUFA production process requires the selection of the proper micro-organism and optimised cultivation techniques (Ratwan 1991). As both, EPA and DHA are important nutritional n-3 PUFAs much effort has been devoted to finding a commercial source of these fatty acids other than from fish oil.

16.3 Cultivation of microalgae for the production of n-3 PUFA

16.3.1 Photosynthetic production systems

At present a few photoautotrophic systems are being used for cultivation of microalgae. The oldest and simplest systems for cultivation of phototrophic algae are open ponds. These cultivation systems, however, are dependent on the weather and climate and therefore the product quantity and quality of separate batches is variable. Processes are time consuming owing to the low specific growth rates of algae, and available light limits the attainable biomass concentrations. Because of contamination with bacteria and predation by protozoa, phototrophic cultivation in open ponds is feasible only when suitable selective environments can be used (e.g. high salinity, high pH). In addition,

Table 16.2 Percentages of specific fatty acids in the lipids of selected marine micro-organisms

Organisms	14:0	14:1	16:0	16:1	18:0	18:1	ω-6 18:2	ω-6 18:3	ω-3 18:4	ω-6 20:4	ω-3 20:5	ω-6 22:5	ω-3 22:6
Thraustochytrium aureum[a] (H)	3		8			16	2	2		3			52
Schizochytrium sp.[b] (H)	3		55		1							6	30
Crypthecodinium cohnii[c] (H)	17		17	1	2	10							44
Amphidinium carterae[d] (H)	8	30	15	5	3	5	6	17					
Isochrysis galbana[e] (P)	12		10	11	2	3	2		11		25		11
Skeletonema costatum[f] (P)	17		17	11		2	1		6		41		7
Amphidinium sp.[g] (P)	5		27		18	17	2	2			8		17
Pavlova lutheri[h] (P)	14		11	10		3					12		7
Monodus subterraneus[i] (P)	6		14	3	1	5	1	1		6	39		
Nitzschia laevis[j] (P)	13	1	10	30	3	3	2			14*	18		
Nitzschia laevis[j] (H)	13	1	22	34	2	3	3	1		5*	15		

* It was not described whether the form was n-3 or n-6.

[a] Singh and Ward (1996); [b] Yokochi *et al.* (1998); [c] de Swaaf *et al.* (1999); [d] Vazhappilly and Chen (1998); [e] Molina Grima *et al.* (1993); [f] Servel *et al.* (1994); [g] Viso and Marty (1993); [h] Meireles *et al.* (2002); [i] Cohen (1999); [j] Wen and Chen (2000).

H and P indicate heterotrophic and phototrophic growth, respectively.

optimal culture conditions are difficult to maintain and, because of the low biomass concentrations, harvesting costs are relatively high (Barclay *et al*. 1994; Molina Grima *et al*. 2003). In closed photobioreactors, made of transparent materials and generally placed outdoors for illumination with sunlight, the environmental parameters can be better controlled, allowing for higher biomass concentrations and a reduced contamination risk. Scale-up of the process is, however, limited by the ability to effectively introduce the light (Pulz 2001) and, in general, the costs of alga production in mass culture in such fermentors are high (Molina Grima *et al*. 2003).

16.3.2 Heterotrophic production systems

For commercial PUFA production, heterotrophic production systems, where microalgae are growing on reduced carbon sources, have been considered for production of specialty SCO (Barclay 1991; Kyle 1994, 1996; Mukherjee 1999). In heterotrophic cultures (i) optimal and axenic conditions can be maintained (Chen 1996), (ii) oil production can be carried out throughout the year as there is no seasonal or climatic dependence, (iii) the process can be controlled and product quality guarantees can be given, (iv) high cell densities, over 100 g dry weight/L, can be achieved (de Swaaf *et al*. 2003a) and (v) technology able to deal with heterotrophic fermentation is widely available.

For n-3 PUFA production by heterotrophic marine micro-organisms, however, several challenges must also be faced:

- At present, only a limited number of heterotrophic species that accumulate n-3 PUFA are available.
- Due to the required rich media and the relatively low growth rates of marine micro-organisms the risk of contamination is an issue.
- Economics of production should be in good proportion to market prices.

Furthermore, for all new products from microalgae legislation and safety items need to be considered.

16.3.3 EPA production

Most of the EPA production processes studied to date have been based on photoautotrophic growth (Qiang *et al*., 1997; Sánches Mirón *et al*. 2002; Molina Grima *et al*. 2003, Table 16.2). Unfortunately, the EPA yield and productivity in photosynthetic systems are low. In a closed flat plate reactor with a narrow light-path and intensive stirring which facilitated high cell concentration, a maximal EPA productivity of 58.9 mg/L/day, corresponding with 2.4 mg/L/h, was produced in *Monodus subterraneus* (Qiang *et al*., 1997). These values, however, are probably far too low in order to establish processes for economically feasible EPA production by photosynthetic microalgae.

Recent advances in heterotrophic production of EPA, with an emphasis on the use of diatoms as producing organisms, were recently reviewed by Wen and

Chen (2003). By using glucose as carbon source and nitrate as nitrogen source for the diatom *Nitzschia laevis*, an optimal EPA yield of 695 mg/L in 14 days of a fed-batch cultivation was reported (Wen *et al*. 2002). Although, compared with batch cultivation the use of a fed-batch cultivation remarkably improved EPA productivity (2.1 mg/L/h), these values are comparable with those reported for *Monodus* under photoautotrophic growth and still rather low for commercial production.

The exploitation of marine micro-organisms for the production of DHA will be discussed in the next section in more detail.

16.4 DHA production from marine micro-organisms

Currently, the production of DHA by marine micro-organisms is subject of intense research and increasing commercial attention (Barclay *et al*. 1994; Kyle 1996, 1997; Ratledge 2001; de Swaaf 2003). In order to select appropriate strains for DHA production, important parameters to be considered include the specific growth rate, the biomass production under optimal culture conditions, the total lipid content and the DHA proportion of the lipid. Furthermore, for downstream processing it is important to know whether DHA is present as part of the membrane structure or as a triacylglycerol and whether or not other PUFA are present. So far, of the more than 30 000 defined species of microalgae, only a limited number have been analysed to determine their lipid composition and fatty acid profiles (Cohen *et al*. 1995). Examples of marine DHA producers are presented in Table 16.2 and include phototrophic as well as heterotrophic strains.

16.4.1 DHA production by heterotrophic marine micro-organisms

In Table 16.2, fatty acid profiles of several heterotrophic and phototrophic marine micro-organisms are shown. *Schizochytrium* spp (Barclay *et al*. 1994) and *Crypthecodinium cohnii* (Kyle 1994, 1996; Ratledge *et al*. 2001a,b; de Swaaf 2003) are currently used in commercial processes for heterotrophic production of DHA.

Schizochytrium sp., a traustrochytrid, is an algae-like micro-organism which can contain over 70 per cent of its weight as lipids and a DHA content of 35 per cent per total fatty acids. Over 90 per cent of the lipids are neutral lipids (Yaguchi *et al*. 1997). About a decade ago, Omega Tech Inc. (recently acquired by Martek Biosciences, Columbia, Maryland, USA) developed a process in which a *Schizochytrium* strain grew to cell densities of 20 g/L in 48 hours. The biomass contained 10 per cent of their weight as ω-3 PUFA (Barclay 1991). This process forms the basis for the current commercial cultivation protocol with an improved strain of the original wild-type culture *Schizochytrium* sp. ATCC 20888 at Martek Biosciences. A related process has been developed in Japan by Nagase Biochemical Industries using a strain named *Schizochytrium* sp. strain

SR21 (Nakahara *et al*. 1996; Yaguchi *et al*. 1997; Yokochi *et al*. 1998). In a bioreactor this strain was able to produce 48.1 g dry cells/L and 13.3 g DHA/L in 4 days (Yaguchi *et al*. 1997). More recently several traustochytrids have been islolated from a variety of biotopes (Bowles *et al*. 1999; Fan *et al*. 2001; Huang *et al*. 2001). These strains produced DHA yields ranging from 2.2 g/L DHA after 107 h cultivation (Bowles *et al* 1999) up to 2.7 g/L in about 52 h (Fan *et al*, 2001). The DHA production rates of different strains and cultivation conditions are presented in Table 16.3.

In addition to DHA, *Schizochytrium* strains also produce relatively high amounts of ω-6 docosapentaenoic acid (DPA) and also some odd fatty acids such as 15:0 (Nakahara *et al*. 1996; Yokochi *et al*. 1998; Ratledge 2001). In mammalian systems ω-6 DPA cannot be converted to DHA and does not have the same functionality as DHA. Research on whether DPA has positive, negative or neutral health effects is still in progress (Gawrisch and Eldho 2002; Zeller *et al*. 2002). So far, *Schizochytrium* strains have been mainly used as poultry feed additives to give DHA-enriched eggs and as a feed for aquaculture. Recently, however, the European Commission approved a request of Martek Biosciences Corporation for the use of DHA-rich oil, derived from *Schizochytrium*, in several products such as dairy products, spreads and dressings, breakfast cereals and food supplements.

The marine dinoflagellate *Crypthecodinium cohnii* can produce high percentages of DHA (25–60 per cent) whereas other PUFA represent less than 1 per cent of the derived oil (Harrington and Holz 1968; Beach and Holz 1973; de Swaaf *et al*. 1999; Ratledge *et al*. 2001a; de Swaaf 2003). Starting in the early 1990s this organism was developed as a commercial source of oil rich in DHA by Martek Biosciences. Since then, several patents have been filed to protect their process and product (e.g. Kyle 1994; Kyle *et al*. 1995, 1998). An overall description of the process was provided by Kyle (1996). An axenic culture of *C. cohnii* is cultivated in large-scale bioreactors while environmental parameters such as temperature, pH, air flow, pressure, agitation and dissolved oxygen are well controlled. Although details of the current commercial process have not been published, the organism is probably cultivated with glucose as the principal carbon source.

De Swaaf *et al*. (1999) described in detail a process for DHA production by *C. cohnii* ATCC 30772 with glucose as carbon source. In these experiments the biomass increased from 1.5 to 27.7 g/L in 74 h and the total amounts of lipid and DHA after 91 h were 3.7. and 1.6 g/L respectively. However, compared with glucose, the use of acetic acid and ethanol as carbon sources proved to be much more efficient in respect to DHA production (Ratledge *et al* 2001a; de Swaaf 2003; de Swaaf *et al*. 2003a,b). In pH-controlled fed-batch cultivations of *C. cohnii* ATCC 30772, a so-called pH auxostat culture, with 50 per cent (w/w) acetic acid as carbon source DHA productivities up to 38 mg/L/h were achieved at laboratory scale (Ratledge *et al*. 2001a; de Swaaf *et al* 2003a). In comparison to several other *C. cohnii* strains, ATCC 30772 appeared to be the best strain in respect to DHA production (Ratledge *et al*. 2001a). The productivity of DHA by *C. cohnii* ATCC

Table 16.3 EPA and DHA productivities of phototrophic and heterotrophic cultured marine micro-organisms

Strain	Cultivation	Device system	Carbon source	Productivity (mg/L/h) DHA	EPA	Reference
Monodus subterraneus	P		CO_2		2.5	Qiang *et al.* (1997)
Nitzschia laevis	H	B	Glucose		2.1	Wen and Chen (2000)
Crypthecodinium cohnii	H	B	Glucose	19		De Swaaf *et al.* (1999)
Crypthecodinium cohnii	H	B	Acetic acid	48		De Swaaf *et al.* (2003a)
Crypthecodinium cohnii	H	B	Ethanol	53		De Swaaf *et al.* (2003b)
Thraustochytrium strain G13	H	B	Glucose	20		Bowles *et al.* (1999)
Schizochytrium sp.	H	S	Glucose	53		Fan *et al.* (2001)
Schizochytrium sp. SR21	H	S	Glucose	33		Yokochi *et al.* (1998)
Schizochytrium sp. SR21	H	B	Glucose	138		Yaguchi *et al.* (1997)

P = phototrophic, H = heterotrophic, S = shake-flask cultivation, B = bioreactor.
Adapted and modified from Sijtsma and de Swaaf 2004.

30772 could be even further increased by use of pure acetic acid and prolonged cultivation periods (de Swaaf *et al*. 2003a). This resulted in cultures, with a dry weight of 109 g/L, 61 g/L lipid and 19 g/L DHA. The maximum overall productivities of lipid and DHA were 152 and 48 mg/L/h, respectively. Vigorous mixing was required to sustain a sufficient oxygen level during these high cell density cultivations. This was complicated by culture viscosity, which resulted from the production of viscous extracellular polysaccharide (de Swaaf *et al*. 2001). Addition of a commercial polysaccharide-hydrolase could decrease the viscosity of the culture and the required stirring (de Swaaf *et al*. 2003a).

A further improvement was achieved by the development of an ethanol fed-batch protocol. In shake-flask cultures the specific growth rate was optimal with 5 g/L ethanol and growth did not occur at 0 g/L and above 15 g/L. In a fed-batch cultivation of *C. cohnii* with pure ethanol as feed 83 g/L dry biomass, 35 g/L lipid and 11.7 g/L DHA were produced in 220 h. The overall volumetric productivity of DHA in this process was 53 mg/L/h, the highest value reported so far for this alga (de Swaaf *et al* 2003b, Table 16.3).

16.4.2 Selection of carbon source for efficient DHA production

Glucose or other sugars are often used in commercial microbial cultivation processes. Glucose is relatively cheap and often readily utilised by heterotrophic micro-organisms. However, compared with glucose usage of acetic acid and ethanol as carbon sources resulted in far superior lipid and DHA productivities by *C. cohnii* (de Swaaf *et al*. 2003 a,b). This may be explained by acetyl-CoA metabolism (de Swaaf 2003). The synthesis of fatty acids is a cytosolic process with acetyl-CoA as the basic building block (Ratledge and Evans 1989). The routes of supply of cytosolic acetyl-CoA depend on the carbon source used for growth and on the organism (Ratledge and Evans 1989). Simplified, the main flux of carbon from glucose to cytosolic acetyl-CoA in oleaginous yeasts (and probably in other oleaginous eukaryotes) involves glycolysis, transport of pyruvate into the mitochondrion, conversion of pyruvate into citrate, transport of citrate into the cytosol and cleavage of citrate by ATP:citrate lyase to yield acetyl-CoA (Fig. 16.2, de Swaaf 2003).

In theory, acetyl-CoA may be supplied in the cytosol in a more direct way by cultivation of the organism on C_2-compounds such as acetate and ethanol. The conversion of acetate into acetyl-CoA involves a one-step enzymatic reaction catalysed by the enzyme acetyl-CoA synthetase. Acetyl-CoA synthetase has been localised in the mitochondrion, microsomes (Klein and Jahnke 1971) and cytosol (Kispal *et al*. 1991) of *Saccharomyces cerevisiae*. Cytosolic activity of acetyl-CoA synthetase has also been detected in mammals (Knudsen *et al*. 1992), insects (Storey and Bailey 1978) and plants (Gerbling *et al*. 1994). Utilisation of ethanol by *C. cohnii* would suggest the presence of an alcohol dehydrogenase, which converts ethanol to acetaldehyde, and an acetaldehyde dehydrogenase that converts acetaldehyde to acetate. As *C. cohnii* is able to use acetate and ethanol for its DHA production (de Swaaf *et al*. 2003b,c), it seems

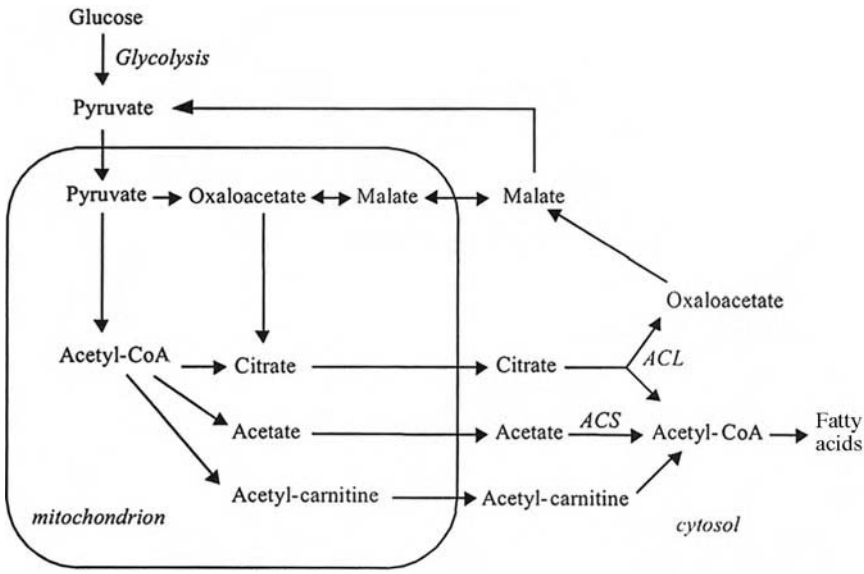

Fig. 16.2 Acetyl-CoA metabolism in oleaginous yeasts (De Swaaf 2003, modified from Ratledge and Evans 1989). ACL = ATP:citrate lyase, ACS = acetyl-CoA synthetase.

interesting to investigate the applicability of C_2-compounds as carbon sources for lipid accumulation by other oleaginous eukaryotes too.

16.5 Applications and future trends

The intake of n-3 PUFA via our diet occurs mainly via the consumption of seafood, which is characteristically rich in n-3 PUFA. The average intake varies among populations. Intake is high by Greenland eskimos (10–14 g/day), intermediate in countries such as Japan and Norway (1–3 g/day) and low in most Western populations (<0.5 g/day) (Schmidt *et al*. 2001). In an expert panel, there was a general agreement that two fish-based meals per week is a healthy dietary habit to obtain sufficient ω-3 PUFA. In practice, this does not often occur in Western diets (Nordøy *et al*. 2001).

In order to provide additional n-3 fatty acids, fish oil capsules are available. Furthermore, PUFA are included in the diet of lifestock to raise the PUFA content of their products. For example, eggs and milk enriched with DHA are on the market (Horrocks and Young 1999). At present, Martek Bioscience Corporation uses *C. cohnii* to manufacture oils that contain high DHA levels for inclusion in infant formulas. Capsules containing DHA are sold as neutraceuticals (Kyle 1996, 1997; Martek 2003).

In addition to the use of DHA for health applications a strong demand for DHA (as well as for other PUFA) results from the introduction of large-scale marine fish farms. The normal growth and development of several marine fish

larvae depend on the supplementation of n-3 PUFA in the diet, particularly DHA and EPA (Rodríguez *et al*. 1998). At present over 1 million tonnes of fish oil are produced of which over 70 per cent is currently used for aquaculture (Tuominen and Esmark 2003). Since these oils would, on average, contain 10 per cent DHA this amount of oil equals 100 000 tonnes of DHA. Based on the best DHA production data described so far (138 mg DHA/l/h, Table 16.3), a bioreactor volume of 200 m^3 and 300 production days per year, one bioreactor could produce 198.7 tonnes DHA annually. These calculations indicate that with 50 large bioreactors, about 10 per cent of the DHA currently available in fish oils could be replaced (Sijtsma and de Swaaf 2004).

The large-scale application of microbial DHA or other PUFA in human nutrition and animal feeds, however, depends on the quality and the production costs of the oils. The quality of microbial oils can be high by proper selection of the micro-organism and by well controlled production methods. Owing to the high quality and relative high cost price, microbial oil rich in DHA is currently applied mainly in infant formula, pharmaceutical and neutraceutical products. Calculated on the amount of DHA, a product such as Neuromin® is currently sold for about €2000–3000 per kg DHA. It is clear that DHA generates the high added value in these products (Sijtsma and de Swaaf 2004).

In order to be or remain competitive with other DHA sources, e.g. obtained from fish oils, and to enlarge the application areas of microbial DHA, the production costs have to be decreased. Cultivation scale and volumetric productivity (r_{DHA}) have been identified as major factors in determining the production costs of fermentative DHA production (Sijtsma *et al*. 1998). Factors that determine r_{DHA} are biomass concentration, lipid content of the cells, DHA content of the lipid and cultivation time. Obviously, a high DHA content of the biomass is also desirable from the viewpoint of product recovery. Subsequently, new and promising processes developed on a laboratory scale, such as the described ethanol process for *C. cohnii*, need to be scaled-up to industrial relevant scales (>50 m^3).

For the future, screening of many of the as yet unknown marine micro-organisms may result in strains that are even better DHA producers than the ones we know now. Once novel strains have been identified more efficient production techniques, which also include detailed knowledge of lipid metabolism and involved genes, should be developed. In addition, it may be possible to modify several very good microbial oil producers (e.g. the yeast *Cryptococcus curvatus*) that do not yet produce the relevant PUFA. Genetic engineering of these organisms may potentially lead to the production of tailor-made oils at costs low enough for application of these fatty acids in a variety of products.

16.6 Acknowledgements

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17

Developments in fat replacers

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17.1 Introduction: the role of fat replacers in reducing cardiovascular disease

There is a general consensus that a high-fat diet is linked with the development of obesity, high serum cholesterol level, and cardiovascular disease. In addition, there is evidence that high fat intake may increase the incidence of breast, colon, and prostate cancers (National Cancer Institute 1984). The relationship between dietary fat and the development of cardiovascular disease has been well documented. Latta (1990) reported that reduction of fat consumption lowered the risk of heart disease by 10 per cent, and the risk of cardiovascular disease by 20 per cent in people who were overweight by losing their weight and altering their diet. Hooper *et al*. (2001) reported that the reduction of dietary fat resulted in the decrease in cardiovascular events by 24 per cent in participants after a period of 2 years. Recent research indicates that the amount and the type of fat in diet are also associated with the prevention of cardiovascular and coronary heart disease. Diets rich in polyunsaturated or monounsaturated fatty acids tend to reduce the risk of cardiovascular and coronary heart disease. Conversely, diets rich in saturated fatty acids increase the risk of cardiovascular and coronary heart disease (Dyerberg *et al*. 1978; Grundy 1994). Therefore, restriction of fatty foods in diet is an effective way of reducing the risk of chronic diseases such as cardiovascular and coronary heart disease. Reducing fat and calories in the everyday diet has become a number one concern for most health-conscious individuals in the US.

Many health-related authorities including the US Dept. of Health and Human Services, US Surgeon General, American Heart Association, American Diabetes Association, American Dietetic Association, American Cancer Society and

National Institutes of Health have recommended dietary energy from fat should be reduced to 30 per cent with saturated fat intake to less than 10 per cent. Fat intake has been generally decreased since the 1970s. A national food consumption survey showed that total fat intake has decreased from 36 per cent in 1978 to 34 per cent in 1990 (Carroll *et al*. 1983; Lenfant and Ernst 1994), but the proportion of energy obtained from fat is still higher than the recommended level (Frazao 1996). The main sources of dietary fat in the US are meat, poultry, fish, baked goods, fats and oils, and dairy products, which accounts for about 90 per cent of total fat intake (Mattes 1998). The decrease in energy and fat consumption may be due to the awareness of health issues and increased availability of low- and reduced-fat products. A national survey in 2000 (Calorie Control Council 2001) showed that 163 million adult Americans (79 per cent of the adult US population) consume low-fat or reduced-fat foods and beverages. The rapid increase in reduced and low-calorie food products has resulted in confusion about labeling standards. Therefore, food labels bearing information on a reduction in fat or calories are important to consumers and food manufacturers. The US nutrition labeling regulations provide claims for the use of reduced fat- and calorie-related terms as shown in Table 17.1 (US Food and Drug Administration 1999). Food manufacturers have developed a number of fat replacers and more than 5000 reduced-fat, nonfat or low-calorie food products have been introduced to the market (Wylie-Rosett 2002). Fat replacers have opened the door for a new age of reduced-fat or fat-free options in variety of foods.

There are some studies in the literature that show that fat replacers provide health benefits to the public, such as weight loss, reduction in cholesterol, and lower incidence of cardiovascular disease. Most of the health benefit studies of fat replacers have been concentrated on one fat replacer, olestra. Patterson *et al*. (2000) reported that subjects consuming olestra had significantly reduced total serum cholesterol levels compared with subjects without consuming olestra. Other studies have shown that olestra has potential to lower total and low-density lipoprotein (LDL) cholesterol levels in both normal and hyper-cholesterolemic individuals (Fallat *et al*. 1976; Glueck *et al*. 1979, 1983; Jandacek *et al*. 1990). Olestra also may be a promising tool for weight reduction.

Table 17.1 Nutrient content claims indicating reduced fat and calorie foods

Claims	Definition
Fat-free	Less than 0.5 g of fat/serving
Low-fat	3 g or less fat/serving
Reduced or less fat	25% or less fat than a reference product/serving
Calorie-free	Fewer than 5 calories/serving
Low-calorie	40 or fewer calories than regular product /serving
Reduced or fewer calories:	25% or fewer calories than regular product/serving
Light	1/3 fewer calories or 50% of the fat in a reference food

Roy *et al*. (2002) reported that a significant weight loss was observed in men and women who replaced one third of dietary fat with olestra during the study period. One study involving obese patients with and without diabetes mellitus was conducted by Grundy *et al*. (1986). He reported that there was a decrease in total and LDL-cholesterol level in people without diabetes and a marked decrease in plasma triglycerides but no uniform change in LDL-cholesterol in people with diabetes when a low-calorie diet with or without olestra was supplied. Considering the report that the annual incidence of cardiovascular disease is increased more than two times in people with diabetes (American Diabetes Association 1996), consumption of foods containing olestra would be advantageous in reducing total and LDL-cholesterol.

17.1.1 Definition of fat replacers

A large number of fat replacers have been developed and are being used in partial or complete replacements for fat in foods. Each fat replacer has unique characteristics and uses. Some of fat replacers have been already approved by the Food and Drug Administration (FDA), while others are under review, and still others are in the developmental stage. Fat replacers represent a diverse chemical structure, functional and sensory properties and food applications.

The term fat replacer is a general term to encompass any ingredients used to replace fat. Generally, fat replacers are categorized into two groups – fat mimetics and fat substitutes. Fat mimetics are substances that imitate organoleptic or physical properties of triacylglycerol (triglycerides, conventional fats, and oils) but that cannot replace fat on a 1:1 weight basis (Shand 1997; McClements and Demetriades 1998; Akoh 2002). Fat mimetics have different chemical structures from triacylglycerols and protein- or carbohydrate-based fat replacers belong to this category. The caloric value of fat mimetics ranges from 1 to 4 kcal/g. Fat mimetics entrap a substantial amount of water and denature or caramelize at high temperatures, so they are not suitable for frying. Fat mimetics carry water-soluble flavors but not lipid-soluble flavor compounds and are generally less flavorful than fat (Akoh 2002). Fat substitutes are ingredients that resemble triacyglycerols chemically and physically. They can replace fat on a 1:1 weight basis and contribute either fewer calories than fat or no calories. Lipid-based fat replacers belong to this category. They are stable to cooking and frying temperatures. The terms fat replacer and fat substitute have been differentiated (Miraglio 1995; Shand 1997; Akoh 2002), but they are used interchangeably to cause confusion and misunderstanding. An ideal fat replacer should be safe with a significant caloric and fat reduction while maintaining the functional and organoleptic properties of a conventional fat (Warshaw and Franz 1996). Since no single ingredient is an ideal fat replacer, several fat replacers are often used in combination as part of a functional blend in one food system. Therefore, the search for an ideal fat replacer continues.

17.1.2 Types of fat replacers

Fat replacers represent a variety of chemical types with diverse physicochemical and sensory properties, so it is not easy to provide a simple classification. Fat replacers may be classified as carbohydrate-based, protein-based, or lipid-based replacers, depending on chemical composition of the ingredients (Hassel 1993; Warshaw and Franz 1996).

17.2 Carbohydrate-based fat replacers

Most of the carbohydrate-based ingredients are classified as generally recognized as safe (GRAS) by the US FDA. Fat-mimicking properties of carbohydrates result from an association of water with the carbohydrate particle, which could provide a sensory and rheological property similar to fat. The major carbohydrate-based fat replacers are starches, maltodextrins, cellulose, fibers, pectins, polydextrose, and gums. Some examples of the products and leading manufacturers of carbohydrate-based fat replacers are shown in Table 17.2.

17.2.1 Maltodextrins

One of the starch-derived fat mimetics that has been extensively studied is maltodextrins. Maltodextrin is defined by the FDA as a nonsweet, saccharide polymer consisting of α-1,4-linked D-glucose units with a dextrose equivalent (DE: a measure of the amount of reducing sugar content) of less than 20 (Roller 1996). In the mid-1970s, it was suggested that maltodextrins with low-DE could be used as fat replacers in a variety of foods (Richter *et al*. 1976; Marchall *et al*. 1999) and low-DE maltodextrins developed for fat replacement were launched on the market in the 1980s. One of the first commercial products introduced into the market was Paselli SA2, a tapioca starch, developed by Avebe America Inc. (Harkema 1996). Maltodextrins are produced by acid-catalyzed or enzymatic hydrolysis of starch with a DE of less than 20. Starch sources are corn, potato, oat, rice, wheat, and tapioca. The DE of maltodextrin has been shown to determine the functional properties of maltodextrins such as viscosity, humectancy and Maillard browning ability (Akoh 1998). Maltodextrins are metabolized in a similar way to starch and provide 4 kcal/g, but when used as fat replacers, they are dissolved in water and used at concentrations of less than 100 per cent and the actual caloric value is about 1 kcal/g. Maltodextrins are available as a powder or concentrated solution.

Maltodextrins with DE below 20 can function more effectively as a fat binder than maltodextrins with high DE (Frye and Setser 1993; Harkema 1996) and form thermoreversible gels that have smooth texure and mouthfeel similar to that of hydrogenated shortenings or oils (Nonaka 1997). Maltodextrins can be used in a myriad of fat-replacing systems including baked goods, extruded snacks, table spreads, margarine, imitation sour cream, salad dressings, frostings, fillings, sauces, processed meats, and frozen desserts. A large number

Table 17.2 Types of carbohydrate-based fat replacers

Trade name	Developer/manufacturer	Composition	Applications
Amalean I	American Maize Products Co.	Modified high-amylose corn starch	Salad dressings, baked goods, sauces
Avicel®	FMC Corp.	Microcrystalline cellulose and carboxymethylcellulose	Baked goods, salad dressings, sauces, ice cream, spreads
C*Pur 01906	Cerestar	Potato maltodextrin	Salad dressings, sauces, margarine
Fibruline	Cosucra	Inulin	Dressings, meat products
Keltrol	CP Kelco	Xanthan	Baked goods, salad dressings, margarines
Maltrin M040	Grain Processing Corp.	Hydrolyzed corn starch	Baked goods, dairy products, spreads, salad dressings, dips
Litesse®	Pfizer Inc.	Polydextrose	Dressings, spreads, bakery fillings
N-Oil	National Starch & Chemical Co.	Tapioca dextrin	Frozen desserts, soups, sauces, puddings, imitation sour creams
Paselli SA 2	Avebe America Inc.	Maltodextrin from potato starch	Baked goods, dips, spreads mayonnaise, dressings, processed meats
Raftiline®	Orafti	Inulin	Baked goods, desserts, beverages, ice cream
Slendid™	Hercules Inc.	Pectin from citrus peel	Spreads, mayonnaise, dressings, frozen desserts, baked goods
Staslim™	A.E.Staley Manufacturing Co.	Modified tapioca/potato starch soups	Salad dressings, soups, baked goods,
Stellar™	A.E.Staley Manufacturing Co.	Corn starch	Baked goods, fillings, sauces, gravies, salad dressings
Oatrim	USDA A.E. Staley Manufacturing Co. (ConAgra Specialty Grain Products)	β-Glucans from oat flour	Dairy products, confectionery, frozen desserts, meat products

of maltodextrins produced from different sources have been developed and are commercially available, which include for example, Maltrin®M040, Paselli SA2, N-Oil® Amalean™, and Stellar™.

17.2.2 Microcrystalline cellulose

Microcrystalline cellulose was manufactured by FMC Corp. and sold by its trade name Avicel®. It has been used as a fiber source and non-caloric bulking and thickening agent for many years. Recently, microcrystalline cellulose has been used as a fat replacer. Microcrystalline cellulose is produced by acid hydrolysis of the cellulose pulp (Humphreys 1996). When microcrystalline cellulose is dispersed in water, it forms 3-dimensional networks of cellulose chains that can mimic the property of fat by providing body, consistency, mouthfeel, viscosity, gloss, and opacity to fat-reduced foods. It is usually used in combination with other hydrocolloids to increase functional properties. Avicel® PH 101, the powdered form of microcrystalline cellulose, was developed first for use in low-calorie food products. The particle size of about 0.2 μm mimics the fat-like mouthfeel of an oil-in-water emulsion (Humphreys 1996). Colloidal microcrystalline cellulose was introduced to the market in 1978 and can be used as a fat replacer in a variety of food products, such as salad dressings, baked goods, dairy products, ice cream, frozen foods, spreads and processed meats. Colloidal microcrystalline cellulose are effective in preventing formation of ice crystal in frozen desserts during freeze–thaw cycles (Clegg 1996). Novage® and Avicel® are examples of commercially available products, both of which are developed by FMC Corp. and have similar food applications.

17.2.3 Pectins

Pectin is a dietary fiber obtained by aqueous extraction of citrus peel and apples (Giese 1996a; Nielsen 1996). Pectin is traditionally used as a gelling agent for jams and jellies and can also act as a thickener. The majority of pectin production are consumed by the fruit processing and confectionery products. It was not until the early 1990s that pectin was considered as a fat replacer. Pectin consists of the partial methyl esters of polygalacturonic acid (Fig. 17.1). Commercial pectins are divided into two groups based on the degree of methyl esterification (DM). DM is defined as the percentage of galacturonic acid units that are methyl esterified. Pectins with a DM below 50 per cent are designated as low-methoxyl (LM) pectin, whereas pectins with DM above 50 per cent are designated as high-methoxyl (HM) pectins. LM pectins require a calcium concentration to form gels with desirable properties, while HM pectins require a certain amount of sugar and acid. Pectin was introduced to the market by Hercules Inc. (Wilmington, DE) under the name of Slendid® since 1991 for fat replacement (Nielsen 1996). Pectin gels can be used to replace fat in frozen desserts, baked goods, mayonnaise, spreads, processed cheese, and soups. The Slendid® line of products is almost calorie-free with a neutral taste and is stable

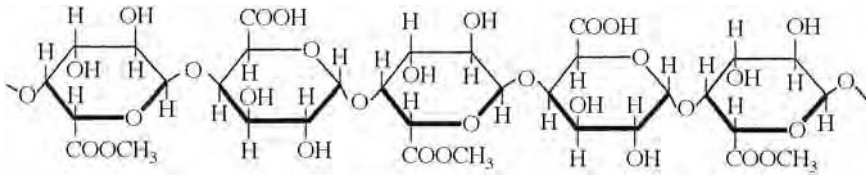

Fig. 17.1 The general chemical structure of pectins.

to heat, pH, shear and salt (Nielsen 1996). There is more than one form available commercially: Slendid®100 and 110 are LM pectins, while Slendid®200 is HM pectin. Slendid® line of products can replace up to 100 per cent of the fat in a wide range of food products including mayonnaise, salad dressings, processed meats, ice cream, processed cheeses, soups and sauces, desserts, and bakery products (Artz and Hansen 1994). The use of Slendid® can reduce the fat content in mayonnaise from 80 per cent to 3 per cent, and in a frankfurter from 25–35 to 3–5 per cent (Nielsen 1996).

17.2.4 Oatrim

Oatrim is a fat replacer developed and patented by the US Department of Agriculture (USDA) in 1991. Oatrim is produced from the partial hydrolysis of oat flour or bran by α-amylase with the β-glucan contents of 1–10 per cent (Cho and Prosky 1999). Oatrim is a soluble, tasteless powder that can be incorporated into food as a dry powder (4 kcal/g) or as a gel (1 kcal/g). Oatrim is heat stable for baking and can withstand pasteurization processing conditions, but is not suitable for frying (Calorie Control Council, 1996; Van der Slvijs *et al*. 1999). Oatrim or its gel gives the sensory property of natural taste and fatty texture to foods. Oatrim applications include pasteurized cheeses, dairy products, confectionery, frozen desserts, cereals, baked goods, and meat products (Inglett 2001). β-Glucan components in the oatrim have been reported to have a serum cholesterol-lowering effect (Inglett 1997, 2001). Oatrim is licensed for commercialization to ConAgra (Omaha, NE), Quaker (Chicago, IL) and Rhone-Poulenc (Cranbury, NJ).

17.2.5 Z-trim

Z-trim was also developed by the USDA. It is made from the high-cellulose portion of the hulls of oats, corn, rice, soybean, and peas, or bran from corn or wheat (Bollinger 1995; Akoh 1998), and is a tasteless, insoluble and indigestible fiber with zero calories. Z-trim gel contributes fiber, moistness, large water-holding capacity, high viscosity, and smooth texture. These properties make it possible for the reduced fat foods to taste like the traditional foods that are rich in fat. Z-trim has food applications in reduced-calorie cheeses, hamburgers and baked goods, but it is not suitable for deep fat frying (Cho and Prosky 1999).

17.2.6 Polydextrose

Polydextrose was invented at Pfizer Inc. in the mid-1970s (Rennhard 1975). Polydextrose has been used primarily as a low-calorie bulking agent, but it is also used as a fat replacer. Polydextrose is made up from randomly cross-linked D-glucose polymers containing a small amount of sorbitol and citric acid (LaBarge 1988). Polydextrose has reducing carbonyl groups that participate in the Maillard browning reaction. It is only partially hydrolyzed by digestive enzymes (Dziezak 1986; Mitchell 1996) and contributes 1 kcal/g, which is quite attractive to health-conscious individuals. However, a laxative effect may be observed from excessive consumption of 90 g/day because a large proportion of polydextrose is excreted intact (Artz and Hansen 1994). Products with more than 15 g of polydextrose per serving must be labeled. Polydextrose is available as a powder with a pH of 2.5–3.5 and a 70 per cent solution with a pH of 5.0–6.0 (Dziezak 1986). Polydextrose is odorless, nonsweet, and highly soluble in water. It exhibits high viscosity when dissolved in water, resulting in creaminess and mouthfeel similar to fat (Dziezak 1986). Polydextrose is commonly used in several food categories, including frozen dairy desserts, baked goods, chewing gums, frostings, salad dressings, puddings, hard and soft candies, spreads, sweet sauces, and syrups (Artz and Hansen 1994). Litesse® is a polydextrose-type product manufactured from Pfizer, Inc. (Mahungu *et al*. 2002) and may be used as a fat replacer, bulking agent and humectant.

17.2.7 Gums

Gums, also often referred to as hydrocolloids, are high molecular weight carbohydrates that have traditionally been used as thickeners, stabilizers, and viscosity enhancers at very low concentrations of 0.1–0.5 per cent to form gels. The type of gum used for a particular food application depends on pH, temperature, and concentration, which can affect viscosity and gel-forming characteristics (Lucca and Tepper 1994). Gums are not used directly as fat replacers, but they are used in formulating low-fat products because they mimic the sensory property of fat such as a slippery and creamy mouthfeel. Agar, alginate, gum arabic, carrageenan, guar gum, locust bean gum, and xanthan gum are frequently used in salad dressings, icings and glazes, desserts, ice cream, dairy products, ground beef, baked goods, soups, and sauces.

Galactomannan gum is most widely used in food products and guar gum and locust bean gum belong to this type. Guar gum is obtained from the seeds of an annual leguminous plant (*Cyamopsis tetragonolobus*). The locust bean gum, also known as carob galactomann, is the common name for the seeds of the carob tree (*Ceratonica siliqua*) and has been used as a food source for thousands of years, whereas guar gum was developed and launched recently to the market owing to a lack of locust bean gum (Clegg 1996). Both gums are neutral polysaccharides composed of a linear chain of α-1,4 linked β-D-mannose to which single α-D-galactose units are attached via α-1,6 linkages (Clegg 1996; Lazaridou *et al*. 2000). Guar gum and locust bean gum are different in their ratio of mannose to galactose

(M:G ratio) and the position of the galactose side chains on the main chain backbone. Guar gum has a highly substituted structure with an M:G ratio of about 1.8–2.0, whereas locust bean gum has an M:G ratio about 3.5–4.0 (Schorsch *et al*. 1997). Guar gum is soluble in cold water and produces highly viscous, pseudoplastic solutions (Clegg 1996; Herald 1986). In contrast, locust bean gum is not easily soluble in cold water and needs heating (80 ºC) for complete hydration to give a highly viscous solution. Locust bean gum gel is not affected by pH change or ionic strength. Galactomannan is not directly used as a fat replacer and the main function of galactomannan gums in low-fat foods is that they control viscosity by holding water (Setser and Racette 1992). This becomes important as the fat level in foods is reduced. Guar gum and locust bean gum have many food applications, including ice cream, frozen desserts, low-fat cheese products, bakery goods, sauces, and dressings. Locust bean gum is preferred in frozen desserts because it retards ice crystal growth. Guar and locust bean gum have a synergistic effect with xanthan gums.

Xanthan gum was discovered about 50 years ago and is produced by fermentation of bacterium *Xanthomonas campestris*. The main polymer chain consists of β-1,4 linked D-glucose units identical to that of cellulose but substituted on every second residue with a charged trisaccharide group. This side group consists of two mannose units separated by a glucuronic acid residue (Clegg 1996; Schorsch *et al*. 1997). Xanthan gum is readily soluble in cold or hot water and exhibits a highly viscous, pseudoplastic rheology. Like galactomannan gums, xanthan gum does not serve as a direct fat replacer, but can be used as a stabilizer in low-fat foods by controlling viscosity and texture. Xanthan gum is stable over a wide range of pH and temperature, whereas other gums lose their viscosity under the same conditions. Such properties persist even at very low concentrations (0.1 per cent) and functions as a very effective stabilizer in low-fat foods such as dressings, sauces and mayonnaises to exploit its weak-gel (Clegg 1996). Kelco (Clark, NJ) has a line of products made from xanthan gum such as Keltrol, Keltrol BT, Keltrol GM, and Keltrol SF.

17.3 Protein-based fat replacers

The protein-based fat replacers include microparticulated protein derived from milk, egg, whey or vegetable proteins. The limitation of protein-based fat replacers is that they cannot be used in high-temperature frying because the protein will denature and lose its creaminess. However, some forms may be used in traditional baking and cooking applications. Common uses are in dairy products such as ice cream, butter and sour cream as well as in oil-based products such as salad dressings and margarines.

17.3.1 Simplesse

Simplesse® (NutraSweet, Deerfield, IL) was the first fat replacer developed with protein. Simplesse received GRAS approval by the FDA in 1990 for use in

frozen desserts (Singer and Moser 1993). It is an all-natural versatile product made from milk and/or egg white protein, sugar, pectin, and citric acid (Gershoff 1995). Simplesse is produced by a patented process known as 'micro-particulation'. Microparticulated protein is created by homogenizing and pasteurizing simultaneously at high temperatures to develop microscopic particles of uniform size of approximately 1 μm in diameter (Singer *et al*. 1988, Singer and Moser 1993). The resultant small spherical particles float over the tongue, giving creamy and smooth texture similar to fat (Gershoff 1995; Warshaw and Franz 1996). Protein particles smaller or larger than 0.5–3 μm in diameter do not provide the fat-like mouthfeel. The nutritive quality of the protein is unchanged during the microparticulation process and the caloric value is 4 kcal/g on a dry basis. However, microparticulate proteins that make up Simplesse are hydrated during manufacturing process and the final caloric value is actually 1–2 kcal/g (Anon 1990; Singer and Moser 1993). Therefore, the use of Simplesse provides a caloric reduction in many food products.

Simplesse is widely used to enhance the quality of low-fat foods. It provides fat-like creaminess in high-moisture foods such as dairy products, baked goods, sour cream, salad dressings, mayonnaise, margarine, sauces, and soups, but is not suitable for use in frying. Simplesse retains the biological property of the protein used, so the individuals who are allergic to egg or milk proteins can experience allergic reactions to it (Singer and Moser 1993; Gershoff 1995). There is more than one form of Simplesse available. Original Simplesse was sold as wet ingredient with solids content about 20–40 per cent. Simplesse D 100, a dry form, is derived from whey protein concentrate without egg protein and is readily hydratable to produce a thixotrophic fluid that can be used in a wide variety of dairy and bakery products.

17.3.2 Other protein-based fat replacers

Most of the protein-based fat replacers are produced in a similar manner in that the protein is shaped into spherical particles under heat and shear or blend at the same time and other components such as starches or gums may be added during the process to prevent extensive coagulation. The products and manufacturers of protein-based fat replacers are shown in Table 17.3. Other protein-based fat replacers include Trailblazer (Kraft General Foods, Glenview, IL), Dairy-Lo (Cultor Food Science, New York, NY), and Dairylight (John Labatt Ltd.'s Ault Foods, Canada), which may be used under the same GRAS approvals as Simplesse. Trailblazer is a blend of protein, starch, gums, and emulsifiers suitable for baked goods. The main limitation of these protein-based fat replacers is that they cannot be used for frying, but can be used to formulate a variety of products including cheesecake, puddings, sauces, pie fillings, cream cheese, ice cream, mayonnaise, dips, and spreads.

Table 17.3 Types of protein-based fat replacers

Trade name	Developer/ manufacturer	Composition	Applications
Simplesse	NutraSweet	Microparticulated egg white and whey protein concentrate	Dairy products, spreads, baked goods, frozen desserts, salad dressings, mayonnaise
Dairy-Lo	Cultor Food Science	Partially denatured whey protein concentrate	Dairy products, spreads
Dairylight	John Labatt Ltd	Partially denatured whey protein coagulate	Frozen desserts, sour cream, dairy products, salad dressings
Trailblazer	Kraft General Foods	Egg white and xanthan gum	Frozen desserts, dressings, sauces, baked goods

17.4 Lipid-based fat replacers

Lipid-based fat replacers are a stable group and include the synthetic compounds. They have chemical structures similar to triacylglycerols, but have reduced or zero caloric content because they are not fully hydrolyzed by digestive enzymes. Examples for lipid-based replacers are sucrose polyester (olestra), sucrose fatty acid esters, structured lipids, caprenin, salatrim, medium chain triacylglycerols, dialkyl dihexadecylmalonate (DDM), esterified propoxylated glycerol (EPG), and trialkoxytricarballylate (TATCA).

17.4.1 Sucrose fatty acid polyesters/olestra

Sucrose polyesters or SPE, commonly known as olestra (Olean, Procter & Gamble Co., Cincinnati, OH), is the common name for the mixture of sucrose esters with the addition of six, seven or eight fatty acids (Fig. 17.2). The common fatty acids are C16:0, C18:0, C18:1, C18:2, and C18:3 fatty acids (Rizzi and Taylor 1978; Akoh 2002). Sucrose polyesters are prepared from the reaction of fatty acids with the hydroxyl groups of sucrose in the presence of catalysts (Gardner and Sanders 1990). The types of fatty acids determine the physicochemical properties of olestra (Akoh 2002) and can be formulated for a variety of foods such as fried foods, cooking oils, shortenings, baked goods and spreads (Giese 1996b; Dziezak 1989). Olestra prepared from saturated fatty acids is solid, whereas olestra prepared from unsaturated fatty acids is liquid at room temperature (Peters *et al*. 1997). Olestra is non-caloric because the molecule is too large to be absorbed or metabolized by pancreatic lipase (Mattson and Nolen 1972; Grossman *et al*. 1994). It has the organoleptic and thermal properties of fat. For this reason, it can be used in high-heat applications such as baking and frying. Olestra was approved by the FDA in 1996 as a food additive. It can be used as a replacement for up to 100 per cent of the fats and oils used in the preparation of savory snacks such as potato, tortilla and

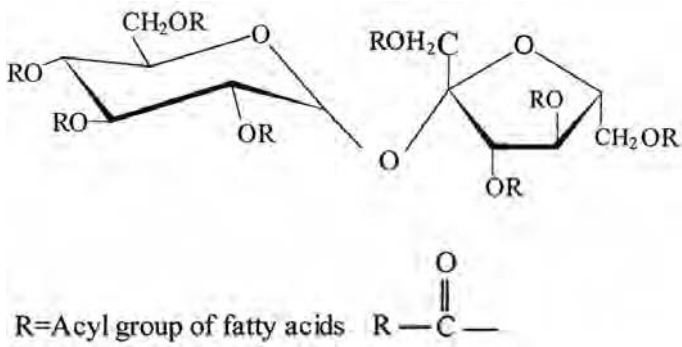

Fig. 17.2 The general chemical structure of sucrose fatty acid polyesters.

corn chips, crisps and crackers (Prince and Welschenbach 1998; Warshaw and Franz 1996). Olestra passes through the gastrointestinal tract without being absorbed and it may be associated with cramping, loose stools and reduced absorption of fat-soluble vitamins and nutrients. Therefore, use of olestra in foods requires the addition of specific amounts of vitamins A, D, E, and K to these foods. Research has shown that olestra does not significantly interfere with the absorption of macronutrients such as carbohydrates, proteins, or water-soluble vitamins and minerals (Bergholz 1992). Olestra is a lipophilic compound and its impact on the absorption and efficacy of lipophilic drugs such as oral contraceptives, diazepam and propranolol was investigated. Results indicate that there was little possibility of interfering with the absorption or bioavailability of lipophilic drugs (Miller *et al*. 1990, Prince and Welschenbach 1998). Toxicologic feeding studies in animals concluded that olestra is not toxic, carcinogenic, mutagenic, or teratogenic (Wood *et al*. 1991; Bergholtz 1992). Gastrointestinal testing showed that olestra has no significant effect on bowel movement, total transit time, or pancreatic response. Olestra is not metabolized by gut microflora in anaerobic conditions, nor does it affect the fermentation of other substrates by microflora in the colon (Nuck *et al*. 1994).

Many clinical studies have shown that olestra has potential to benefit some individuals. For example, replacement of conventional fat with olestra can benefit people at high risk of cardiovascular disease, coronary heart disease, obesity, and colon cancer by helping them to lower total fat intake and blood cholesterol level, and to lose weight (Crouse and Grundy 1979; Glueck *et al*. 1979, 1983; Grundy *et al*. 1986; Jandacek *et al*. 1990; Patterson *et al*. 2000; Bray *et al*. 2002; Roy *et al*. 2002).

17.4.2 Sucrose fatty acid esters (SFEs)

Sucrose fatty acid esters (SFEs) are mono-, di- and triesters of sucrose with fatty acids (Osipow *et al*. 1956). The structure of a sucrose monoester is shown in Fig. 17.3. Unlike olestra, SFEs are easily digested by pancreatic lipases, so they provide calories. SFEs have hydrophilic and lipophilic properties because of the

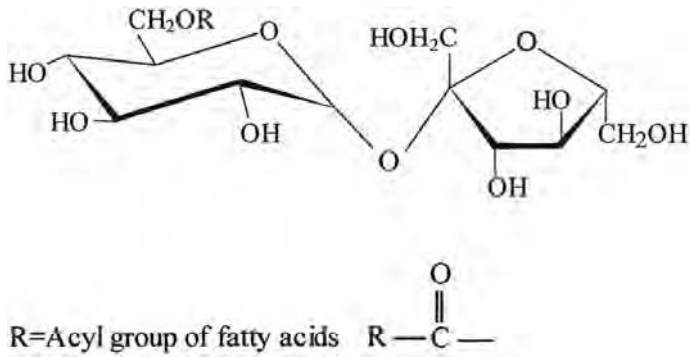

Fig. 17.3 The general chemical structure of sucrose fatty acid esters.

five to seven free hydroxyl groups with one to three fatty acid esters, which give the compound an emulsifying and surfactant functionality. Therefore, SFEs have been approved in the US for use as emulsifiers, texturizers, and stabilizers in a variety of foods such as margarines, shortenings, baked goods, frozen desserts and spreads. They are also used as coating components to retard ripening and spoilage of fruits, anticaking agents in dry soup mixes, and antimicrobial agents in hot canned drinks (Harrigan and Breene 1989, Marshall and Bullerman 1994). SFEs are not used directly as a fat replacer, but can function as part of a fat replacer by reducing fat and calorie contents (Shand 1997).

17.4.3 Structured lipids

Structured lipids (SL) are triacylglycerols containing short-chain fatty acids (S) and/or medium-chain fatty acids (M) and long-chain fatty acids (L) (Fig. 17.4). Structured lipids are prepared by hydrolysis and random transesterification of medium-chain triacylglycerols (MCTs) and long-chain triacylglycerols (LCTs) (Heird *et al*. 1986; Kennedy 1991; Akoh 2002). Structured lipids have been used for treatment of disorders of lipid absorption (LaBarge 1988). MCTs contain saturated fatty acids of chain length of C8:0 (caprylic) and C10:0 (capric), which are obtained from vegetable oils such as coconut and palm kernel oils (Babayan and Rosenau 1991; Megremis 1991). LCTs are composed of fatty acids with chain lengths greater than C12:0 (lauric). MCTs exhibit functional properties that are different from conventional fats and oils. Since MCTs contain saturated fatty acids, they are stable at high and low temperatures and do not readily undergo oxidation (Babayan and Rosenau 1991). They are used to replace liquid vegetable oils in low- and reduced-calorie foods to carry flavors, colors, and gloss and to prevent sticking on confectionery products (Babayan and Rosenau 1991; Akoh 1998). MCTs are absorbed intact in the intestine as free fatty acids and they are transported to the liver where they are oxidized to produce energy of about 8.3 kcal/g. MCTs are much more readily utilized as a source of energy, but not a source of essential fatty acids. Therefore, LCTs that are rich in linoleic

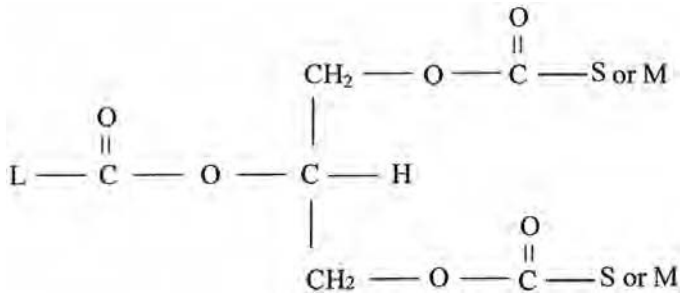

Fig. 17.4 The general chemical structure of structured lipids.

acid are still needed to meet the need of essential fatty acid requirements (Matthews and Kennedy 1990; Megremis 1991).

17.4.4 Caprenin

Caprenin (caprocaprylobehinic triacylglycerol) is a structured lipid introduced into the market by the Procter & Gamble Company. Caprenin is synthesized by esterification of three molecules of fatty acids, caprylic (C8:0), capric (C10:0), and behenic (C22:0) fatty acids to one molecule of glycerol. Caprylic and capric acids are obtained from coconut and palm oils, whereas behenic acid is from peanuts and marine oils (Artz and Hansen 1994). Caprenin provides 5 kcal/g instead of 9 kcal/g because caprylic and capric acids are metabolized less efficiently than common fatty acids and behinic acid is partially absorbed in the intestinal tract. Food applications include dairy products, confections, and baked products (Giese 1996a).

17.4.5 Salatrim

Salatrim (short and long acyltriglyceride molecule) is another structured lipid with a reduced-calorie fat similar to caprenin. Salatrim is composed of a mixture containing one short-chain fatty acid (acetic, propionic and butyric acid) and one long-chain fatty acid (stearic acid) randomly attached to the glycerol molecule (Kosmark 1996). Short-chain fatty acids contribute less energy than long-chain fatty acids and as stearic acid is partially absorbed, the calorie content of Salatrim is approximately 5 kcal/g. Salatrim compositions with differing amounts of short-chain fatty acid and long-chain fatty acid determine the functional and physical properties such as melting points, hardness, and appearance. Salatrim is a product of the Nabisco Foods Group (East Hanover, NJ) and has the same application as caprenin in reduced fat systems. Salatrim may be used to replace fat in chocolate-flavored coatings, toffees, fillings, baked goods, peanut spreads, dressings, dips and sauces, cheeses, sour cream, and frozen dairy desserts (Kosmark 1996). Salatrim, however, is of little use for deep-fat frying applications. Benefat 1 (Cultor Food Sci.) was the first Salatrim

product developed primarily to replace cocoa butter in confectionery applications.

17.4.6 Other synthetic fat replacers

Synthetic fat replacers have functional properties similar to conventional triacylglycerols, but their caloric values are reduced. They can replace fat on a 1:1 weight basis. Strategies for developing a synthetic fat replacer were suggested by some researchers (Ham 1984; Singhal *et al*. 1991; Akoh and Swanson 1994), which are based on reducing the susceptibility to enzymatic hydrolysis while maintaining the functional properties of conventional fats and oils. Strategies include (i) the replacement of the glycerol moiety of conventional triacylglycerol with alternate alcohols, (ii) replacement of fatty acids with alternate acids, (iii) reduction of the ester linkage of glycerol moiety to an ether linkage, and (iv) reversal of the ester linkage by replacing the glycerol moiety with a polycarboxylic acid or amino acid and esterify with a suitable long-chain alcohol.

Dialkyl dihexadecylmalonate (DDM) is synthesized from malonic acid, fatty acids, and hexadecane. DDM is noncaloric because it is not digested and is suitable for high-temperature application (Akoh 2002). The blend of DDM and soybean oil provided a sensory property of potato chips equally crisp and less oily than those prepared with conventional oils (Anon 1990).

Esterified propoxylated glycerols (EPG) are synthesized from glycerol and proplylene oxide to form a polyether polyol, which is then esterified with fatty acids. The resultant product exhibits an oil-like property with no caloric value (Artz and Hansen 1994). EPG is heat stable for baking and frying (Artz and Hansen 1994; Akoh 1998) and may be used to replace fat in foods such as desserts, salad dressings, and baked goods.

Trialkoxytricarballylate (TATCA) is similar to a triacylglycerol in that two to four carboxylic acid groups are esterified with saturated or unsaturated alcohols (Ham 1984; LaBarge 1988). TATCA could be used in margarine, mayonnaise, and salad dressings as a vegetable oil substitute. TATCA is not digested, but anal leakage and fatalities were observed in feeding experiments in rats (LaBarge 1988). The products and manufacturers of lipid-based fat replacers are shown in Table 17.4.

17.5 Safety and regulatory issues

Manufacturers of fat replacers must be aware of existing legislative requirements to obtain approval for the use of fat replacers as well as the labeling of foods. Safety of fat replacers are regulated under two FDA approval categories, GRAS and food additives. GRAS substances are defined as ingredients that are produced from common food components and generally recognized by experts qualified by scientific training or by scientific procedures to be safe under

Table 17.4 Types of lipid-based fat replacers

Trade name	Developer/manufacturer	Composition	Applications
Olestra	Procter & Gamble Co.	Sucrose with 6–8 fatty acids	Cooking/frying oil, baked products, margarine, processed meats, chips, crackers
Sucrose fatty acid esters	Mitsubishi-Kasei Food Corp. America, Inc., Crodesta Inc.	Sucrose with 1–3 fatty acids	Baked goods, margarine, shortenings
Caprenin	Procter & Gamble Co.	Caprocaprylobehenin, structured triacylglycerol	Chocolate, baked goods, margarine
Salatrim/Benefit	Nabisco Foods Group/Cultor Food Science	Structured triacylglycerol	Chocolate, baked goods, fillings, dressings
TATCA	CPC International Co.	Trialkoxytricarballylate	Mayonnaise, margarine, dressings
DDM	Frito-Lay, Inc.	Dialkyl dihexadecylmalonate	Mayonnaise, margarine
EPG	ARCO Chemical Co. CPC International Co.	Esterified propoxylated glycerols	Salad dressings, ice cream, mayonnaise, baked goods

conditions of its intended use (Middlekauff 1974; Warshaw and Franz 1996). GRAS category was established in 1958 and many new substances including fat replacers have been approved (Middlekauff 1974, 1989; Vanderveen 1994). There are two approaches to submit GRAS affirmation petition to the FDA. The first is a manufacturer's self-determination of the substance claiming as GRAS. The other is petitioning FDA to grant the ingredient as GRAS. The majority of fat replacers have received GRAS status by FDA (Warshaw and Franz 1996; Clydesdale 1997). The determination of the ingredients as GRAS is based on long history of safe use or scientific data that support the safety of the ingredients for specific applications (Warshaw and Franz 1996; Mattes 1998). Examples of fat replacers approved as GRAS are starches, gums, and microparticulated proteins.

Obtaining a new ingredient approval as a food additive is more complex than a GRAS affirmation petition. A food additive is defined as a substance the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of any food (Middlekauff 1974). Food additives, unlike a GRAS substance, cannot be added in the foods or marketed before the FDA approves their use. Therefore, a food additive petition requires submission of extensive research data on the new ingredient's safety and intended use. Only olestra has received food additive approval for a lipid-based fat replacer.

Fat replacers could substitute a significant proportion of fat in the diet and be consumed in gram quantities each day by some individuals, so their safety must be examined more carefully. Traditional safety evaluation methods are not adequate to evaluate the fat replacers because most other food additives are consumed in only 1–2 per cent of food products, whereas fat replacers are consumed in large amounts. Because of this, a specially designed safety evaluation program must be developed with the consideration of exact chemical structure, stability during production, and identification of degradative byproducts of fat replacers. Consideration also should be made in the toxicological, physiological, and nutritional testing (Artz and Hansen 1994; Borzelleca 1996). Safety testing of fat replacers should be conducted on animals and the results from animal testing could be confirmed in human studies. If the fat replacers are not absorbed, effects on gastrointestinal tract, colonic microflora ecology, toxicity of degraded metabolites, and laxative effects should be considered (Gershoff 1995; Mahungu *et al*. 2002). If the fat replacers are absorbed, their absorption and elimination pathway must be assessed.

Since dietary fats are a source of essential fatty acids and a carrier for fat-soluble vitamins, reduced consumption of traditional fats by consuming fat replacers could lead to a depletion of these nutrients. So the studies to investigate the long-term effects of fat replacers on the absorption or utilization of essential fatty acids and fat-soluble and water-soluble nutrients should be conducted. It has been shown that olestra-containing products lowered the serum concentration of carotenoids and fat-soluble vitamins (Westrate and van het Hof 1995; Schlagheck *et al*. 1997; Broekmans *et al*. 2003). With the exception of

olestra, few studies have been undertaken that relate consuming fat replacers to the nutritional status of dietary components. In addition, the effects of the interaction of the fat replacer or its metablite with orally dosed drugs also should be considered (Vanderveen 1993, 1994; Mahungu *et al*. 2002). The effect of fat replacers on selected population groups with health problems such as obesity, cardiovascular disease, and diabetes as well as with healthy people should be considered.

The majority of fat replacers approved by the FDA are GRAS substances because they are created from common food components of carbohydrate or protein, so the minimal requirement for safety testing was required. However, olestra has received food additive approval and more extensive research data including toxicological, clinical, nutritional, and gastrointestinal testing was required for the food additive petition.

Considering both the scientific literature and the FDA review process, the fat replacers that are currently available in the market are safe. As new fat replacers are developed, their safety and availability will be regulated by the GRAS or food additive petition process.

17.6 Future trends

Most health-care professionals believe that a high-fat diet is closely linked with the development of cardiovascular disease, obesity, and some types of cancer, so they continue to advise consumers to reduce fat consumption. Consequently, the message from health groups and government agencies has driven the public to consume foods that are low in fat and calories. Owing to the increasing demand for low-fat and low-cholesterol products, reduced-calorie and low-fat food markets showed dynamic growth and the market is expected to continue growing in the coming years. The potential for growth of low-fat foods depends on the palatability of the products. Marketing studies indicate that consumers will not compromise product quality: although they want a healthy product, if low-fat product is unacceptable in sensory aspect it will be rejected. Therefore, further development in fat replacement is needed to improve taste and texture of low-fat foods to be equally as good as full-fat counterparts. Recent research is being focused on the development of heat-stable fat replacers that are suitable for frying and baking with reduced calories while maintaining the taste and texture of traditional high-fat foods. Efforts are being made by food manufacturers to develop fat replacers that do not interfere with the absorption of nutrients or the utilization of drugs. Research is conducted to ascertain health and nutrition benefits of fat replacers for the people with cardiovascular disease, obesity, and diabetes. The labeling system of foods may be revised to give more information to consumers of health implication and benefits for fat replacers.

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Part IV

Starch and other functional ingredients

18

Starch in food diabetes and coronary heart disease

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18.1 Introduction: starch digestion and health

More than half of human energy consumption comes from starch. Different starchy foods are digested at different rates, which are related to the glucose and insulin responses they elicit. The major carbohydrate sources in a Western diet contain rapidly digestible starch. Consequently, many common starchy foods such as bread, breakfast cereals and potato products produce high glycaemic responses. There are strong indications that the large amounts of rapidly available glucose derived from starch and free sugars in the modern diet lead to periodic elevated plasma glucose and insulin concentrations that are detrimental to health in many contexts, including diabetes and coronary heart disease. Many recent epidemiological studies have shown a reduced risk of type 2 diabetes in persons having high intake of wholemeal products. This review will focus on the role of food structure, and possibilities to use structure engineering for developing cereal-based products with low starch digestibility.

There is not a consensus regarding the use of glycaemic index (GI) for guiding the food choice of people with diabetes or risk groups. Because GI is measured in individual foods, the ability to predict glycaemic responses to mixed meals is one area of discussion. There is also need for long-term clinical trials of low-GI diets in the prevention and treatment of relevant diseases.

18.2 How starchy foods are digested

18.2.1 Degradation in mouth

In the mouth, food is disrupted by chewing and salivary α-amylase. The ease of disruption of plant food depends on its integrity. Food eaten in large pieces without chewing can produce much lower blood glucose levels than the same foods chewed completely (Read *et al.*, 1986). Starch in foods is partly hydrolysed in the mouth to maltose, isomaltose and glucose (Linke *et al.*, 1997). Cariogenicity studies of starchy foods have shown that these sugars are converted by bacterial fermentation into acids. Acid formation can start very quickly after starchy food has interacted with the dental plaque (Linke *et al.*, 1997). Lingström *et al.* (1993) have shown that acid formation in plaque after chewing soft bread and potato chips was more intense than after intake of sucrose. Rice and pasta products decreased mouth pH very slightly. High-amylose starch gel did not decrease mouth pH (Vesterinen *et al.*, 2002), indicating a slower rate of hydrolysis.

Recent studies in our own laboratory have shown that some foods such as pasta and breads, are swallowed partly in very large pieces, 2–20 mm in diameter (Liukkonen *et al.* unpublished). Accessibility of salivary α-amylase to pasta starch is smaller than to wheat bread starch owing to the more compact structure of pasta, which explains the slower hydrolysis and degradation of pasta in the mouth.

18.2.2 Stomach and gastric emptying

In the stomach, food pieces are subjected to the action of pepsin, acid conditions and to the vigorous grinding action of gastric motility. Gastric emptying is affected by particle size (Thomsen *et al.*, 1994). The only exit from the stomach to small intestine is the polyrus, which allows food pieces less than 2 mm in diameter to exit. Before solid emptying can take place, the liquid must be emptied from the stomach (Houghton *et al.*, 1988). Gastric emptying half-time has been calculated for some food systems: for spaghetti 75 min and for mashed potato 35 min (Mourot *et al.*, 1988). Gastric emptying half-time correlates with blood glucose and insulin values (Mourot *et al.*, 1988). This is due to the fact that size reduction in stomach for foods, which leave the mouth as coherent and large particles, will take a longer time and the blood sugar values will be increased gradually.

In recent investigations large differences were observed in the particle size of different breads after mastification and treatments that mimic stomach phase (Liukkonen *et al.* unpublished). Rye breads remained as coherent, large particles (largest in the range 2–20 mm in diameter), whereas wheat breads, which still exist as large pieces after mastification, were ‘in the stomach conditions’ broken down to very small particles (< 3 mm in diameter). Investigations with healthy persons have shown that ryebreads produce clearly lower insulin index than white wheat breads (Juntunen *et al.*, 2002). Very acidic barley breads have

reduced the rate of gastric emptying in reference to white wheat breads (Liljeberg and Björck, 1996). Acid in such concentration will make the bread structure very firm. Also studies with pasta products have shown that after mastification and the stomach stage pasta residues exist in large pieces (Liukkonen *et al.*, unpublished).

Viscous polysaccharides often slow gastric emptying by reducing the movement of the liquid phase. Viscous polysaccharides have also been reported to speed the emptying of compact solids (Meyer and Doty, 1988). With some viscous polysaccharides, accelerated gastric emptying of liquids has been observed (Edwards *et al.*, 1987; Potkins *et al.*, 1991).

18.2.3 Digestion in small intestine

A high amount of rapidly digestible carbohydrates, including starch, is typical for the current Western diet. These carbohydrates are absorbed very quickly in the upper part of the small intestine. Shifting the absorption further down in the small intestine, while still maintaining the palatability of foods, is a major challenge for the food technologists.

The major part of starch digestion occurs in the small intestine by pancreatic α-amylase. Tissue integrity and other structural characteristics of foods, to be discussed in more detail further on, influence the rate of starch digestion. The products of digestion must move through the bulk phase to the mucous layer, and further to the brush border of the mucosal epithelium, where the final digestion to glucose takes place. The enterocytes lining the small intestine produce the amylolytic enzymes needed for the hydrolysis of oligosaccharides to glucose, and also are the sites of absorption.Viscous polysaccharides in the diet slow down the rate of carbohydrate and lipid digestion and absorption. One suggested main reason for the slower carbohydrate responses in the presence of viscous polysaccharides is the hindered mixing of luminal contents, causing retarded diffusion and contact of gastrointestinal enzymes and their substrates. High viscosity also causes reduced absorption of glucose because of retarded transport, as the high viscosity is suggested to increase the thickness of the unstirred layer on the absorbing surface (Edwards *et al.*, 1988; Lund *et al.*, 1989). The decreased mass transfer slows down the movement of starch hydrolysis products through the bulk phase to the brush border of the mucosal epithelium. Both soluble (Begin *et al.*, 1989) and insoluble (Satchitanandam *et al.*, 1990) dietary fibres have also been reported to increase mucin production, which increases in addition to that of fibre.

Oat β glucan has been reported to reduce postprandial glucose and insulin responses after an oral glucose load both in healthy (Braaten *et al.*, 1991, 1994; Hallfrisch *et al.*, 1995; Wood *et al.*, 1994) and diabetic (Braaten *et al.*, 1994; Tappy *et al.*, 1996) subjects. The effect has been observed both with isolated oat gum (Braaten *et al.*, 1991; Wood *et al.*, 1994) and with oat bran containing β-glucan (Braaten *et al.*, 1994; Tappy *et al.*, 1996). In both cases, the attenuated responses have been related to the viscosity caused by oat β-glucan. The action

of viscous, soluble polysaccharides is dependent on their concentration at each site of the gut, ionic environment, and shear rate, etc. Predicting the action of a polysaccharide on the basis of preingestion viscosity can be misleading (Edwards *et al.*, 1987).

18.2.4 Digestion in large intestine

Dietary fibre including resistant starch will enter from small intestine to large colon, and is subjected to fermentation by colonic bacteria. Carbohydrates and oligosaccharides are fermented to short-chain fatty acids (SCFA), acetic, propionic and *n*-butyric acid and gases (Cummings, 1995).

18.3 Factors affecting starch digestion, glucose and insulin response

18.3.1 Tissue integrity

A linear relationship has been found between the proportion of barley kernels in bread and the glycaemic response in humans (Liljeberg and Björck, 1994). When barley flours were milled to flour, the corresponding wholemeal bread produced equally high glucose and insulin responses, as did the white bread reference product. Similar results have also been shown with wheat, rye and oats. The kernels in breads are whole although they are softer due to processing and subsequent swelling in comparison to native grains. Wholemeal rye flour contains whole cell structures even after baking to bread (Autio *et al.*, 1997). The sizes of endosperm and aleurone tissue structures in wholemeal breads are in the range 250 μm to 1 mm. These sizes do not influence the gastric emptying times, since particles smaller than 2 mm are easily passed from the stomach without size reduction. Whole tissue structures originated from whole kernels or wholemeal flour in breads can decrease enzymatic hydrolysis due to limited accesibility.

Most legume products prepared by conventional cooking produce low glucose and insulin responses (Jenkins *et al.*, 1980; Brand *et al.*, 1990; Wolever, 1990). Great differences in GI (12–74) have been observed between different legume products (Jenkins *et al.*, 1988; Wolever *et al.*, 1990). The botanical origin has an effect and the processing, especially canning and mechanical disruption, produces higher GI values. Also in the case of legumes, the tissue integrity and softness of the product seem to be important factors.

18.3.2 Porosity

Many porous foods, such as puffed rice and wheat, corn flakes and corn chips, produce high glucose and insulin responses (Brand *et al.*, 1985). In very porous bread, starch granules were broken down during mastification, whereas in a very dense bread baked without yeast, some starch granules even after mastification

had a microstructure similar to the original bread (Autio *et al.*, 2003). White wheat bread has a very porous structure, because gluten has the ability to expand during proofing and to retain gases. Saliva will easily penetrate through the pores inside the pieces and hydrolysis of starch will begin in the mouth. Rye bread is much less porous, which might be a reason for its reduced insulin responses (Juntunen *et al.*, 2003b).

18.3.3 The continuous food matrix

After mastification, the pieces of white wheat bread are large, since the continuous gluten phase is still cementing the dispersed starch phase. Microstructural studies have shown that after mastification the pieces of white wheat bread are easily broken down by pepsin under conditions mimicking the stomach (Liukkonen *et al.*, unpublished). The low glycaemic response of spaghetti is due to the dense texture based on gluten (d'Emden *et al.*, 1987; Colonna *et al.*, 1990; Bornet *et al.*, 1990). High-temperature drying increases firmness and starch–protein interactions of pasta (Resmini and Pagani, 1983; Pagani *et al.*, 1986). In rye breads, the continuous matrix is composed of swollen starch granules, leached amylose and swollen cell walls. Saliva together with chewing can produce 'chewing gum'-type large particles from rye breads, which are not easily broken down.

18.3.4 Structural properties of starch

Native starch granules are generally digested slowly by α-amylase (Holm *et al.*, 1988). As a result of gelatinization, the *in vitro* rate of amylolysis increases dramatically (Holm *et al.*, 1988). It has been shown with wheat starch gels that the starch hydrolysis rate will decrease markedly when amylose leaches out of the granule (Slaughter *et al.*, 2000). It is possible that amylose, when located near the granule surface, also retards hydrolysis of amylopectin.

Resistant starch is defined as starch products that are not absorbed in the small intestine of healthy individuals (Asp, 1995). Resistant starches are divided into three different categories. Physically inaccessible starch is classified as type 1. Banana, potato and high-amylose maize contain high amounts of resistant starch (54.1–61.4 per cent; Englyst *et al.*, 1992). These starches are type 2. Retrograded amylose, which is found in cooled, cooked potato, bread and corn flakes, is identified as resistant type 3. Resistant starch content can be increased by prolonging the wet stage after cooking, and heating or freezing the cooked food.

Studies made with high-amylose maize starch gels at different starch concentrations and pure amylose and amylopectin gels showed that the higher the amylose content, the higher the gel rigidity and the lower the hydrolysis rate *in vitro* and *in vivo* (Vesterinen *et al.*, 2002; Autio *et al.*, 2002). The lowered rate of amylolysis can also be due to higher rigidity of high-amylose starch gels.

Incomplete gelatinization will have also firmness-increasing effect. A higher amylose content in starch granules will significantly increase the temperature of starch swelling. Studies with high-amylose maize starch (amylose content 70 per cent, Hylon VII) have shown that temperatures higher than 145 ºC are needed to break the granule structure (Vesterinen *et al.*, 2001).

18.3.5 Viscosity

An inverse highly significant linear relationship has been shown between the postprandial blood glucose and insulin responses and the viscosity of the liquid mixtures consumed (Wood *et al.*, 1994, 2000). The viscosity of β-glucan, as measured in an assay, simulating the physiological conditions, has also been emphasised in the case of oat bran-containing extruded breakfast cereals (Tappy *et al.*, 1996). The importance of viscosity has also been verified with other fibres or fibre analogues, such as guar, pectin, and methylcellulose (Jenkins *et al.*, 1978).

18.4 Analysing the health effects of foods: the use of glycaemic index (GI) and other measurements

18.4.1 The concept of the glycaemic index

When foods containing digestable carbohydrates are eaten, they cause a rise in blood glucose level, i.e. a glycaemic response.The concept of the glycaemic index (GI) was developed over 20 years ago (Jenkins *et al.*, 1981) to classify foods on the basis of their ability to increase the blood glucose level. This concept ranks individual foods according to their postprandial rate of carbohydrate digestion and absorption.

The GI is defined as the incremental area under the postprandial blood glucose curve (the change in blood glucose level 3 hours after a meal) after the consumption of 50 g (digestable) carbohydrates from a test food, divided by the area under the corresponding curve after a meal containing a similar amount of the reference food, normally white bread or glucose (Wolever *et al.*, 1991). Thus the reference is given the value 100, and the lower the response, the smaller the GI. When white wheat bread is used as a reference, the GI values are higher than when using glucose as reference. Recently, it has been suggested that white rice could be used as the reference food especially for the Asian population (Sugiyama *et al.*, 2003).

During the past 20 years the GI of a variety of foods has been determined, and summary tables of the GI of of over 750 different food items are available (Foster-Bowell and Brand Miller, 1995; Foster-Powell *et al.*, 2002). The GIs of starchy foods range from over 100 to as low as below 40 (with white bread as reference), with several potato and bread products having high values and unprocessed grains, pasta and legumes being in the lower range. The effect of low-GI foods on glucose and insulin balance has been shown to extend to the

next meal, so that foods eaten during dinner might influence the glycaemic response even at breakfast (Wolever *et al.*, 1988). Some low-GI foods eaten at breakfast (e.g. pasta) have been reported to maintain a net increment in blood glucose and insulin at the time of lunch, thus reducing postprandial glycaemia and insulinaemia (Liljeberg and Björk, 2000).

Recently it has been suggested that in addition to the slow-release properties, the dietary fibre content of a food would be important for the second-meal effect. A low-GI (53) barley meal rich in dietary fibre eaten in the evening improved glucose tolerance at breakfast, whereas an evening meal with pasta (GI 54) had no effect (Björk and Liljeberg, 2003). The responsible factors for the extended effect remain to be defined, but obviously it is beneficial that low-GI cereal foods would also be rich in dietary fibre.

Glycaemic index has in numerous studies been associated with reduced risk of developing diabetes and cardiovascular disease, as reviewed recently by Augustin *et al.* (2002), Jenkins *et al.* (2002a, b), Ludwig (2002a, b), and Willet *et al.* (2002). Beneficial effects have also been shown with respect to insulin resistance, blood lipids, satiety and obesity. The mechanisms of action will be further discussed later in this chapter. The use of GI for guiding the food choice of people with diabetes and/or at risk is, however, still a contraversial issue (Ludwig and Eckel, 2002). Because GI is measured in individual foods, the ability to predict glycaemic responses to mixed meals is one area of discussion. There is also need for long-term clinical trials of low-GI diets in the prevention and treatment of relevant diseases.

18.4.2 Insulin index

Insulin secretion is largely assumed to be proportional to postprandial glucose responses. The extent to which other food characteristics affect postprandial insulinaemia is less well known. Other nutrients have also been suggested to influence the overall level of insulinaemia (Holt *et al.*, 1997). For example protein may have an insulinotropic effect, increasing the uptake of circulating glucose (van Loon *et al.*, 2000).

Some foods, such as high-amylose rice (Van Amelswoort and Weststrate, 1992; Goddard *et al.*, 1984) and rye breads (Leinonen *et al.*, 1999; Juntunen *et al.*, 2003b) decrease insulin responses of healthy persons, but have small or no effect on glucose responses. Consumption of barley-containing pasta with β-glucan also led to more blunted insulin response than after wheat pasta in healthy men, although the plasma glucose did not differ significantly (Bourdon *et al.*, 1999). The insulin index has been suggested as a concept for dietary management for people with diabetes (Holt *et al.*, 1997). Further research is needed to understand better the influence of food quality on postprandial insulinaemia, and the usefulness of the concept in relation to the treatment of metabolic syndrome.

18.4.3 Comparing foods using GI and GL

As stated above, the GI was introduced to compare foods at a standard dose (50 g) of absorbable carbohydrates. As not only the availability and absorption rate, but also the content of carbohydrates are important for the postprandial fluctuations of blood glucose, the concept of glycaemic load (GL) was introduced and proved useful in epidemiological studies (Salmeron *et al.*, 1997a,b; Liu *et al.*, 2000a). GL, calculated by multiplying the GI of a food with its amount of total dietary carbohydrate per serving, assesses the total glycaemic effect of the diet (Fig. 18.1). In the recent international tables of GI, the corresponding GLs per serving have been listed, calculated by multiplying the amount of carbohydrate in a typical portion and dividing by 100 (Foster-Powell *et al.*, 2002). The GL is thus the best estimate for the expected postprandial elevation of blood glucose and insulin. Its relevance to the prevalence of chronic diseases has just been reviewed by Brand-Miller *et al.* (2003). Some examples of GI and GL of some cereal products are shown in Table 18.1.

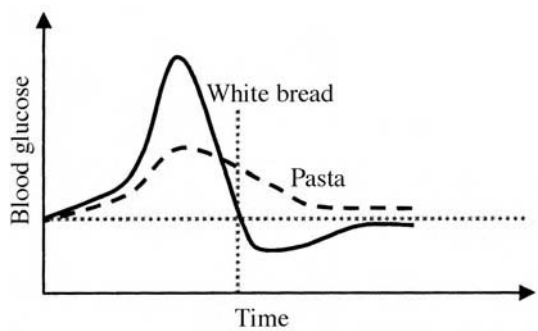

Fig. 18.1 A schematic presentation of the blood glucose response after digestion of white bread and pasta.

18.4.4 *In vitro* methods

The rate of starch digestion in foods has been investigated by *in vitro* hydrolysis using alimentary amylolytic enzymes (Jenkins *et al.*, 1980; Snow and O'Dea, 1981; Bornet *et al.*, 1989). An *in vitro* method based on chewing was developed by Granfeldt *et al.* (1992), to predict the metabolic responses to different starchy foods. An equivalent amount of potentially available starch of the test foods was chewed *in vivo* prior to *in vitro* hydrolysis with pancreatic alpha-amylase in a dialysis tube for 3 hours. A hydrolysis index (HI) was calculated as the area under the hydrolysis curve of the test food as a percentage of the reference (white wheat bread). By using 17 food items the authors obtained a good correlation between GI and HI ($r = 0.877$, $P < 0.001$) and between the insulin index (II) and HI ($r = 0.647$, $P < 0.01$). The authors also emphasised that this method is not suitable for ranking foods having differences in the rate of gastric emptying.

Table 18.1 Examples of GI values of cereal-based products

Sample	GI (wheat bread =100)	GL
Rye kernels	42–56	11–15
Wheat kernels	43–69	11–16
Parboiled rice, low amylose	73–124	19–34
Parboiled rice, high amylose	39–69	11–19
Commercial muesli	61–94	7–17
Cornflakes	103–123	18–24
All-bran	43–72	4–9
Oat porridge	76–106	14–25
Spaghetti	38–97	14–28
Pumpernickel rye bread	58–78	5–7
Commercial rye bread	79–123	6–12
Spelt wheat bread	77–105	9–17
Barley bread	76-100	9–14
Wheat biscuits	81–103	9–15
Cookies	40–113	6–23
Crackers	79–124	9–19

Source: Foster-Powell *et al*. (2002).

Another similar *in vitro* starch hydrolysis method was also shown to have a good correlation with the *in vivo* GI assay (Goni *et al.*, 1996). Different cereal, legume and vegetable foods were studied, and the HI values were calculated. Starch hydrolysis at 90 min correlated even slightly better ($r = 0.909$, $P < 0.05$) with *in vivo* glycaemic responses than the HI values ($r = 0.894$), and was suggested as a simpler way of predicting the GI.

Analytical methods have been developed to classify starch to rapidly available glucose (RAG) and slowly available glucose (SAG) (Englyst *et al.*, 1999, 2003).The enzymatic procedure included incubation with both pepsin and and a mixture of amylolytic enzymes, and pH, temperature, viscosity and mechanical mixing were adjusted to mimic the gastrointestinal conditions (Englyst *et al.*, 1999). It was concluded that these parameters can explain the GI and II values of the cereal foods studied (Englyst *et al.*, 2003). SAG and fat content together accounted for 73.1 per cent of the variance in GI, and RAG and protein content together 45.0 per cent of the variance in II.

The chewing/dialysis method does not mimic the events of gastric emptying. A method has been developed that would better take into account the particles size of the chewed food, and the gastric emptying (Liukkunen *et al.* unpublished). The samples are chewed in the mouth (four persons). Then the masticated residues will be treated with pepsin, mimicking the stomach conditions (mixing, acid, pepsin, +37 ºC). After dilution, the particles are photographed by camera and the particle size distribution is determined from all the masticated material by image analysis. This method gave good correlation with II (Liukkonen *et al.* unpublished). The particles originating from pasta were clearly largest, and those of normal wheat bread smallest.

18.5 Starch digestion, diabetes and cardiovascular disease: the metabolic syndrome

The metabolic syndrome as a concurrence of disturbed glucose and insulin metabolism, overweight and abdominal fat distribution, dyslipidaemia and hypertension constitutes a major threat to public health because of its association with increased risk of type 2 diabetes mellitus and cardiovascular disease. The hallmark of the syndrome is resistance to biological effects of insulin in target tissues, and is also known as the insulin resistance syndrome (Reaven, 1988). The ultimate pathogenesis of this syndrome is unknown, but obesity and sedentary lifestyle in association with a Western type of diet and genetic factors clearly interact to produce it. With increasing occurrence of overweight and obesity, the problem of the metabolic syndrome as a threat to public health will increase.

Despite the abundant research that has been published on the metabolic syndrome, definitions of the metabolic syndrome have been numerous. The World Health Organization (WHO, 1999) consultation for the classification of diabetes and its complications, and the National Cholesterol Education Program Expert Panel (2001), have recently published definitions of the metabolic syndrome. The manifestations of cardiovascular risk factors such as dyslipidaemia, hypertension, endothelial dysfunction, inflammation, hypercoagulability and impaired fibrinolysis, obesity and abnormal insulin and glucose metabolism predispose persons with the metabolic syndrome to another major consequence of the metabolic syndrome, cardiovascular disease. In the Finnish population-based prospective study applying defined criteria of metabolic syndrome the overall mortality was also nearly two times higher in men with the metabolic syndrome (Lakka *et al.*, 2002). Adjustment for conventional and non-conventional risk factors that were not part of the definition of the metabolic syndrome did not attenuate the risks.

The pathogenesis of the metabolic syndrome is poorly understood. Skeletal muscle is a major determinant of whole-body glucose disposal and the defects in insulin signalling in muscle tissue contribute to lowered insulin-stimulated glucose uptake (Kelley and Mandarino, 2000). Further, an abdominal distribution of fat is particularly deleterious. Abdominal fat can also be divided into subcutaneous and visceral compartments. Abdominal obesity has been hypothesized to mediate its deleterious effects on carbohydrate and lipid metabolism through the increased lipolytic activity of especially omental fat, which drains directly into the portal-venous system (Björntorp, 1991). This in turn results in higher non-esterified fatty acid concentrations, with consequent insulin resistance in the liver and skeletal muscle. In addition to abdominal subcutaneous and visceral fat, the lipid accumulation in skeletal muscle and liver has also been shown to be powerful determinants of insulin sensitivity (Ravussin and Smith, 2002).

As the metabolic syndrome becomes more severe, interplay between genetic susceptibility, insulin resistance and dietary patterns may lead in susceptible

individuals to progressive β-cell failure and impaired insulin secretion capacity. As β-cell function declines, impaired glucose tolerance (IGT) develops (Kahn, 2003). Roughly 5–10 per cent of persons with IGT convert to type 2 diabetes yearly (Edelstein *et al.*, 1997). With time, hyperglycaemia leads to further loss of pancreatic β-cell function. It has not been fully resolved whether this loss of pancreatic function results primarily from excessive secretion of insulin (i.e., β-cell exhaustion) or toxicity to β-cells because of hyperglycaemia. However, from this mechanism one may anticipate that a diet that produces higher plasma glucose concentrations and greater demand for insulin would increase the risk of type 2 diabetes. By definition, high-GI forms of carbohydrate produce high concentrations of plasma glucose and increased insulin demand and may therefore contribute to an increased risk of type 2 diabetes. The individual response to a given carbohydrate load is influenced by the degree of underlying insulin resistance, which is, in turn, determined primarily by degree and type of adiposity, physical activity, genetics and other aspects of diet. Thus, it might be expected that the adverse metabolic effects of high-GI foods would be pronounced in sedentary, overweight or genetically susceptible persons and be quite modest in healthy graduate students participating frequently in metabolic studies.

One of the problems with the GI concept is that the insulin response is not proportional to the glucose response. Holt *et al.* (1997) compared the effect of isoenergetic amounts of foods on the insulin secretory response and found that the postprandial insulin responses were not closely related to the carbohydrate content or to the glycaemic effects of the foods. Whereas the glycaemic response was a significant predictor of the insulin response, it accounted for only 23 per cent of the variability in the insulinaemia. This is in line with our experience in which in healthy subjects with normal glucose tolerance there was no difference in the glycaemic responses between wheat or different rye breads, whereas insulin responses of rye breads were markedly different so that the same amount of carbohydrate required less insulin. Therefore it seems that in healthy persons the use of GI is hampered by many difficulties, and insulin responses could be used instead. However, even if the total glucose responses did not differ between the different types of breads (Juntunen *et al.*, 2002), the shapes of the glucose curves showed interesting patterns – after 3 hours from ingestion of the wheat bread plasma glucose levels had declined below the fasting level whereas after rye bread ingestion glucose levels were still above fasting level. The rapid decrease of blood glucose may increase the feeling of hunger and may cause more frequent eating.

18.6 The role of low-GI carbohydrates in treating and preventing disease

Recent nutritional guidelines usually recommend high intakes of carbohydrate. The main reason was that high-fat diets enhanced the development of

cardiovascular diseases. Recently, it has been acknowledged that the type of fat is at least as important as the quantity of fat. It seems that we are encountering the same phenomenon as regards to type of carbohydrates in the diet; the quality is at least as important as quantity. Epidemiological studies addressing the association of dietary factors with the metabolic syndrome using the WHO or NCEP definitions have not yet been published and there is an urgent need for controlled randomized studies in these group of subjects. However, the large cohort studies assessing the association of incidence of diabetes mellitus, or cardiovascular disease nonetheless suggest that a diet that has a low glycaemic index, high in fibre or high in fruit and vegetable intake may decrease the risk for obesity, type 2 diabetes or cardiovascular disease and its risk factors. Herein we review the effects of low-GI diets and their effects on the treatment of overt diabetes, prevention of type 2 diabetes, obesity and serum lipoproteins.

18.6.1 The low-GI carbohydrates in the treatment of diabetes

Low-GI carbohydrates may have acute effects on the clinical management of people with diabetes, e.g. patients treated with short-acting prandially administered insulin analogues may need to inject their insulin after eating. However, the main goal of treatment of diabetes is to prevent long-term complications by improving metabolic control, which is commonly assessed by glycated proteins, such as fructosamine or haemoglobin A1c. In studies conducted mostly in patients with type 2 diabetes the low-GI diets have shown an improvement of 10 per cent as compared to high-GI foods (FAO/WHO, 2003). This effect is clinically significant and comparable to the effects of monounsaturated fat compared with high-carbohydrate diets.

Recently, a meta-analysis identified 14 studies, comprising 356 participants, that met strict inclusion criteria and that showed that low-GI diets reduced HbA1c by 0.43 per cent points above that produced by high-GI diets (Brand-Miller *et al.*, 2003**)**. Taking both HbA1c and fructosamine data together and adjusting for baseline differences, glycated proteins were reduced 7.4 per cent more on the low-GI diet than on the high-GI diet. Therefore, choosing low-GI foods in place of conventional or high-GI foods has a small but clinically useful effect on medium-term glycaemic control in patients with diabetes and the benefit is similar to that offered by pharmacological agents that also target postprandial hyperglycaemia.

In a carefully conducted Swedish study (Järvi *et al.*, 1999) in patients with type 2 diabetes the baseline diet for both study groups was in line with the current recommendations but differed in GI. Although both diets improved insulin sensitivity and serum lipids, food modification by lowering GI about 30 per cent resulted in lower LDL-cholesterol, glucose and insulin responses. Low-GI has been shown to have beneficial effects on LDL-cholesterol levels in patients with type 2 diabetes during weight-losing diets (Heilbronn *et al.*, 2002). In subjects with type 1 diabetes EURODIAB study showed that low-GI was

associated with better metabolic control, more favourable lipoprotein pattern and smaller waist circumference (Buyken *et al.*, 2001).

18.6.2 The low-GI carbohydrates in the prevention of type 2 diabetes

Findings from epidemiological studies suggest that total carbohydrate intake is not associated with increased risk of type 2 diabetes (Feskens *et al.*, 1995; Salmeron *et al.*, 1997a, b; Meyer *et al.*, 2000) whereas the intakes of whole grain cereal products, such as ryebread, have been shown to reduce the risk (Meyer *et al.*, 2000; Liu *et al.*, 2000b; Montonen *et al.*, 2003), and highly refined grain products such as white wheat bread and white rice to increase the risk (Salmeron *et al.*, 1997a, b). The results are conflicting concerning the risk increase due to refined grain products (Liu, 2003).

Multifactorial prevention of type 2 diabetes including increasing fibre intake as a component has been shown to prevent the development of type 2 diabetes. In the Finnish Diabetes Prevention Study (DPS) 523 obese persons with IGT were randomized in five centres into an intervention and control group. The study was stopped prematurely after consultation with an expert panel because of highly significant difference in the incidence of diabetes between the intervention and control groups (Tuomilehto *et al.*, 2001). During the trial the risk reduction of diabetes was 58 per cent. When the results were analysed according to the success score, none developed diabetes in either group if they achieved four or five intervention goals (weight loss at least 5 per cent, physical activity at least 4 h/week, fibre intake > 15 g/1000 kcal, intake of total fat less than 30 per cent of energy and that of saturated fat less than 10 per cent of energy).

What are the theoretical mechanisms by which low-GI foods prevent development of type 2 diabetes? The fundamental defects are insulin resistance and impaired β-cell function. In a study by Frost *et al.* (1996) women were randomly assigned to consume high- or low-GI diets for 3 weeks. Insulin resistance measured *in vivo* and in cultured adipocytes was greater in women consuming the high-GI diet. The adverse effects of the high-GI diet appeared to be due to an increased production of free fatty acids in the late postprandial state. In a study by Kiens and Richter (1996) an increase in insulin resistance was not found in seven healthy, lean young men after the subjects had consumed a high-GI diet. It should be noted that subjects were quite healthy and had normal insulin sensitivity, emphasizing the concept that metabolic effects of GI are difficult to observe in healthy population. A recent crossover study involving 11 overweight subjects showed that insulin sensitivity measured by the euglycaemic hyperinsulinaemic clamp improved after subjects consumed a whole-grain diet compared with a refined-grain diet for 6 weeks, independent of body weight (Pereira *et al.*, 2002). Thus there is suggestive evidence of relationship of GI with insulin resistance but further studies are required.

β-Cell failure may be induced by high-GI foods by repeated postprandial hyperinsulinaemia leading to its overstimulation and exhaustion (Ludwig,

2002b; Grill and Bjorklund, 2001). In a crossover study of six healthy adults, Jenkins *et al.* (1987) found that a low-GI diet containing mainly intact whole grains reduced C-peptide concentrations (a 32 per cent reduction) compared with a high-GI diet containing primarily refined grain products. Recently, we showed that long-term ingestion of rye bread in elderly women enhanced acute insulin response to intravenous glucose stimulus (Juntunen *et al.*, 2003a), which could be a novel mechanism for the effects of low GI on glucose and insulin.

18.6.3 The low-GI carbohydrates and obesity

In theory, low-GI foods may benefit weight control by promoting satiety and fat oxidation at the expense of carbohydrate oxidation. Most studies have suggested that low-GI meals increase fullness to a greater extent than do comparable high-GI meals (Ludwig, 2002a). This may be due to 'ups and downs' or hyperglycaemic and hypoglycaemic effects of high-GI foods that could partly explain the lower satiety observed in the postprandial period. Further, prolonged transit time in the gastrointestinal tract may have a prolonged stimulation of receptors signalling to central satiety centres. Slabber *et al.* (1994) studied 30 obese females with two hypocaloric diets (high-GI versus low-GI) in a 12-week study that was followed by a 12-wk crossover study. Both diets produced weight loss during the first 12 weeks (9.4 compared with 7.4 kg, $p = \text{NS}$). During the follow-up crossover study, the low-GI diet produced greater weight loss (7.4 compared with 4.5 kg; $P = 0.04$) than did the high-GI diet. The further satiety mechanism may be due to the second-meal effect; studies with variable GI and load have suggested that the lower the glycemic index and load of the first meal, the less food is consumed in the subsequent meal (Wolever *et al.*, 1988; Liljeberg and Björck, 2000).

Low-GI foods may also have relevance with fuel oxidation. Postprandial rises in glucose and insulin concentrations increase carbohydrate oxidation and decrease fatty acid oxidation but during later postprandial phases increased release of counter-regulatory hormones restores euglycaemia and elevates free fatty acid (FFA) levels. Increased availability and oxidation of fatty acids may in turn decrease carbohydrate oxidation (Jenkins *et al.*, 2002b). Whether high-GI diets, which induce chronic hyperglycaemia and hyperinsulinaemia, can reduce the body's capacity to oxidize fat and significantly increase body fat storage remains unresolved. There is an urgent need for randomized, controlled, multicentre intervention studies comparing the effects of conventional and low-GI diets on body weight, composition and fuel metabolism to confirm or cancel the hypothesis that the faster digestion and absorption and higher insulin responses after high-GI meals affect in different ways satiety and energy partitioning that over the long term favour expansion of the fat stores.

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18.6.4 The low-GI carbohydrates and the serum lipids and lipoproteins

High carbohydrate consumption is associated with increased serum triglyceride and low HDL-cholesterol levels, both of which are hallmarks of the metabolic syndrome and increase the risk of cardiovascular diseases. Serum triglycerides change more rapidly in metabolic studies, whereas epidemiological studies have shown an association between low HDL-cholesterol and GI.

A retrospective analysis of 7-day weighted records of 1420 British adults performed in 1986–1987 and showed a significant negative correlation between serum HDL cholesterol and GI ($P < 0.0001$ for women, for men $P = 0.02$) (Frost *et al.*, 1999). The difference in the HDL-cholesterol between the highest and lowest GI quintiles was 0.25 mmol/L, which is a clinically significant difference. Data from United States of 14 000 subjects showed consistent findings in this regard: dietary GI and plasma HDL-cholesterol concentrations showed an inverse association and the differences in HDL-cholesterol concentrations between the lowest and highest GI quintiles was 0.10 mmol/L (Ford and Liu, 2001).

There has been an enormous amount of work concerning the impact of longer-term consumption of carbohydrate-rich diets on blood lipids, which has been reviewed recently (Parks and Hellerstein, 2000). When the content of carbohydrates is increased above 50 per cent of the daily intake of energy at the expense of fat, and especially monounsaturated fat, it has been repeatedly shown to induce hypertriglyceridaemia coupled with lowered HDL-cholesterol. Even if the mechanisms for hypertriglyceridaemia are unresolved, it has been suggested to result from the overproduction of both VLDL triglycerides and VLDL particles together with impairment of VLDL clearance.

However, if high-carbohydrate diets, which are composed of natural foods, have been rich in starchy unrefined and whole products, they have not shown elevations in triglyceride concentrations as compared with high-fat, low-carbohydrate diets. In a recent meta-analysis of 11 studies triglycerides and total cholesterol were reduced on average by 9 and 6 per cent, respectively, coupled with improvements in glycaemic control and insulin sensitivity when low- *vs*. high-GI diets were compared. While changing only around half the carbohydrates from conventional foods to low-GI ones, which is around 25–30 per cent from dietary calories, measurable gains have been observed (Miller, 1994). It is therefore appealing to suggest that intake of low-GI foods overcomes the adverse changes in lipid metabolism induced by high-carbohydrate diets, and offers a practical means to treat and prevent diseases linked to metabolic syndrome.

18.6.5 The low-GI carbohydrates and cardiovascular diseases

The Nurses Health Study, consisting of 10 years follow-up on 75 000 women aged 38–63 years, showed that a high glycaemic load from dietary carbohydrates increases risk of coronary heart disease (Liu *et al.*, 2000a). The glycaemic load was obtained by using a food-frequency questionnaire. During the follow-up, 763 myocardial infarctions occurred. Defined glycaemic load was

associated with risk of myocardial infarction after adjustment for other risk factors, like age, smoking, total energy intake, hypertension and dyslipidaemia. The relative risk of myocardial infarction and glycaemic load was most pronounced in overweight women. However, this large study has been criticized for the food-frequency data used (Pi-Sunyer, 2002).

18.6.6 The low-GI carbohydrates and other health implications

Low-grade inflammation is a rather novel characteristic of obesity, metabolic syndrome and atherosclerosis (Ridker *et al.*, 2003). The GI may have a role in the liberation of proinflammatory cytokines and acute phase proteins and a high-GI diet may induce the generation of reactive oxygen species and lowering of serum antioxidants, thus contributing to oxidative damage (Jenkins *et al.*, 2002b). The relation of GI to cancer has been also been suggested (Terry *et al.*, 2003). Mental performance should also be a subject of further studies and recently it has been shown in elderly people with type 2 diabetes that acute ingestion of high-GI carbohydrate contributes to the memory impairment (Greenwood *et al.*, 2003).

18.7 The manufacture of cereal-based products that produce low blood postprandial insulin responses

Findings from different studies of diverse populations suggest that intake of whole-grain products can lower the risk of type 2 diabetes (Salmeron *et al.*, 1997a, b; Meyer *et al.*, 2000; Liu *et al.*, 2000b; Fung *et al.*, 2002; McKeown *et al.*, 2002; Montonen *et al.*, 2003). There is a need to develop greater variation of low-GL commercial cereal-based products to the market, but the greatest challenge is the management of good perceived texture. If a product contains fibre, the starch content will be lower and the blood glucose increasing potential lower than without fibre. Breads that have beneficial effects on glucose and insulin responses can be baked by either adding fibre or by lowering rate of starch digestibility.

18.7.1 Cereals containing high-amylose starches

Mutations that increase the ratio of amylose to amylopectin in starch-containing plants can be used for developing new cereal-based ingredients that induce low glycaemic responses. Maize varieties homozygous for the amylose extender or '*ae*' gene, with amylose levels between 50 and 80 per cent, have been commercially available since the 1950s (Vineyard *et al.*, 1958). High-amylose barley, maize and rice have been studied in relation to digestibility *in vitro* and *in vivo*.

A lower glycaemic and insulin index and higher content of resistant starch have been reported for a barley bread baked from a high-amylose barley genotype by using long-time/low-temperature baking conditions (Åkerberg *et*

al., 1998). Recent studies have shown that breads baked from high-amylose wheat flour had significantly lower loaf volume than breads baked from normal flours (Morita *et al.*, 2002). Concerning bread products the great challenge is to combine low starch digestibility with good sensory characteristics (Autio *et al.*, unpublished).

In many studies rice products based on high-amylose varieties were shown to lower GI (Goddard *et al.*, 1984; Juliano and Goddard, 1986; Miller *et al.*, 1992). A study including 12 different rice products, found lower GI and II only for high-amylose rice products. With cooked rice, hardness increased and stickiness decreased with an increase in the amylose content (Kohyama *et al.*, 1998). Hardness is also greatly dependent on the degree of cooking, and water content. High amylose rice is a potential new raw material, as amylose also improves the textural properties of cooked rice products, such as rice noodles (Yoenyongbuddhagal and Noomhorn, 2002).

High-amylose maize is available as a cereal grain and as a starch. Reduced postprandial responses of glucose and insulin in healthy subjects following ingestion of crackers made from high-amylose maize starch compared with a corresponding product made from low-amylose starch has been reported (Behall *et al.*, 1988). Autoclaved high-amylose maize starch when incorporated in hot mixed lunches had beneficial effects on glucose and insulin responses (Van Amelsvoort and Weststrate, 1992).

18.7.2 Soluble fibres

The use of soluble fibres as ingredients for low-GI foods presumes that they are soluble and have high viscosity, which can be achieved by combining high-molecular weight gum with low concentration or low-molecular weight gum with high concentrations. Oat and guar gum are possible candidates for low GI foods. Brennan *et al.* (1996) have shown that the rate of starch hydrolysis was retarded significantly when the starch granules and surrounding bread matrix were coated with a layer of galactomannan. It is easier to solubilize gums with higher amounts of water and thus foods, such as drinks, soups and sauces could be more suitable than foods with lower water content. With an oat β-glucan preparation of high-molecular weight, the maximum practicable concentration of β-glucan in the soup was 0.5 per cent (Lyly *et al.*, unpublished). With a lower molecular weight preparations of oat and barley, it was possible to add up to 2 per cent β-glucan to the soup. The viscosities of 0.5 per cent high molecular weight oat, 2 per cent lower molecular weight oat and barley, at $50\,s-1$ were 347, 1575 and 343 mPa s respectively. From the point of view of flavour release, barley β-glucan was better than oat.

18.7.3 Texture management by processing tools

Bran supplementation or use of wholemeal flours that contain bran usually weakens the bread volume, loaf structure and mouthfeel (Rao and Rao, 1991;

Zhang and Moore, 1999; Salmenkallio-Marttila *et al.*, 2001). Prefermentation of wheat bran with yeast and with yeast and lactic acid bacteria improved the loaf volume, crumb structure and shelf-life. The bread had good flavour and homogeneous pore structure. A combination of commercial baking enzymes also had a positive effect on the volume, structure and flavour of bread containing wheat bran (Salmenkallio-Marttila *et al.*, unpublished). The best result was obtained by combining the use of prefermentation and addition of baking enzymes. In another study, three different wholemeal breads (60 per cent wholemeal flour on the basis of flour weight) baked from oat, rye and wheat were fed to 15–20 healthy subjects, and their II and GL were determined (Autio *et al.*, unpublished). The texture of wholemeal breads was improved by gluten addition in order to improve sensory characteristics of the breads. All wholemeal breads had an II about the same or slightly higher than normal wheat bread. All breads contained 5.9–7.0 per cent fibre, their GL were for oat 9.7, for rye 10 and for wheat 11 in comparison to 14.2 obtained for normal wheat bread.

18.7.4 Structure engineering of protein matrix

In a preliminary study (only five breads), a very good correlation was obtained between bread hardness and insulin index (Autio *et al.*, unpublished). Bread hardness reflects many properties, such as porosity, hardness of continuous and dispersed phases. Bread hardness can be increased by cross-linking enzymes, such as transglutaminase (Gerrard *et al.*, 1998). Transglutaminase increases gluten fibre interactions and elasticity of dough, and as a result harder and less porous crumb structure will be formed (Autio *et al.*, 2003). The palatability of these types of breads can be increased by better taste (e.g. longer fermentation) and by optimizing the moisture content.

18.8 References

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19

The use of cereal beta-glucans to control diabetes and cardiovascular disease

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19.1 Introduction: the health benefits of soluble fibre/beta-glucans in cereals

As early as 1913 it was reported that intake of oats could be beneficial in the treatment of diabetes (Allen 1913). A positive effect of oats in the control of cardiovascular disease was demonstrated in the early 1960s, when subjects were given rolled oats incorporated in bread, which led to decreased blood cholesterol levels (DeGroot *et al.* 1963). Since then many human studies have confirmed these health effects and suggested that they are related to the soluble fibre in oats, the beta-glucans. Another cereal rich in beta-glucans is barley, while wheat and rye contain lower amounts. Suggested mechanisms for the physiological effects are that the beta-glucans delay the intestinal absorption of glucose and lipids and inhibit the absorption and reabsorption of cholesterol and bile acids (Anderson & Bridges 1993). The reduced absorption is probably mainly due to the high viscosity of beta-glucan solutions which increases the viscosity of the contents in the intestine (Mälkki 2001). Other factors can also be important. One of these relates to the fermentation of the beta-glucans in the colon during which short-chain fatty acids are produced, and these could have different physiological effects.

The interest in using cereal beta-glucans as an ingredient in foods was increased when a health claim was approved by US Food and Drug Administration (FDA, 1997) that 'a diet high in soluble fibre from whole oats and low in saturated fat and cholesterol may reduce the risk of heart disease'. The recommended minimum dosage is 3 grams of beta-glucan per day.

In this chapter the development of new food products enriched with beta-glucan are discussed. First, current limitations in using beta-glucans are

presented. Different available beta-glucan concentrates are thereafter reviewed and the documentation behind the effects on the lipid and glucose metabolism is evaluated. At the end of the chapter future foods enriched with beta-glucan and new techniques (nutrigenomics) to document the health effects are discussed.

19.2 Current limitations in using beta-glucans as food ingredients

19.2.1 Beta-glucans in whole kernels and bran

The oat and barley hull remains attached to the kernel during threshing. Oat hulls are the most concentrated source of fibre (80–95 g/100 g) but these fibres lack soluble dietary fibre and beta-glucans (Vollendorf & Marlett 1992). The hull is therefore normally removed mechanically before oats and barley can be used for human consumption. The remaining kernel is similar to other common cereals in general morphology. An oat kernel consists of about 12 per cent bran (pericarp, testa, aleurone), 84 per cent starchy endosperm and 4 per cent germ (Kent 1983).

The nutritional composition of oat kernels from cultivars grown in Sweden over 3 years has been investigated (Asp *et al.* 1991). On average, the kernels contained 15.9 per cent protein, 7.0 per cent fat and 63.2 per cent starch (percentage of dry matter). The samples contained 9.7 per cent (5.0−13.4) total dietary fibre and 3.5 per cent (2.0−5.0) soluble dietary fibre (mean and range).

Both oats and barley are rich sources of beta-glucans. In different oat varieties harvested in Sweden in three different years the beta-glucan content varied from 3.5 to 5.7 per cent of the dry matter (Asp *et al.* 1991). Barley normally contains beta-glucans in about the same amount as oats (3.5−5.9 per cent of the dry matter; Oscarsson *et al.* 1998) but higher amounts have been detected in some varieties (up to 11 per cent). Beta-glucans are linear polymers composed of glycosyl residues linked via a mixture of β-(1→3) and β-(1→4) linkages (Fig. 19.1). The beta-glucan in oats and barley are similar in structure but differences in the ratio of β-(1→3) and β-(1→4) linkages, molecular weight and possibly solubility have been reported (Wood and Beer 1998). The oat beta-glucan molecular weight can reach 3×10^6 while the molecular weight for barley beta-glucans is usually lower ($2–2.5 \times 10^6$).

The beta-glucans are the main component in the endosperm walls. The distribution of beta-glucans in the kernel of hull-less barley was investigated by Zheng *et al.* (2000). The beta-glucan content was very low in the outermost 20 per cent of the kernel for all varieties. For low beta-glucan varieties, the beta-glucan content was largest in the subaleurone region and declined slightly toward the inner layers. For high beta-glucan varieties, the beta-glucan was more evenly distributed throughout the kernel. The same beta-glucan distribution has also been found for oats using microspectrofluorometry (Miller & Fulcher 1994). Comparing central cross-sections of five cultivars of differing beta-glucan content (3.7−6.4 per cent), there was a trend for the high concentration

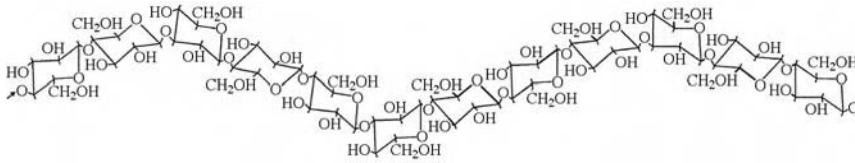

Fig. 19.1 The structure of mixed linked (1→3) (1→4)-β-D-glucan (beta-glucan).

of beta-glucan in the subaleurone layer to become less distinct as the total beta-glucan content of the cultivars increased. Åman *et al.* (1989) showed that cultivar, stage of development and growing conditions influence the beta-glucan content while storage time has little effect.

Oat bran is a product where the beta-glucans have been concentrated by sieving. It is defined as an oatmeal material with a beta-glucan content of at least 5.5 per cent and a total dietary fibre content of at least 16.0 per cent (dry matter basis; AACC 1989). Oat bran has thus normally a higher content of fibre than oat kernels but both contain relatively similar amounts of various fibre components (Vollendorf & Marlett 1992).

19.2.2 Important properties of beta-glucans

If barley or oats is to be incorporated into food systems, it is important to ensure that the beta-glucans are intact and have not lost their viscosity during the food preparation, processing or storage. The viscosity of beta-glucans is mainly determined by their concentration and molecular weight and the larger the molecular weight, the higher the viscosity (Autio *et al*. 1992; Wood *et al.* 2000). The linear structure of beta-glucans is, however, very susceptible to degradation during processing and parameters that can affect the beta-glucans are temperature, pressure, pH, moisture, enzymes and heating time. The effects of molecular size on the rheological properties of oat beta-glucans have been studied by Lazaridou *et al.* (2003). Five highly pure (93–97 per cent) beta-glucan preparations with a peak molecular weight of 35 000, 65 000, 110 000, 140 000 and 250 000 were analysed. All beta-glucans, except the one with the highest molecular weight, were able to form gels and with increasing molecular weight the gelling time increased and the gelation rate decreased. The melting temperature of the gel network became higher with increasing molecular weight of the beta-glucans. It seems from this study that beta-glucans with higher molecular weights increase the gelling time of a solution compared with the ones with a low molecular weight but the stability of the gel formed is higher.

Another important prerequisite to get a viscous solution is that the beta-glucans should be soluble. The solubility of the beta-glucans in four types of hull-less barley has been investigated by Izydorczyk *et al.* (2000). The highest content of beta-glucans was found in high amylose (7.5 per cent), followed by waxy (6.9 per cent), zero amylose waxy (6.3 per cent) and normal (4.4 per cent) barley varieties. However, the solubility (extractability) of beta-glucans was

lower in the high amylose barley (20–30 per cent) in comparison with the other barley genotypes (30–50 per cent). The viscosity was affected by the content of soluble beta-glucans, beta-glucanase activity and molecular weight of the beta-glucans. Hydrothermal treatments (autoclaving and steaming) did not increase the extractability but prevented the enzymic hydrolysis. The addition of enzymes such as protease and esterase during the extraction increased the extractability of beta-glucans from barley. The disruption of either covalent or noncovalent bonds within barley cell wall material might bring about the release and solubilisation of the initially insoluble beta-glucans. Processing of barley into ready-to-eat cereal products is another way to increase the solubility (Marlett 1991). In this study it increased the solubility of the beta-glucans from 48–57 to 90 per cent.

Thus, to be physiologically active the beta-glucans should be soluble and viscous but this leads then to limitations regarding the kind of foods to which they can be added and the amounts used. Another problem is that when beta-glucans are soluble they are probably also much more easily degraded during processing.

19.2.3 Methods for the isolation of beta-glucans

It is possible to isolate preparations with a high concentration of beta-glucans, but care must be taken during the isolation process so the beta-glucans remain physiologically active. Bhatty (1993) used distilled water adjusted to pH 10 with 20 per cent sodium carbonate to extract beta-glucan from barley and oat bran. The yield of beta-glucan from barley bran was 52–55 per cent and the purified fraction contained 72–81 per cent beta-glucans. The yield from oat bran was higher, 61 per cent, and the separated fraction contained 84 per cent beta-glucan. Beer *et al.* (1996) used a similar method to extract oat beta-glucans and purified the preparations further with either dialysis, ultrafiltration, or alcohol precipitation. It was possible to produce preparations with a beta-glucan content of 60–65 per cent with all three methods. However, dialysis gave a preparation with a higher viscosity than the other methods. The authors concluded that alcohol precipitation would be the method of choice if large quantities of oat beta-glucans are to be prepared.

The beta-glucans can also be somewhat enriched by fine grinding and air classification (Wu and Doehlert 2002). Defatted oat bran was ground in a pin mill and the highest beta-glucan content was found in fractions containing particles larger than 30 μm (18.8 per cent). Further fractionation of this preparation to fractions greater than 90 μm increased the beta-glucan content somewhat (20 per cent).

Methods used to produce concentrated beta-glucan preparations on a commercial scale are presented in Section 19.3.

19.3 Developing new oat functional products enriched with beta-glucan

The interest in the development of functional foods containing oat beta-glucans has increased and thus there are several beta-glucan concentrates available commercially that can be added to different foods (Table 19.1). Two new functional foods rich in oat beta-glucans have been developed: a breakfast cereal and a snack bar (Jenkins *et al.* 2002). The products were sweetened with fructose and had a beta-glucan content of 6.5–8 per cent. The beta-glucan was added to the functional foods as a cooked-extruded oat bran. They were highly acceptable to the volunteers and taken as breakfast they gave a glycaemic index of 52 (beta-glucan breakfast cereal) and 43 (snack bar), which was significantly lower than that of an oat bran breakfast cereal (86) and white bread (100). The beta-glucan concentrate used in the breakfast cereal and the snack bar was an OatWell ingredient (http://www.creanutrition-sof.com). This ingredient is produced from oat kernels that are milled, sieved and extracted with ethanol. OatWell ingredients are rich in beta-glucans, up to 22 per cent concentration, and have also been included in bread, biscuits, a powder drink and pasta. Several other human studies have been made with OatWell ingredients, and the effect on glucose metabolism in diabetic subjects has been investigated in detail (Tappy *et al.* 1996, Pick *et al.* 1996, Kabir *et al.* 2002).

A company from Finland have used special milling techniques to make a product named Natureal® Oat Bran (http://www.natureal.fi). The beta-glucan content is at least 15 per cent (dry matter basis) and it is used by the food industry in bakery products, snacks, bars, performance products, drinks and breakfast cereals.

Another product that could be added to different foods is Oatrim. Oatrim is produced from oats or oat bran that is digested with alpha-amylase in hot water (US patent no. 4996063). The product contains amylodextrins and soluble beta-glucans of between 1 and 10 per cent. It was developed to be used as a fat replacer and to increase the dietary fibre content in food. It is used in a wide variety of foods in the United States such as fat-reduced cookies, fat-free cheeses, cereal bars, muffins, beverages and meats. The physiological effects of Oatrim have been documented both in animal and human studies (Inglett & Newman 1994; van der Sluijs *et al.* 1999).

Table 19.1 Examples of oat preparations available commercially

Oat preparation	Beta-glucan content (%)
Oatrim	1–10
Natureal® Oat Bran	≥15
OatWell®	22
Oatvantage™	50

The commercial product with the highest level of beta-glucans (50 per cent) so far is called Oatvantage™ and it is produced in United States (http:// www.nurture-inc.com). The beta-glucans are extracted with an alkaline solution (US patent no. 6323338). Then the extract is acidified or neutralised and heated to between 60 and 100 ºC. During cooling a flocculate is formed and removed. The remaining beta-glucan solution can be further purified with ultrafiltration or evaporated. Suggested applications for this product are liquids, semi-solid foods and beverages, functional foods, nutraceuticals and dietary supplements.

There are also possibilities to directly make foods enriched with beta-glucan through new processing techniques. An oat-based milk been developed based on new technology (US patent no. 5686123). The oat milk is made from steam-prepared or heat-treated oat flakes or oat flour and is rich in beta-glucans. The manufacturing process includes dry or wet milling of the flakes at 60 ºC followed by an enzymatic reaction step using beta-amylase. In this way maltose and beta-limit dextrins, the main carbohydrate species in the final product, are formed from the starch. After the enzymatic step, insoluble fibres can be optionally separated using a decanting step. The product obtained, high or low in insoluble fibre, can be further modified by addition of oil, nutrients and flavours. The oat milk is produced in Sweden and sold commercially today in 15 countries, including Sweden, Great Britain, France, Germany and Australia (http:// www.oatly.com). Products other than oat milk have also been developed, such as ice cream, fruit beverages and sauces. Another process is used to isolate beta-glucan preparations that can be added into different foods. Preparations with a content of 25 per cent oat beta-glucans have been attained (R Öste and A Öste Triantafyllou, EP 1124441).

We have studied the physiological effects of oat milk in several human trials. The oat milk was well tolerated and got a high rating in the sensory evaluation. One trial compared the oat milk with soya milk and cows' milk (Önning *et al.* 1998). Twenty-four participants took the oat milk for 4 weeks and the other test drink for 4 weeks in a crossover design (12 subjects in each group). The participating women consumed 0.75 l oat milk (3.4 g beta-glucan) while the men took 1 l oat milk (4.5 g beta-glucan) per day. The oat milk lowered the plasma cholesterol level by 4 per cent and the low-density lipoprotein (LDL) cholesterol level by 9 per cent compared with the baseline values. No significant differences in total cholesterol and LDL-cholesterol between the oat and cows' milk periods were seen, which can be explained by the relatively small numbers of participants in this study. In another trial a larger number of subjects participated and the oat milk was compared with a control drink low in beta-glucans (Önning *et al.* 1999). Fifty-two participants completed the study. They took the test drinks for 5 weeks with a 5-week wash-out period between the test periods. Compared with the control drink, the intake of oat milk (3.8 g beta-glucans/day) resulted in significantly lower cholesterol (6 per cent) and LDL-cholesterol (6 per cent) levels. Analysis of the beta-glucans in the oat milk revealed that they were of a rather low molecular weight (peak molecular weight 82 400; Biliaderis, unpublished) but

still the oat milk had the expected quantitative cholesterol-lowering effects as compared with less processed products.

The sensory quality of new oat-based functional foods is an important factor that should be considered. The viscosity of beta-glucans is expected to influence the sensory quality. Lyly *et al.* (2003) investigated the influence of oat beta-glucans with different molecular weight on the perception of mouthfeel in beverages. Trained test panellists used 11 attributes to describe the mouthfeel and flavour of different oat beta-glucan beverages. The mean molecular weight range of the beta-glucan preparations added into the beverages varied between 60 000 and 2 000 000. Beverages made with the preparations with higher molecular weight beta-glucans (bran-type) were more viscous and had higher perceived thickness than beverages made with more pure and low molecular weight beta-glucans. Technologically, the more pure beta-glucans were easier to add to a beverage in sufficient amounts.

Another way to increase palatability is to agglomerate the beta-glucans in the presence of maltodextrins (Wood *et al.* 1994). When mixed in a drink the agglomerated oat beta-glucan concentrate will disperse smoothly without formation of lumps, and the development of the maximum viscosity is delayed.

Cooked oat meal flakes have also been studied by sensory profiling (Lapvetelainen and Rannikko 2000). Different parameters that were evaluated were thickness, adherence to spoon and perceived size of the swollen oat flake in the mouth. The cooking conditions affected the texture of the cooked product markedly and the variety of the rolled oat used also influenced the sensory profile.

19.4 Testing the effectiveness of beta-glucans in preventing cardiovascular disease and diabetes

Several human studies have been made on the physiological effects of oats but also of barley (Fig. 19.2). A Medline search combining the words oats or barley and cholesterol or glucose was performed in May 2003. The search was limited to include human clinical trials made from 1980 until May 2003. Fifty-five studies were detected on the effect of oat products on serum cholesterol levels in humans. Most of the studies focused on long-term effects and in more than half of the studies the participants had increased cholesterol levels. Barley is believed to have the same cholesterol-lowering effects as oats but only five human studies were found on that topic in this search. The effect of barley on the glucose metabolism has been studied more frequently and in total over 30 studies on barley and oats were found, mainly on their postprandial effects.

19.4.1 Cardiovascular disease: oats

The main biomarkers for cardiovascular diseases are total and LDL-cholesterol concentrations. Epidemiological data and clinical trials suggest that each

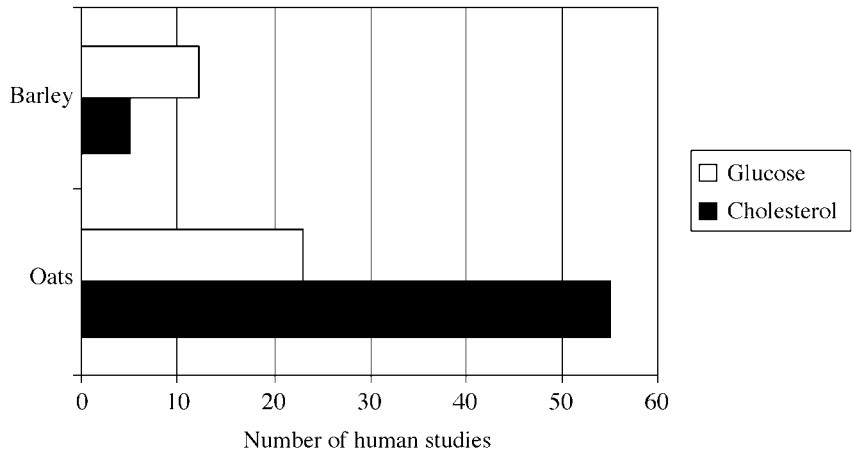

Fig. 19.2 Number of human clinical trials investigating the effect of oats or barley on the blood cholesterol or the glucose levels. Data from a Medline search made in May 2003 covering studies made from 1980 to May 2003.

0.026 mmol/L increment in LDL-cholesterol causes an increase in coronary risk of 1 per cent (Mensink & Katan 1992). Law *et al.* (1994) found that a prolonged difference in serum cholesterol with 0.6 mmol/L was associated with an almost 30 per cent reduction in the risk of coronary heart disease.

Kestin *et al.* (1990) compared three different cereal brans (wheat, rice, oats) in mildly hypercholesterolaemic men. The bran was incorporated in bread and muffins and was given to the subjects for four weeks in a crossover design. In comparison with wheat and rice bran, oat bran significantly reduced the plasma cholesterol concentration with 5.6 and 3.8 per cent, respectively. The main difference between the test products was that oat bran contained twice as much water-soluble fibres as rice and wheat bran.

The dose–response effect of oat bran and oatmeal was studied in hypercholesterolaemic subjects by Davidson *et al.* (1991). The oatmeal or oat bran were given in doses of 28, 56 and 84 g/day for six weeks. Oat bran in doses of 56 and 84 g and oat meal in a dose of 84 g significantly reduced the total and LDL-cholesterol concentration compared with a control group given 28 g farina. The higher efficiency of the oat bran is probably due to its higher beta-glucan content. The conclusion that beta-glucans is the active component was further confirmed in a study by Braaten *et al.* (1994a). They gave a purified preparation containing 80 per cent beta-glucans mixed in a beverage to hypercholesterolaemic participants for 4 weeks. The preparation significantly reduced the total and LDL-cholesterol levels without changing HDL-cholesterol in comparison with a maltodextrin placebo drink. In this study blood samples were taken each week and it was thus possible to follow the hypocholesterolaemic effects developed in more detail. When the participants took the beverage containing beta-glucan, the LDL-cholesterol level was

reduced almost linearly during 4 weeks, and when the intake was stopped, the LDL-cholesterol level went back to the baseline value in 2 weeks. Other, longer, studies have indicated that the cholesterol-lowering effects of an intake of beta-glucans can diminish with time (Uusitupa *et al.* 1992). In this study there were significant cholesterol-lowering effects after 4 weeks but not after 8 weeks. Oat bran can also alter the postprandial effects after a meal in normolipidaemic humans (Dubois *et al.* 1995). The oat bran was added to a test meal when the subjects have been on a low-fibre diet or a diet supplemented with oat bran (40 g/day) for 2 weeks. No change in fasting blood lipid values or plasma insulin was observed after the 2-week oat bran period compared with the low-fibre period. Adding oat bran to the test meal markedly reduced the postprandial insulin rise. The postprandial effects were enhanced after 14 days of oat bran feeding and increased plasma phospholipids, increased plasma and HDL-free cholesterol, decreased plasma and HDL-cholesterol esters, were observed.

Some studies did not show significant effects on the blood cholesterol levels when oats was consumed. An oat bran concentrate was baked into a bread, one roll containing 11.2 g beta-glucan (Törrönen *et al.* 1992). The bread was consumed for 8 weeks by 13 men with mild to moderate hypercholesterolaemia. Another group of 15 men instead took a control product (wheat bread). No significant blood cholesterol-lowering effect was observed even if the cholesterol level for the oat group was reduced from 6.30 to 6.05 mmol/L after 4 weeks of consumption. The authors conclude that the insignificant effect could be due to a poor solubility of the beta-glucan preparation, enzymatic hydrolysis after ingestion and thus a low viscosity in the intestine. The number of subjects in the study was also rather low which could have contributed to the insignificant result. Another study on healthy young men for 2 weeks did not detect any cholesterol-lowering effects of an oat beta-glucan concentrate (9 g beta-glucan/day) with a peak molecular weight of 1 000 000 (Beer *et al.* 1995). The authors point out that to be able to estimate the physiological effects of the beta-glucans not only the content but also the solubility and the viscosity should be measured. Fourteen days may also be too short a time to detect any significant effects of the beta-glucan diet. In another study the cholesterol lowering effect of 3 g oat beta-glucan/day, the level prescribed as the minimum in the FDA health claim, was investigated (Lovegrove *et al.* 2000). The study had a parallel design and as a control wheat bran was used. The subjects were asked to eat the supplement together with low-fat yoghurt or low fat milk each day for 8 weeks. The beta-glucan diet did not reduce total or LDL-cholesterol, despite having a high viscosity.

There are thus conflicting results concerning the hypolipidaemic effects of beta-glucans but most of the studies with a good design indicate that oats have a cholesterol-lowering effect. This was confirmed by Ripsin *et al.* (1992) who made a meta-analysis to estimate the effect of soluble fibre from oats on the blood cholesterol levels. They included studies that had different designs

(parallel, crossover), intervention times (2.5–12 weeks), background diets (usual, American Heart Association step 1 diet (AHA-1), low fibre, low fat), study subjects (sex, age, cholesterol levels), selection of control product (low fibre, wheat bran) and soluble fibre dose (1.1–7.6 g/day). When 12 well-designed studies were included in the meta-analysis, the soluble oat fibre reduced the blood cholesterol with 0.13 mmol/L, a modest reduction. The reduction was larger if the subjects had higher blood cholesterol values (>5.9 mmol/L) and if the dose was over 3 g/day.

A later meta-analysis (Brown *et al.* 1999) concluded that soluble fibre is associated with a small but significant decrease in total cholesterol (−0.045 mmol/L/g) and LDL-cholesterol (−0.057 mmol/L/g). The effects of soluble fibre from oats, psyllium or pectin were not significantly different.

To be able to document the relatively small cholesterol-reducing effects of beta-glucan containing functional foods it is important to use an appropriate study design (see Chapter 2). It is also important to check that the beta-glucans are soluble. In a recent study (Lia Amundsen *et al.* 2003), an oat bran concentrate were added to food products such as bread, teacake, muesli, muffins, macaroni, pasta and apple drink. The authors also checked how much of the beta-glucans that were soluble with the method of Åman and Graham (1987). It was found that the solubility of the beta-glucans in the products was surprisingly low (50 per cent) but the daily dose of soluble beta-glucans consumed by hypercholesterolaemic subjects (2.7 g) was still high enough to decrease the blood cholesterol levels significantly compared with a control diet.

Tables 19.2 and 19.3 summarise clinical studies on hypercholesterolaemic subjects given oats. The reduction in blood lipids are compared with a control group given no oat supplement, wheat bran, wheat, rice, maltodextrins, farina or corn flakes. For some of the studies the oat supplement was consumed for 8 weeks but to make a more similar comparison a study length of 4 weeks was selected. There are large variations in the type of oats (oat bran concentrate, oat bran, oats), the preparation (hot, cold, cereal, beverage, bread, cookies) and in the daily doses (2.2–13.4 g soluble fibre or beta-glucan) in the studies. However, all studies except one seems to reduce the total and LDL-cholesterol level in comparison with the control group. The reduction in total cholesterol varied between 0 and 12.6 per cent and for LDL-cholesterol between 0 and 16.5 per cent. The effect on the HDL-cholesterol and triglyceride levels was more variable. No clear correlation between the dose of beta-glucan/soluble fibre and the reduction in blood lipid values was found. Also it is difficult to conclude which preparation that is the most efficient one. In a study by Kerckhoffs *et al.* (2003) a larger reduction of the cholesterol values was observed when the oat bran was mixed in a juice compared with when it was incorporated in bread and cookies.

19.4.2 Cardiovascular disease: barley

Only few studies so far have investigated the effects of an intake of barley on the lipid metabolism in humans. There are somewhat more animal studies in this

Table 19.2 Selection of clinical studies on hypercholesterolaemic subjects given oats. The study with the largest total blood cholesterol-lowering effect in comparison with the control group is listed first

Entry	Literature	Type of oats	Preparation	Beta-glucan or soluble fibre* (g/day)	Control diet	Number of subjects	Duration (days)
1	Gerhardt and Gallo 1998	Oat bran	Cereal crisp	3.3*	Rice starch	13	42
2	Davidson *et al*. 1991	Oat bran	Hot cereal, muffins, shakes	4.0	Farina	20	42
3	Braaten *et al*. 1994a	Oat gum	Mixed in non-carbohydrate drink/water	2.9	Maltodextrin	19	28
4	Anderson *et al*. 1991	Oat bran	Hot cereal, muffins	13.4*	Wheat bran	10	21
5	Davidson *et al*. 1991	Oatmeal	Hot cereal, muffins, shakes	3.6	Farina	20	42
6	Turnbull & Leeds 1987	Rolled oats	Porridge, biscuits	5.4*	Wheat	17	28
7	Uusitupa *et al*. 1992	Oat bran	Added to juice, yoghurt, porridge, dessert	10.3	Wheat bran	20	28
8	Kestin *et al*. 1990	Oat bran	Bread, muffins	5.8*	Wheat bran	24	28
9	Önning *et al*. 1999	Oat bran, oat flakes	Oat milk without insoluble fibre	3.8	Rice milk	52	35
10	Anderson *et al*. 1990	Oat bran, oat bran concentr.	Flakes, biscuits, chex	3.5*	Corn flakes	12	14
11	Lia Amundsen *et al*. 2003	Oat bran concentr.	Muesli, bread, extruded flakes, muffins, pasta, apple drink	5.1	No oat suppl.	16	21
12	Törronen *et al*. 1992	Oat bran concentr.	Bread	11.2	Wheat	13	28
13	Whyte *et al*. 1992	Oat bran	Porridge, processed	10.3*	Wheat bran	23	28
14	Van Horn *et al*. 1991	Oats, instant	Not controlled	2.2*	No oat suppl.	42	28
15	Kerckhoffs *et al*. 2003	Oat bran, oat bran concentr.	Bread, cookies	5.9	Wheat fibre	25	28
16	Poulter *et al*. 1993	Oat bran	Oat bran crispies	2.2*	Cereal without oats	59	28
17	Lovegrove *et al*. 2000	Oat bran concentr.	Cereal cold	3	Wheat bran	31	28

Table 19.3 Blood lipid values in the end of the diet period in different clinical studies on hypercholesterolaemic subjects given oats. The study with the largest total blood cholesterol-lowering effect in comparison with the control group is listed first[1]

Entry	Cholesterol		LDL-cholesterol		HDL-cholesterol		Triglycerides	
	Control value (mmol/L)	Change %	Control value (mmol/L)	Change %	Control value (mmol/L)	Change %	Control value (mmol/L)	Change %
1	7.20	−12.6	5.02	−13.0	1.30	−1.4	2.04	−19.9
2	6.79	−10.1	4.79	−16.5	1.34	+2.3	1.42	+14.0
3	6.77	−9.2	4.62	−10.0	1.29	−1.5	1.86	−15.1
4	6.26	−8.4	3.18	−6.6	0.77	−4.3	2.16	−0.2
5	6.79	−7.7	4.79	−10.7	1.34	+0.6	1.42	+0.9
6	6.00	−6.7	4.20	−16.3	1.30	+16.7	1.10	+8.2
7	7.40	−6.2	5.36	−8.2	1.33	+ 1.5	1.58	−1.9
8	6.39	−5.6	4.54	−6.6	1.09	0	1.66	−6.2
9	6.58	−5.6	4.38	−5.8	1.39	−9.0	1.85	−12.2
10	6.71	−5.4	4.64	−8.5	0.99	−3.3	2.28	+8.7
11	7.34	−5.4	5.11	−7.5	1.53	−3.2	1.68	+10.6
12	6.42	−4.9	4.27	−3.4	1.44	−1.0	1.92	−13.9
13	5.89	−4.1	4.11	−5.6	1.32	−2.5	1.24	+2.9
14	6.39	−3.2	4.61	−2.6	1.26	−1.7	1.12	−15.1
15	6.04	−2.7	4.11	−3.0	1.41	−4.2	1.12	+2.7
16	6.01	−2.3	3.78	−4.6	1.55	−2.7	1.50	+13.0
17	6.50	0	4.40	0	1.40	0	1.40	0

[1] The percentage change was calculated as:

$$\frac{\text{baseline control} - \text{end value control}}{\text{baseline control}} \times 100 - \frac{\text{baseline oats} - \text{end value oats}}{\text{baseline oats}} \times 100$$

area. In a study in hamsters, barley were given in doses of 0, 25, 50 and 75 per cent of the diet and it was shown that barley lowered the total cholesterol concentration but no dose–response was found (Ranhotra *et al.* 1998). The cholesterol-lowering effects of beta-glucan fractions from barley and oats were also compared in hamsters (Delaney *et al.* 2003). The cereals were given in three doses; 2, 4 and 8 g/100 g diet. The diets gave a clear dose-dependent decrease in the total cholesterol level but no differences in the cholesterol-lowering effects between barley and oats were observed. Chicken has also been fed beta-glucans from barley and the effect on the blood cholesterol concentration was followed (Fadel *et al.* 1987). A non-waxy (Franubet) and a waxy (Washonupana) starch genotype was compared and it was shown that only the waxy genotype had an effect on the blood cholesterol. This was probably due to the waxy genotype having a higher viscosity when mixed with water, a greater average degree of polymerisation and a lower endogenous beta-glucanase activity (Bengtsson *et al.* 1990).

In a human study, McIntosh *et al.* (1991) investigated the cholesterol-lowering effects of barley bran and barley flakes using a 4-week crossover design. The bran and the flakes were incorporated in different foods: bread, muesli, spaghetti and biscuits. Wholewheat flour was substituted for barley in the control products. The intake of beta-glucan was about 8 g/day in the barley period and 1.5 g/day in the wheat period. Consumption of barley led to a significant fall in total (6 per cent) and LDL-cholesterol (7 per cent) compared to the wheat diet. It was also shown by Lupton *et al.* (1994) that barley bran flour enhanced the cholesterol-lowering effect of the National Cholesterol Education Program (NCEP) step 1 diet in individuals with hypercholesterolaemia. In another study barley was cooked with rice (50/50) and consumed by people with normal or increased lipid levels (Ikegami *et al.* 1996). In the participants with normal lipid levels no effects on the cholesterol level was seen when barley was included in the diet, while for those with hypercholesterolaemia the total and LDL-cholesterol levels were decreased significantly. In a recent study no significant effects on the total and LDL-cholesterol levels were observed in a group of mildly hypercholesterolaemic men that were given 8.1–11.9 g barley beta-glucans/day (Keogh *et al.* 2003). More human studies are needed to confirm if beta-glucans from barley have similar cholesterol-lowering properties to oat beta-glucans.

19.4.3 Diabetes

The main biomarkers for the efficiency of a food to control diabetes are measurement of blood glucose and insulin after a standardised meal (glycaemic index, GI, postprandial effects) or the fasting glucose, insulin or HbA1c levels (long-term effects). Both metabolic and epidemiological evidence suggests that replacing high-GI forms of carbohydrates with low-GI forms of carbohydrates will reduce the risk of acquiring type 2 diabetes (Willett *et al.* 2002).

Granfeldt *et al.* (1995) investigated the postprandial effect of two oat products, flaked oats (muesli) and boiled oat flakes (oat porridge) in healthy subjects. Both these products had a similar GI as white bread, while intact boiled oat kernels tested at the same time gave lower glucose and insulin responses. The same is also valid for barley that consumed as porridge gave a similar postprandial response as white bread in healthy subjects (Liljeberg *et al.* 1996). A porridge with a high fibre barley genotype gave however a lower glycaemic and insulin response. Other studies (van der Sluijs *et al.* 1999) also showed that if a more concentrated oat extract (Oatrim) is consumed in a cooked, boiled or baked form, it lowers the glucose and insulin responses. This effect of more concentrated forms of oats (oat bran and oat gum) seems to be valid both for people with type 2 diabetes and for healthy people (Braaten *et al.* 1994b). Tappy *et al.* (1996) gave diabetic subjects a cooked extruded oat bran concentrate for breakfast in different doses (4.0, 6.0, 8.4 g beta-glucan). The maximum increase in plasma glucose for the oat bran meals were 67, 42 and 38 per cent compared with a continental breakfast (35 g available carbohydrates).

Battilana *et al.* (2001) have studied the mechanism of action of beta-glucans on postprandial glucose metabolism. Healthy men were given a diet with (8.9 g/day) or without beta-glucans for 3 days. On the third day the diet was administered as fractionated meals ingested every hour for 9 hours. In this way it was possible to study effects on the metabolism that were unrelated to a delayed carbohydrate absorption, for example fermentation effects. However, the glucose metabolism (glucose and insulin concentrations) was similar for the diet with or without beta-glucans. Thus, the main effect of the beta-glucans seems to be a delayed intestinal absorption of carbohydrates.

Wood *et al.* (1994, 2000) suggested that the reductions in glucose and insulin responses after a meal are mainly due to the viscosity of oats. They studied mixtures of oat beta-glucans with different viscosity and there was a highly significant linear relationship between the viscosity and the glucose and insulin responses. This can be explained by a delay in the carbohydrate absorption due to the high viscosity. Würsch and Pi-Sunyer (1997) have in a review concluded that a concentration of 10 per cent beta-glucan in a cereal food gives a 50 per cent reduction in the postprandial glucose peak.

The effect on the glucose metabolism of a long-term intake of oat beta-glucans has also been investigated. An intake of oat beta-glucans (3 g in muesli) taken for breakfast for 4 weeks in men with type 2 diabetes led to a decreased cholesterol level and lower postprandial glucose peaks but no effects on the fasting plasma glucose, insulin and HbA1c were observed (Kabir *et al.* 2002). The intervention period of 4 weeks may be too short to detect a change in the glucose metabolism in the fasted state. In a longer 12–week pilot study, a bread containing an oat bran concentrate (9 g soluble fibre/day) improved the postprandial glucose metabolism in partcipants via type 2 diabetes (Pick *et al.* 1996).

19.5 Future trends

19.5.1 New functional foods enriched in beta-glucans

Cereals rich in beta-glucans may be a useful nutritional tool to control the metabolic disorders hyperlipidaemia and type 2 diabetes mellitus. One problem, however, is that such products may be unacceptable to many consumers. This can be improved by offering a wider range of foods enriched in beta-glucans. This is the goal for an ongoing EU-funded project coordinated by the author Gunilla Önning 'Design of foods with improved functionality and superior health effects using cereal beta-glucans (QLR1–2000–00535)'. In the project beta-glucans are isolated and incorporated in different foods that normally do not contain cereals, such as ready-meals. The sensory and functional properties are followed carefully in the designing of the new, food enriched with beta-glucan. This and other projects will lead to enhanced possibilities for the consumer to select from a wider range of beta-glucan containing foods in the future.

19.5.2 Usage of new techniques: nutrigenomics

The main part of the studies on the health effects of beta-glucans has been devoted to their effects on glucose and lipid metabolism. In the future other physiological aspects will certainly attract increased attention. An interesting area is, for example, the importance for gut health of the beta-glucans. To reach a deeper understanding of the health effects of beta-glucans, newly developed nutrigenomic techniques can be used. From a nutrigenomic perspective, nutrients are dietary signals that are detected by the cellular sensor systems that influence gene and protein expression and, subsequently, the production of metabolites. It is known that nutrients can be potent dietary signals that influence the metabolic programming of cells and thus have an important role in the control of homeostasis (Müller & Kersten 2003). Transcription factors are probably the main agents through which nutrients influence the gene expression. For example, dietary polyunsaturated fatty acids potently repress the hepatic expression of several genes involved in fatty acid synthesis by binding to the receptor family of PPARs (Kersten *et al.* 2000). By using nutrigenomics it is possible to measure the response of thousands of actively transcribing genes in a cell.

So far, only a few human trials in the nutrigenomic area have been done. In a study by Vidon *et al.* (2001), a high-carbohydrate diet and a high-fat diet gave the same mRNA concentration for the LDL receptor in blood lymphocytes. Other studies have analysed the gene expression profile and investigated the effect of diabetes (Sreekumar *et al.* 2002; Maier & Olek 2002) and the function of short-chain fatty acids in the colon (Mariadason *et al.* 2000).

19.6 Sources of further information and advice

The American Association of Cereal Chemists (AACC) has published two books on oats. The titles of the books are *Oats: Chemistry and Technology* (Webster

1986) and *Oat Bran* (Wood 1993). The first book is very comprehensive and gives a good review of oats chemistry, usage, and nutritional value including health aspects and one chapter on oat beta-glucans. *Oat Bran* focuses on the dietary fibre components, including the beta-glucans. Later, another book on oats was published: *The Oat Crop: Product and utilization* (Welsch 1995), including chapters presenting the botany, production, processing and food uses of oats. The AACC has also published one corresponding book on barley entitled *Barley: Chemistry and Technology* (MacGregor and Bhatty 1993). The book covers aspects such as the use of barley in malting, feed and as a human food.

There are also several recent review articles in this area. Mälkki has written a chapter in the *Handbook of Dietary Fibre* (2001) with the title 'Oat fibers: production, composition, physico-chemical properties, physiological effects, safety and food applications'. Wood and Beer have written a chapter about 'Functional oat products' included in *Functional Foods. Biochemical & processing* (1998). A review of the role of viscous soluble fibre in the metabolic control of diabetes with special emphasis on cereals rich in beta-glucans has been published by Würsch and Pi-Sunyer (1997). A more recent review on the influcence of beta-glucans on the human serum lipoproteins has been made by Kerckhoffs *et al.* (2002). This article also reviews the effect of other dietary components such as soy protein, plant sterols and isoflavones.

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20

Grain legumes and the prevention of cardiovascular disease

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20.1 Introduction

Ripe seeds of the plant family *Fabaceae*, known commonly as 'legumes' or 'pulses', are major foodstuffs in most countries and are an indispensable supply of proteins for the third world population, as they contain the highest amount of proteins in edible vegetables (Belitz and Grosch, 1999).

In spite of their positive features, the consumption of grain legumes has decreased during the twentieth century in most industrialised countries: for example it was 7.3 kg per person per year in 1920 in France, whereas it is now less than 1 kg (Champ, 2001). The low consumption in Europe is due to a number of reasons, such as difficult digestibility, at least for some individuals, taste and, critically important, the long cooking time, in a society in which most people can dedicate to cooking only a very short period of time. However, in the last few years, a reduction of the consumption of food items of animal origin and a moderate increase in the consumption of vegetables, grain legumes included, has resulted as a positive consequence of the crisis of the meat market, mostly due to bovine spongiform encephalopathy (BSE).

As the incidence of cardiovascular disease, obesity and type II diabetes is increasing, it has become urgent to promote a more appropriate diet for the populations of industrialised countries. Pulses may have a very important role in this direction. In fact, based on a number of studies reporting beneficial effects induced by the use of soy proteins *vs*. animal proteins in the prevention of cardiovascular disease, the US Food and Drug Administration (FDA) recently validated (FDA, 1999) the health claim about the role of soy protein in reducing the risk of coronary heart diseases, mainly by reducing cholesterolaemia (Anderson *et al.*, 1995; Bakhit *et al.*, 1994; Sirtori *et al.*, 1998).

This chapter will provide data on the nutritional value of major pulses, will discuss some problems related to the presence of antinutritional factors, and will discuss in detail the experimental and clinical literature supporting the beneficial role of soybean and other legumes in the prevention of cardiovascular diseases.

20.2 The main components of grain legumes

The composition of major grain legumes is shown in Table 20.1. Soybean and lupin are particularly rich in protein, poor in starch and have a rather high content of unsaturated lipids; peanut is definitely rich in lipids, whereas the most abundant constituents of all the other legumes are digestible carbohydrates. The protein content of the least protein-rich legumes, runner bean and chickpea, is anyhow higher than 22 per cent, i.e. a value very seldom found in vegetables.

20.2.1 Proteins

Legume proteins are an important complement to cereal proteins in vegetarian diets or diets poor in animal proteins, because they have a satisfactory content of lysine and other essential amino acids, their only defect being the relatively low content of sulphur-containing amino acids (Gueguen and Cerletti, 1994). The bioavailability of legume proteins has been studied in detail especially with reference to their use as feed for monogastric animals. Their protein efficiency ratios (PER) are lower than those of animal proteins, such as casein and lactalbumin (Bhatty *et al.*, 2000; Cuadrado *et al.*, 2002; Marzo *et al.*, 2002; Urbano *et al.*, 2003). Indeed, when animal feeding is considered, a high value for this parameter is critically important, because increasing body weight in the shortest time is the main target. However, when dealing with human adults, the philosophy may be the opposite and a lower PER may mean less difficulty in maintaining a correct caloric intake and consequently correct body weight.

Purified legume storage proteins (chickpea 11S and 7S globulins, faba bean globulins and lupin globulins) and casein have been subjected to an *in vitro* enzyme (pepsin + pancreatin) digestion process. Protein digests were then used in a bicameral Caco-2 cell culture system to determine amino acid transport across cell monolayers. With digests from legume proteins, absolute amounts of aspartate, glycine and arginine transported were higher than those found in digested casein. These results confirm the *in vivo* observations that amino acids from legume proteins are probably absorbed at rates different from those in other proteins of animal origin such as casein (Rubio and Seiquer, 2002).

Fractionation of legume proteins using procedures based on differential solubility yields three fractions: albumins, globulins and glutelins, with globulins being predominant (Brooks and Morr, 1992). The total lack of prolamines makes legumes particularly useful for preparing gluten-free foods for coeliac individuals. Globulins in seeds function mostly as storage proteins, to be mobilised during the course of germination. Globulins can be separated by

Table 20.1 Percentage composition of main grain legumes

Systematic name	Common name	Crude protein	Lipids	Digestible carbohydrates	Crude fibre	Minerals
Arachis hypogea	Peanuts	27.4	50.7	9.1	7.5	2.7
Glycine max	Soybeans	39.0	19.6	7.6	16.6	5.5
Lupinus albus	White lupins	44.7	12.5	13.6	38.3	5.0
Lupinus angustifolius	Narrow-leaf lupins	31.1	6.0	5.3	14.7	3.5
Lupinus luteus	Yellow lupins	50.2	6.1	8.4	30.7	4.1
Cicer arietinum	Chickpeas	22.7	5.0	54.6	10.7	3.0
Lens culinaria	Lentils	28.6	1.6	57.6	11.9	3.6
Phaseolus coccineus	Runner beans	23.1	2.1	55.2	4.5	3.9
Phaseolus lunatus	Lima beans	25.0	1.6	57.8	15.0	3.9
Phaseolus vulgaris	Common beans	24.1	1.8	54.1	19.2	4.4
Pisum sativus	Peas	25.7	1.4	53.7	18.7	3.0
Vicia faba	Broad beans	25.7	1.5	46.5	10.3	5.6
Vigna unguiculata	Cowpeas	24.6	1.8	52.0	5.0	3.4
Vigna radiata	Mungo beans	26.9	1.6	46.3	5.1	3.6

Sources: Belitz and Grosch (1999); Chango *et al.* (1998); Gueguen and Cerletti (1994)

ultracentrifugation or chromatography into two major components present in all legumes: legumins (approximately 11S) and vicilins (approximately 7S). In soybean, legumins derive from a protein precursor, which is split into an acidic polypeptide A (p$I \approx 5$) and a basic polypeptide B (p$I \approx 8.2$). These two peptides are linked by a disulphide bridge between Cys (92) and Cys (424) and are regarded as subunits. Six subunits are assembled to give the 11S globulin (Belitz and Grosch, 1999). Strong homology exists between the 11S globulins of different legumes, although variable regions are found mostly in the A peptide, and the A/B cleavage site is highly conserved. Generally legumins are not glycosylated, but there are exceptions, for example the 11S fraction of lupin (Gueguen and Cerletti, 1994).

The sequences of many vicilins are well known too: they derive from the post-translational cleavage of a single precursor in some polypeptides. In the case of soybean (β-conglycinin), these peptides are three, α, α' and β and vicilins consist of three polypeptides, not linked by a disulphide bridge, which can be different or identical (hetero- and homopolymeric forms). Taking into consideration other legumes, the sequences of precursor proteins are highly homologous, but the cleavage sites are variable. The vicilins are always glycosylated, but at a different extent in different legumes (Gueguen and Cerletti, 1994).

Under non-denaturating conditions, the 11S and 7S globulins exhibit a tendency towards reversible dissociation/association, depending to a large extent upon the pH value and ionic strength. The 11S globulins are relatively more stable than the 7S globulins. The lower stability of 7S globulins is evident also during industrial processing. For example a comparison of native globulins of soybean with some industrial soybean protein isolates by 2D electrophoresis and MALDI-TOF (matrix-assisted laser desorption/ionisation time of flight) has shown that fragments deriving from the legumins are much more recognisable than those deriving from vicilins (Gianazza *et al.*, 2003). This is crucially important, as vicilins are the major responsible components for the hypocholesterolaemic activity of soybean proteins (Lovati *et al.*, 1992).

20.2.2 Carbohydrates

Most cultivated legumes contain 39–51 per cent starch in raw seeds, with the exception of soybean and lupin. Legume starch has a higher content of amylose (30–45 per cent) versus cereals and potato (20–30 per cent). A higher amylose content means: (a) a higher temperature for completing gelatinisation of starch granules, and (b) a higher susceptibility to retrodegradation with the consequence of increasing resistance to endogenous amylases (Champ, 2001). Starch remains undigested in the small intestine, and is fermented by the colonic microflora when it reaches the large intestine. One characteristic of the fermentation of resistant starch is the formation of short-chain fatty acids, such as butanoate, known as the main nutrient of the colonocyte and potentially involved in the protection of colon from some diseases, particularly cancer (Bird *et al.*, 2000).

Pulses are also a source of non-starch polysaccharides (NSP), for example lentils and beans contain 10.6 and 17.3 per cent NSP (on dry seed), 12.4 and 26.3 per cent being soluble NSP, respectively (Champ, 2001). They are contained in part in the hull of seeds rich in insoluble polysaccharides, mostly cellulose, and in part in cell walls more rich of soluble fibres, such as peptic polysaccharides. These fibres decrease the bioavailability of starch determining the low glycaemic index (GI) of legumes (Guillon and Champ, 2002), varying between 18 and 56, whereas food based on cereals have GI values in the range 65–95. This is beneficial in diabetes and may be beneficial also for the prevention of cardiovascular disease. For example, a decrease of the GI of diets of hyperlipidaemic patients from 82 to 69 units for a 1-month period resulted in a significant reduction in total and low-density lipoprotein (LDL) serum cholesterol and triglycerides, compared with mean lipid values for the preceding and following months (Jenkins *et al.*, 1985).

20.2.3 Lipids

Peanuts, soybeans and lupin seeds are also interesting sources of lipids (Table 20.1), a fact that for peanuts and soybean has a significant economic impact. The average composition of the oil of these seeds is reported in Table 20.2. White lupin oil seems particularly promising because it has a composition close to dietary recommendations for cardiovascular prevention (>50 per cent oleic acid, >17 per cent linoleic acid, >7 per cent linolenic acid), however, to our knowledge, it is not commercially available. The importance of lipids for the prevention of cardiovascular disease is discussed in detail in Chapter 11 of this book.

20.3 The non-nutritional components of legumes

Unlike animal proteins, whose nutritional value is largely determined by their amino acid composition, the full nutritional potential of legume protein is

Table 20.2 Triglyceride percentage composition of oil-rich legumes

Fatty acid	White lupin (%)	Yellow lupin (%)	Narrow leaf lupin (%)	Soybean (%)	Peanut (%)
16:0	7.8	4.8	11.0	10	10
18:0	1.6	2.5	3.8	5	3
18:1 (n-9)	53.0	21.0	38.2	21	41
18:2 (n-6)	17.2	47.5	37.1	53	35.5
18:3 (n-3)	9.5	7.5	5.3	8	0
20:0 & 20:1	5.5	4.5	1.2	4.0	2.5
22:0 & 22:1 (n-11)	5.8	7.9	1.9	–	3
n-3/n-6	*0.55*	*0.16*	*0.14*	*0.15*	*0*

Source: data from Hudson *et al*. (1983); Belitz and Grosch (1999).

attained only after a certain amount of heat has been applied (Liener, 1994; Wang and McIntosh, 1996). In fact there are a number of components in legumes that can exert a negative impact on the nutritional quality of proteins and have to be inactivated by heat at least in part. The heat-labile factors are protease inhibitors, lectins, goitrogens, antivitamins; the heat-stable compounds saponins, tannins, phytoestrogens, flatulence factors, phytate, allergens (Champ, 2001). In addition, the industrial procedures may produce some new compounds with toxicological relevance, such as lysinoalanine (Arnoldi, 2002).

20.3.1 Inhibitors of protease

Inhibitors of proteases are present in many vegetables and in all legume seeds. In soybean there are two main inhibitors, the Kunitz inhibitor (MW 21 500 da) and the Bowman-Birk inhibitor (8 000 da), but other inhibitors have been characterised in peanuts, chickpea, common bean, runner bean, lima bean, broad bean and pea. Trypsin and α-chymotrypsin are the main targets of this activity and the inhibitor content depends on the variety, degree of ripeness and storage time. The probable function of these inhibitors in the seeds is the protection against damage by higher animals, insects and micro-organisms. They have to be destroyed by heat treatments, which improve considerably the PER determined on the growth of rats. Moreover, soybean proteases inhibitors increase the size of the acinar cells of pancreas as well as their number (hyperplasia) (Liener, 1994).

20.3.2 Lectins

Although protease inhibitors are generally considered to be the main antinutritional factors in legumes, there is clear evidence that other components are responsible for growth inhibition. They are lectins, a class of proteins widespread in vegetables, which have the unique property of binding carbohydrate-containing substances. In particular they have the ability to agglutinate the red blood cells from various animal species, because of the interaction of multiple binding sites on the lectin molecules with specific glycoconjugate receptors on the surface of cell membranes. Most lectins are glycoproteins. When their molecular weight exceeds 30 kda, they consist of several subunits (Belitz and Grosch, 1999). Lectins bind to the epithelial cells on the intestinal wall, causing deleterious nutritional effects by interfering with nutrient absorption. The lectins of soybean and common bean are particularly toxic, whereas other legumes, such as lupin, have very small amounts of these factors (Muzquiz *et al.*, 1998).

20.3.3 α-Galactosides

All legumes contain a certain amount of α-galactosides of the raffinose family, composed of a sucrose molecule linked to 1–3 molecules of galactose (raffinose,

stachiose, verbascose). The α-galactosidase, necessary to hydrolyse the α1–6 linkage, is not available in the small intestine. As a consequence, these compounds reach the large intestine, where they are fermented to produce gases, giving the well-known flatulence experienced by many people when eating legumes. In this respect α-galactosides are rated as antinutrients; however, they are also prebiotic because they stimulate the growth of lactic bacteria, especially bifidobacteria, in the colon (Champ, 2001).

20.3.4 Other constituents

Legumes contain other minor constituents, which contribute to defend the seeds from insects or fungi, such as saponins or isoflavonoids, rather common in many legumes, or quinazolidine alkaloids, specific of lupin. They are a family of about 100 bitter compounds containing a rather uncommon bicyclic structure. Wild species may contain more than 600 mg/kg of alkaloids, but modern domesticated varieties are called 'sweet' because, by careful breeding, the alkaloid content has been reduced to less than 130 mg/kg, well below the current maximum concentration permitted in Australia of 200 mg/kg. The Australia New Zealand Food Authority has proposed a provisional tolerable daily intake of 0.035 mg/kg/day for these substances (Australia New Zealand Food Authority, 2001). Lupins are an interesting source of protein concentrates and isolates: the acidic work-up during their separation from the flour reduces their presence to a minimum.

Isoflavonoids are congeners of genistein that have a protective role in seeds and plants and are extensively biosynthesised in response to an abiotic or biotic stress (phytoalexins). Although they have been rated as useful components of legumes for the prevention of some diseases, such as breast cancer, osteoporosis and menopausal hot flushes, in the last few years a number of papers have raised the issue of potentially serious toxicological problems (Fort *et al.*, 1990; Kulling *et al.*, 1999; Kumi-Diaka *et al.*, 1999; Newbold *et al.*, 2000; Sirtori, 2001). These data have suggested several legislative interventions to reduce their consumption (Working Group UK, 2002). However, it should be emphasised that high amounts of isoflavone are typical of soybean, while other legumes have a much lower content (Table 20.3).

20.4 The use of soybean protein in the prevention of hypercholesterolaemia

Legume proteins, particularly soy proteins, reduce plasma cholesterol both in animals, when it is elevated by dietary means (high cholesterol intake, semi-synthetic diets, etc.) (Kim *et al.*, 1980; Terpstra *et al.*, 1982), and in patients with hypercholesterolaemia of monogenic or polygenic origin (Sirtori *et al.*, 1998).

The soybean diet, as of now, is certainly the most effective dietary tool for treating hypercholesterolaemia and provides a unique opportunity for the management of very young patients and also for exploring new mechanisms in

Table 20.3 Isoflavone content of grain legumes

Legume	Daidzein μg/g	Genistein μg/g
Arachis hypogea	0.50	0.82
Glycine max	105–560	268–841
Lupinus albus	ND	trace
Lupinus luteus	ND	trace
L. angustifolius	ND	trace
Cicer arietinum	0.11–1.92	0.69–2.14
Lens culinaria	0.03	0.07
Phaseolus lunatus	0.12–0.89	0.10–0.19
Phaseolus vulgaris	0.07–0.40	0.07–5.20
Pisum sativum	0.04–0.08	ND–0.23
Vicia faba	0.16–0.32	trace
Vigna radiata	0.30–0.36	0.16–0.60
Vigna unguiculata	0.21–0.30	0.11–0.56

ND = not detectable.
Source: Mazur *et al*. (1998); Katagiri *et al*. (2000).

plasma cholesterol regulation. The validity of this therapeutic approach was recently supported by the US FDA approving the health claims about the role of soy protein in reducing the risk of coronary heart disease (FDA, 1999).

The earliest studies by Sirtori and co-workers (Sirtori *et al.*, 1977) clearly established that patients with elevated cholesterolaemia (total cholesterol above 7.8 mmol/L) show the most favourable response to the substitution of animal proteins with isoflavone-free soybean proteins. The initial study, a crossover trial under metabolic ward conditions, showed a 20–22 per cent reduction of total cholesterol, with no change of triglycerides (TG) and a 22–25 per cent fall of low-density lipoprotein-cholesterol (LDL-C) (Sirtori *et al.*, 1977). In a small group of patients the effect of the addition of cholesterol to the soybean protein concentrate was also investigated. They received 500 mg of cholesterol daily, either in the first 3 weeks or in the last 3 weeks of administration of the diet, this addition did not appear to influence the hypocholesterolaemic response.

The results of these metabolic ward studies were confirmed, later on, in a large investigation on 127 outpatients treated for 8 weeks with a similar soy protein regimen within a low lipid diet (Descovich *et al.*, 1980). A mean reduction of cholesterolaemia of 23.1 per cent in the 67 participating males and of 25.3 per cent in the 60 females was detected. Again, no significant changes in plasma TG, high-density lipoprotein-cholesterol (HDL-C) or body weight were recorded. In this study, it was possible to monitor the trend for the successive return of cholesterolaemia to baseline. This occurred in most patients 6–8 weeks upon switching to a low-lipid diet with animal proteins, and was definitely accelerated in patients with familial hypercholesterolaemia (FH).

Anderson *et al.* (1995) have analysed a total of 38 studies, both in participants with elevated plasma cholesterol and in normolipidaemic volunteers, all treated for a variable length of time with a diet with partial or total substitution of

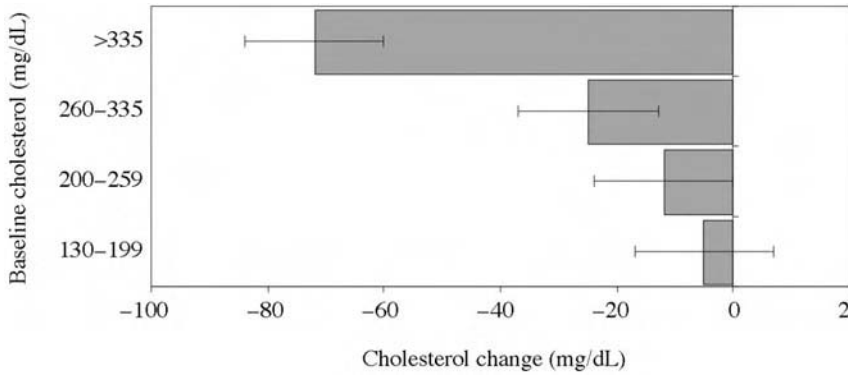

Fig. 20.1 Change of cholesterol levels induced by the consumption of soybean proteins *vs*. baseline cholesterol levels (data from Anderson *et al*., 1995).

animal proteins with soy proteins. The reviewed studies ranged from the evaluation of fibre-like properties of soybean proteins, to hormonal studies, to the more recent clinical reports linking LDL receptor activation to the remarkable cholesterol-lowering properties of this diet. The conclusions of this meta-analysis, summarised in Fig. 20.1, confirm that serum and LDL-C concentrations are modified according to baseline cholesterolaemia, from a minimum of −3.3 per cent in subjects with cholesterol in the normal range, way up to −19.6 per cent (LDL-C −24 per cent) in people with clear-cut hypercholesterolaemia. Normolipidaemic individuals do not respond to the cholesterol-lowering effect of soy protein (Anderson *et al.*, 1995).

An interesting area for the use of the soybean protein diet has been the treatment of pediatric hypercholesterolaemia and of hypercholesterolaemia secondary to kidney disease. In an Italian multicentre study on 18 pre-puberal children a reduction of cholesterolaemia around 25 per cent or more was constantly achieved (Gaddi *et al.*, 1987). These findings were later confirmed by Widhalm *et al.* (1993), who evaluated a similar regimen in 23 children with familial or polygenic hypercholesterolaemia. The LDL-C reduction was 22 per cent when the soy protein diet preceded the standard lipid-lowering diet, and 25 per cent, when it was given as the second treatment.

20.4.1 Mechanism of action

Two studies have addressed, both in a direct and an indirect way, the potential of the soy protein diet to increase LDL receptor expression in humans. In the first study, with a similar protocol as in previously described investigations in rodents (Sirtori *et al.*, 1984), hypercholesterolaemic patients were treated in a crossover protocol, with either animal proteins or soy protein concentrates (this time with the addition of cholesterol, in order to normalise dietary fat intake) (Lovati *et al.*, 1987). Besides plasma lipid changes, LDL degradation by mononuclear cells after each diet was monitored. During the animal protein intake, there were

hardly any changes in LDL-C levels or receptor activity, but with the soy protein diet, in the presence of an elevated cholesterol intake, treated patients showed a consistently raised degradation of LDL by mononuclear cells, about 8-fold higher than the reference diet. Therefore this study confirms that some factor/s in soy proteins may exert an up-regulation of LDL receptors.

It seems, therefore, reasonable to suggest that the mechanism of action of the soy protein diet in humans is not at the intestinal or endocrine level, but rather that it involves a stimulatory effect on LDL receptors, chronically depressed in hypercholesterolaemia. This is one further reason why normolipidemic individuals respond to a limited extent to the regimen; in the presence of LDL receptor down-regulation, e.g. by cholesterol loading in normolipidemics, the hypocholesterolaemic activity becomes apparent.

Based mainly on studies in monkeys (Anthony *et al.*, 1996), the 1995 meta-analysis (Anderson *et al.*, 1995), suggested that up to 60 per cent or more of the dietary effect on cholesterolaemia might be linked to the presence of isoflavones, i.e. genistein and daidzein, and/or their conjugates (aglycones and glucosides) (Wang and Murphy, 1994). However, the same author has very recently changed his opinion (Anderson, 2003)

The majority of the reported clinical studies in the same meta-analysis were carried out with soy concentrates or isolates, i.e. with dietary formulations containing minimal amounts of isoflavones, i.e. less than 40 μg/g (Sirtori *et al.*, 1995, 1997). Many studies have compared soy protein isolates or concentrates with a normal isoflavone content, soy protein isolates or concentrates depleted in isoflavones and other proteins (mainly casein or lactalbumin) added with isoflavones, giving controversial results (Adlercreutz *et al.*, 1987; Crouse *et al.*, 1999; Greaves *et al.*, 1999). A general problem of these studies is that none reports a detailed investigation of the consequences induced on the structure of the proteins by the processing used to eliminate the isoflavones (mostly extraction with boiling ethanol). A different approach has been used by Fukui *et al.* (2002) in order to investigate whether isoflavones are responsible for the hypocholesterolaemic effect of soy protein. These authors prepared an isoflavone-free soy protein isolate (IF-SPI) by column chromatography. The study was conducted in rats in comparison with casein and a standard soy protein isolate (SPI). Plasma total cholesterol concentrations of rats fed SPI and IF-SPI were comparable and significantly lower than those of rats fed casein. Thus, the cholesterol-lowering effect of SPI in rats can be attributed entirely to their protein content.

Final confirmation of the unacceptability of the isoflavone hypothesis has come from the repetition of the primate experiments in more appropriate conditions. Greaves *et al.* (1999) showed that addition to a casein diet of a semipurified ethanol extract of soy, rich in isoflavones, failed to improve cholesterolaemia in ovariectomised cynomolgus monkeys *vs*. intact soy proteins. The same authors more recently confirmed that a soy protein diet reduces cholesterolaemia in ovariectomised adult female cynomolgous monkeys, also by partially inhibiting cholesterol absorption, whereas a semipurified soy extract,

rich in isoflavones, added to a casein diet does not exert any lipid lowering effect (Greaves *et al.*, 2000). The recent lack of confirmation of the hypothesis of positive vascular effect of an isoflavone-rich diet in postmenopausal women (Simons *et al.*, 2000), provided definitive evidence against any role of isoflavones in the beneficial effects of soy proteins, possibly suggesting that these products, of very dubious benefit and associated with potential risk, should not be freely available (Ginsburg and Prevelich, 2000).

Other studies have been devoted to evaluating the possible responsibility of proteins *per se* in the reduction of cholesterolaemia. This hypothesis seems reasonable in the face of evidence (Potter *et al.*, 1996), indicating that the presence of additional components, besides protein in the diet, might not affect in a significant way the plasma cholesterol reduction achieved with the diet.

In agreement with the results of animal and human studies, a number of experiments have been performed in a hepatoma cell line (HepG2), an *in vitro* model of human liver cells, highly sensitive to factors regulating LDL receptor expression and cholesterol biosynthesis/breakdown, by tracking the uptake and degradation of labelled LDL, in order to identify the soy protein component/s potentially responsible for the cholesterol-lowering effect. It has been concluded that the 7S globulin directly up-regulates the LDL receptor. This effect is exerted both on HepG2 cells and also, albeit to a lesser extent, on human skin fibroblasts (HSF), and is paralleled by an enhanced LDL degradation (Lovati *et al.*, 1992).

Another study by the same group has examined the effect of 7S soy globulin subunits *vs*. the whole 7S on the up-regulation of lipoprotein uptake and degradation in Hep G2 cells. This experiment clearly indicated that incubation of cells with purified $\alpha + \alpha'$ subunits from 7S markedly increases uptake and degradation of ^{125}I-LDL, whereas the β-chains are ineffective (Lovati *et al.*, 1998). These experiments may also open the way to the development of soybean varieties with different ratios among the major globulins, possibly resulting in cultivars with improved cholesterol-lowering potential. Interestingly, a soy cultivar mutant, Keburi, devoid of the α' subunit, had no activity of this sort, thus possibly suggesting development of soy cultivars rich in the α' subunit (Manzoni *et al.*, 1998). This indirect result has been recently confirmed by a direct methodology. In an experiment with HepG2 cells, the up-regulation of LDL receptors by the α' subunit was significantly greater than that found in control cells. In addition, this study revealed a potentially interesting association of soybean 7S globulin with proteins, such as thioredoxin 1 and cyclophilin B, both involved in cell protection against oxidative and other stresses (Manzoni *et al.*, 2003).

In order to assess the final identity of the putative peptide/s responsible for the biochemical effect, experiments have been performed in Hep G2 cells, exposed either to synthetic peptides corresponding to specific sequences of 7S soy globulin, or to peptides coming from the *in vitro* digestion of Croksoy^{R}70, a commercial isoflavone-poor soy concentrate, routinely used by Sirtori and co-workers in the dietary treatment of hypercholesterolaemic patients. Increased

^{125}I-LDL uptake and degradation vs. controls were shown after Hep G2 incubation with a synthetic peptide (10^{-4} mol/L, MW 2271 Da) corresponding to the 127–150 positions of the α' subunit of 7S globulins (Lovati *et al.*, 2000). Cells exposed to Croksoy^{R}70 enzyme digestion products showed a more marked up-regulation of LDL receptors than controls (Lovati *et al.*, 2000). These findings support the hypothesis that if one or more peptides can reach the liver after intestinal digestion, they may elicit a cholesterol-lowering effect. Evaluation of the LDL receptor stimulatory activity of the major soy isoflavone, genistein, up to concentrations of 1 mg/mL, failed to demonstrate any evident change.

In view of these results, it would be very useful to evaluate in detail whether the ethanol extraction of isoflavones from the soy protein isolate also removes some biologically active peptides. From a practical point of view, it is important to underline that these results have stimulated the industrial interest for patents for soy protein isolates and concentrates with a very high content of β-conglycinin (Bringe, 2001).

20.5 The hypocholesterolaemic activity of other legumes

In view of the high homology of legume vicilins, it is reasonable to foresee that other legumes may exert a biological activity similar to soy proteins. Experimental data in this field are rather scarce, especially considering the vastness of the literature dealing with soybean. In part this may be due to the prejudice of the need of isoflavones that are generally rather scarce in the other legumes (Table 20.3). However, especially in the last few years some investigations have been published both on experimental animals and in humans. Most studies have been performed on growing rats fed a normal diet or on adults fed a hypercholesterolaemic one (Nath *et al.*, 1959). These papers have different characteristics, not always homogeneous, as shown in Table 20.4, whereas major results are summarised in Table 20.5, which includes also some data on soybean for comparison.

The effects of *Lupinis angustifolius* has been studied by Rahman et al. (1996). In rats pair-fed for 10 days on cholesterol-free diets containing lactalbumin, raw lupin seed meal or five different semi-purified lupin fractions, a significant lowering effect on total plasma cholesterol was observed in growing rats fed the seed meal fractions compared with the value obtained from the lactalbumin control. In particular a fraction, containing γ-conglutin lowered total plasma cholesterol by 34 per cent compared with the lactalbumin-fed group. Liver lipid and cholesterol were also found to be decreased in rats fed *L. angustifolius* seed meal and its fractions.

Yellow and white lupin meals have been studied by Chango *et al* (1998) in rats fed cholesterol-rich diets. Differences among the total blood serum cholesterol levels of rat groups fed these diets for 28 days were not significant. Compared to casein and yellow lupin diets, the white lupin diet decreased

Table 20.4 Characteristics of studies on rats fed pulses

Entry	Literature	Type of pulse	Preparation	Amount (%)	Duration (days)	Control ingredient	Cholesterol content
1	Mokady & Liener, 1982	*Glycine max*	Meal	10	28	Casein	2%
2a	Lovati *et al*., 1992	*Glycine max*	7S globulins	(a)	14	Casein	1%
2b	Lovati *et al.*, 1992	*Glycine max*	11S globulins	(a)	14	Casein	1%
3	Fukui *et al.*, 2002	*Glycine max*	Protein isolate (c)	20	14	Casein	0.5%
4	Sirtori et al., 2004	*Lupinus albus*	Protein isolate	(b)	21	Casein	1%
5a	Chango *et al.*, 1998	*Lupinus albus*	Meal	45	28	Casein	1%
5b	Chango *et al.*, 1998	*Lupinus luteus*	Meal	40	28	Casein	1%
6a	Rahman *et al.*, 1996	*Lupinus angustifolius*	Meal	36	10	Lactalbumin	0%
6b	Rahman *et al.*, 1996	*L. angustifolius*	Protein isolate	15	10	Lactalbumin	0%
6c	Rahman *et al.*, 1996	*L. angustifolius*	Conglutin-γ	12	10	Lactalbumin	0%
7	Zulet *et al.*, 1999	*Cicer aretinum*	Meal	70	16	Casein	1%
8a	Lasekan *et al.*, 1995	*Pisum sativum*	Protein isolate	24	28	Casein	1%
8b	Lasekan *et al.*, 1995	*P. sativum*	Protein isolate	24	28	Casein	0%
9	Alonso *et al.*, 2001	*P. sativum*	Meal	57	15	Casein	0%
10a	Macarulla *et al.*, 2001	*Vicia faba*	Meal	68	14	Casein	1%
109b	Macarulla *et al.*, 2001	*V. faba*	Protein isolate	23	14	Casein	1%
11a	Dabai *et al.*, 1996	*Phaseolus vulgaris*	Meal	33	56	Casein	1%
11b	Dabai *et al.*, 1996	*Pisum sativum*	Meal	33	56	Casein	1%
11c	Dabai *et al.*, 1996	*Lens culinaria*	Meal	33	56	Casein	1%
11d	Dabai *et al.*, 1996	*Phaseolus lunatus*	Meal	33	56	Casein	1%

(a) 30 mg/kg by gavage.
(b) 50 mg/kg by gavage.

Table 20.5 Studies on rats fed diets containing pulses: serum lipids, glucose, insulin and liver cholesterol

Entry	Cholesterol		LDL-cholesterol		HDL-cholesterol		Triglycerides	
	Baseline value	Change (%)	Baseline value	Change (%)	Baseline value	Change (%)	Baseline value	Change (%)
1	1.65	−48.04	ND	ND	0.21	− 5.00	2.25	−16.08
2a	4.19	−34.80	ND	ND	ND	ND	0.61	+11.47
2b	4.19	−32.70	ND	ND	ND	ND	0.61	+ 4.92
3	3.84	−22.70					1.65	−10.30
4	5.60	−22.69	4.90	−30.16	0.70	+29.63	0.84	−16.22
5a	2.47	−17.81	ND	ND	ND	ND	1.11	−49.13
5b	2.47	−11.34	ND	ND	ND	ND	1.11	−12.31
6a	1.96	−15.82	ND	ND	ND	ND	0.19	−10.53
6b	1.96	−29.08	ND	ND	ND	ND	0.19	+ 5.26
6c	1.96	−34.18	ND	ND	ND	ND	0.19	+ 5.26
7	3.64	−34.06	2.27	−43.17	0.90	−17.78	2.36	−20.34
8a	4.14	−26.88	ND	ND	1.17	−57.78	1.78	−40.13
8b	3.97	−61.43	ND	ND	1.35	−30.67	1.51	−47.37
9	2.07	−17.50	0.54	−66.67	1.45	+5.66	0.795	−24.28
10a	3.48	−36.78	2.54	−56.30	0.92	+3.26	1.20	−39.17
10b	3.48	−29.89	2.54	−36.61	0.92	−9.78	1.20	−10.83
11a	6.72	−36.31	4.87	−52.99	0.98	+41.84	0.818	−39.12
11b	6.72	−13.99	4.87	−26.90	0.98	− 8.16	0.818	−11.37
11c	6.72	− 6.70	4.87	−32.24	0.98	−2.04	0.818	−33.25
11d	6.72	−23.96	4.87	−38.81	0.98	+40.82	0.818	−2.81

plasma triglyceride levels and the insulin/glucagon ratio, as well as non-esterified liver cholesterol and plasma LDL triglycerides levels. The yellow lupin diet increased plasma glucose and insulin, as well as liver total cholesterol compared to the casein and white lupin diets.

A protein isolate from *Lupinus albus* was analysed by Sirtori *et al.* (2004) using a pharmacological approach. Rats fed a hypercholesterolaemic diet containing 20 per cent casein and treated daily by gavage with 50 mg/rat of a lupin protein isolate for 14 days compared with vehicle only. Lupin-treated rats had 167 mg/dl total cholesterol and 62 mg/dL triglycerides, versus 216 mg/dL total cholesterol and 74 mg/dL triglycerides of controls, whereas glucose was unaffected. The daily dose given to animals is particularly low, comparable to that of some well-known lipid-lowering drugs, such as fibrates (Staels *et al.*, 1992). Isolated lupin protein fractions were also able to up-regulate the LDL receptors in HepG2 cells (Sirtori *et al.*, 2004). White lupin proteins seem therefore promising hypocholesterolaemic nutraceuticals.

The hypercholesterolaemic rat model was also used to study yellow pea (*Pisum sativum*): the diet contained 20 per cent pea proteins or casein. Pea proteins reduced cholesterol and triglycerides by 27 per cent and 40 per cent respectively, when cholesterol was included in diets. Plasma glucose and insulin levels were slightly lower in rats fed pea proteins versus those fed casein, apo Al level were also lower in rats fed pea proteins (Lasekan *et al.*, 1995).

Broad beans were studied for the first time in 1985 (Mengheri *et al.*, 1985). Recent work has compared the hypocholesterolaemic efficiency of a *Vicia faba*-protein isolate compared with the intact legume (Macarulla *et al.*, 2001). The protein isolate was prepared by isoelectric precipitation and spray dried. Rats fed on *Vicia faba* diets showed significantly lower body weights and energy intakes than rats fed casein. The whole seed diet induced a significant reduction in plasma triglycerides. Feeding dietary hypercholesterolaemic rats with diets containing faba bean seeds, or the protein isolate, induced a significant decrease of plasma (LDL+VLDL)-cholesterol (from 2.54 mmol/L of the casein + cholesterol diet to 1.11 mmol/L and 1.61 mmol/L respectively), but not of HDL-cholesterol. Liver cholesterol and triglycerides were also reduced. The faba bean-protein isolate was useful in improving the metabolic alterations induced by feeding a hypercholesterolaemic diet, compared with casein, but the effectiveness of whole seeds was higher as that of the protein isolate (Macarulla *et al.*, 2001).

Another legume that has been studied in detail is chickpea (Zulet *et al.*, 1999). The study was performed in rats fed a cholesterol-rich diet for 42 days. Lipid levels were markedly improved by feeding a chickpea diet for 16 days and liver glycogen deposition was also re-established. Data concerning carbohydrate utilisation indicated potential positive effects for diabetes therapy.

Dabai *et al.* (1996) have compared the hypocholesterolaemic effects of diets containing four different legumes: baked beans (*Phaseolus vulgaris*), marrowfat peas (*Pisum sativum*), lentils (*Lens culinaria* Medik) or butter beans (*Phaseolus lunatus*) in hypercholesterolaemic rats fed for 8 weeks. All experimental diets

were effective, but diets containing baked beans and butter beans were more potent at lowering raised cholesterol levels than diets based on marrowfat peas and lentils. Differences in cholesterol-lowering capacity of the various legume diets in this experiments were not associated with larger concentrations of faecal bile acids or neutral sterols. However, there was evidence that the inclusion of legumes in the diets reduced fecal excretion of secondary bile acids.

The general impression is that most legumes have an effectiveness very similar to soybean in rats fed hypercholesterolaemic diets and that this area is worthy of more detailed investigations in order to single out the bioactive component(s) of each legume.

Another very useful model is the pig as developed by Kingman *et al.* (1993). Thirty-six growing boars were randomly allocated, in groups of six to six diets, eaten continuously for 42 days. The diets fed were: (1) a semipurified (SP; control group 1) diet, (2) SP + 10 g cholesterol/kg (control group 2), and (3), (4), (5) and (6) SP + cooked legumes (70:30, wt./wt.; baked beans (*P. vulgaris*), peas (*P. sativum*), lentils (*L. culinaria*), and butter beans (*P. lunatus*)] + 10 g cholesterol/kg. Fasting blood samples were taken on days 0, 14, 28 and 42 for the detection of total plasma cholesterol, VLDL-, LDL- and HDL-cholesterol, and triglycerides. Between days 7 and 11 and days 28 and 32 complete 5-day faecal collections were made for the measurement of neutral, acidic and conjugated steroids. After 42 days, total cholesterol and VLDL + LDL-cholesterol levels (Figs 20.2) were raised significantly in all groups, but to different extents. Compared with control group 2, diet-induced hypercholesterolaemia was significantly inhibited in the groups consuming baked beans, peas, and butter beans, although HDL-cholesterol levels were unchanged. Faecal steroid excretion by the legume groups was not significantly different from that of control group 2. This agrees with the results in rats (Dabai *et al.*, 1996) and suggests that the mechanism for the hypocholesterolaemic effect does not involve increased hepatic bile acid synthesis and increased cholesterol clearance via the intestinal route, but probably rather involves a cellular mechanism, i.e. LDL-receptor up-regulation as already observed for soybean (Lovati *et al.*, 1987) .

20.5.1 Clinical studies

Anderson and Major (2002) have very recently published a meta-analysis of all published clinical studies on pulses, in total 11. The reader is recommended to read this paper to have a complete overview of available data in this field. As already indicated above, the meta-analysis on soy (Anderson *et al.*, 1995) has clearly shown that a clear-cut hypercholesterolaemia, related to a chronic depression of LDL receptors, is necessary to achieve an evident hypocholesterolaemic effect. Taking this into consideration, studies on normolipidaemic subjects will not be considered here. The experimental designs of these studies are quite varied (Table 20.6), but a common feature is that whole seeds are considered, thus not helping in singling out which component(s) is

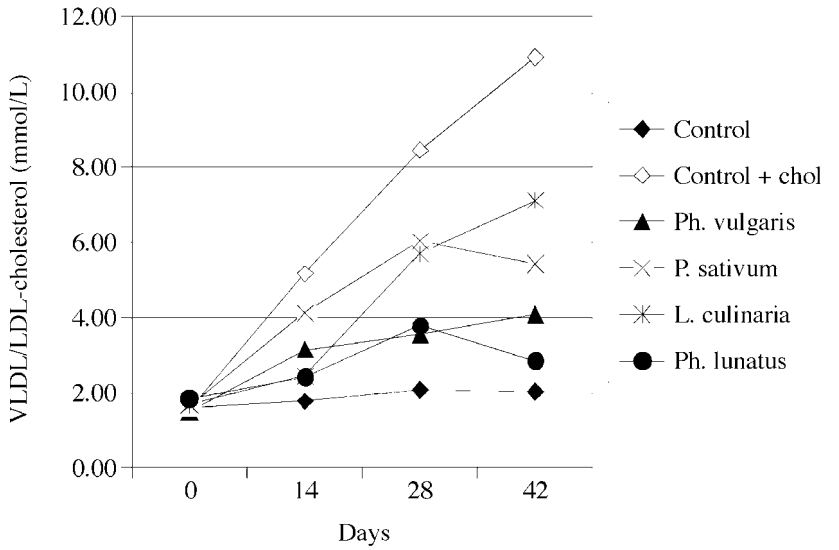

Fig. 20.2 Mean values for very low-density lipoprotein and low-density lipoprotein-cholesterol (VLDL + LDL-cholesterol) for four groups of pigs fed on different legume-containing diets and two control groups.

(are) responsible for the observed effects. In addition several of these studies take into consideration mixed legumes or beans and oat-bran. The most relevant results of these studies are summarised in Table 20.7.

Study 1 (Anderson *et al.*, 1984) was based on oat-bran and beans. After a control diet, 20 hypercholesterolaemic men (average cholesterol value 298 mg/dL) were randomly allocated to oat-bran or bean-supplemented diets for 21 days on a metabolic ward. Control and test diets provided equivalent energy, fat and cholesterol, but test diets had twice more total and 3-fold more soluble fibre. Bean diets decreased total cholesterol concentration by 18.5 per cent and LDL-cholesterol by 23 per cent. Triglycerides were in contrast unchanged.

Study 2 (Anderson *et al.*, 1990) was carried out on canned beans; 24 hyperlipidaemic men (average cholesterol value 295 mg/dL) ate one of three bean diets for 21 days in a metabolic ward. Diets A and B included 227 g canned beans (120 g beans with 107 g tomato sauce) daily, in a single dose for diet A and in a divided dose for diet B. Diet C included 182 g canned beans (162 g beans with 20 g tomato sauce) daily in a divided dose. Diets B and C, the most effective, lowered total and LDL-cholesterol and triglycerides by about 10 per cent.

In study 3 (Jenkins *et al.*, 1983) seven male mildly hyperlipidaemic patients (average cholesterol value 268 mg/dL) substituted approximately 140 g dried beans daily for other sources of starch in their diet over a 4-month period. After this, mean fasting triglycerides were reduced by 25 per cent, while total and LDL-cholesterol levels were 7 per cent lower than values during the previous five clinic attendances. While taking beans, a nonsignificant fall (0.7 kg) was

Table 20.6 Characteristics of clinical studies on hypercholesterolaemic or mild hypercholesterolaemic subjects on diets containing pulses

Entry	Literature	Type of pulse	Preparation	Amount (g/day)	Number of subjects	Duration (days)
1	Anderson *et al*., 1984	Beans	Cooked	120	10	21
2	Anderson *et al*., 1990	Beans	Canned	69	24	21
3	Jenkins *et al*., 1983	Mixed beans	Cooked/canned	140	7	120
4	Cobiac *et al*., 1990	Beans	Canned	145	20	28
5	Mackay and Ball, 1992	Beans + oat bran	Cooked	80	39	42
6	Fruhbeck *et al*., 1997	Field beans	Raw/cooked	90	10	30
7	Oosthuizen *et al*., 2000	Common beans	Extruded	110	22	28

Table 20.7 Clinical studies on hypercholesterolaemic or mild hypercholesterolaemic subjects: serum lipids responses

Entry	Cholesterol		LDL-cholesterol		HDL-cholesterol		Triglycerides	
	Control value (mmol/L)	Change (%)	Control value (mmol/L)	Change (%)	Control value (mmol/L)	Change (%)	Control value (mmol/L)	Change (%)
1	7.73	−18.5	5.71	−23.12	0.83	−12.05	2.63	−3.04
2	7.63	−10.4	5.19	−8.4	1.08	−6.9	2.89	−10.80
3	6.95	−7.1	4.88	−4.80	0.97	+14.50	3.03	−25.00
4	6.32	−0.47	4.63	−0.43	1.26	−3.17	1.25	+0.80
5	6.27	−0.80	4.19	0.00	1.15	+10.43	1.52	−0.66
6	6.23	−6.90	4.37	−7.32	1.05	+15.23	1.78	−32.58
7	6.15	0.00	4.87	+4.52	0.89	−5.62	1.64	+6.70

seen in body weight. Nevertheless no change was seen in macronutrient intake determined by 1-week diet histories recorded both before and four times during the study, although cholesterol intake decreased by 80 mg.

In study 4 (Cobiac *et al.*, 1990) the plasma cholesterol-lowering potential of canned baked beans was examined in a crossover comparison with canned spaghetti. The difference in total dietary non-starch polysaccharide (NSP) of 12 g daily (6.6 g difference in soluble NSP), was insufficient to alter cholesterol, HDL-cholesterol, triglyceride and glucose concentrations in 20 borderline hypercholesterolaemic men (average cholesterol 244 mg/dl). Thus, eating an average of six 440 g cans of this source of baked beans per week, large servings, does not lower plasma cholesterol when the intake of foods of animal origin is not decreased.

The effects of consuming oat-bran or beans were examined in 40 mildly hypercholesterolaemic men and women (average cholesterol 242 mg/dL) in study 5 (Mackay and Ball, 1992). The subjects were initially established on a low-fat background diet (29 per cent of energy from fat) and then 55 g low-fibre oat bran, 55 g high-fibre oat bran or 80 g mixed cooked beans were added to their diet in random order for 6-week periods. Total and LDL-cholesterol and triglycerides were unchanged.

In study 7 (Oosthuizen *et al.*, 2000) 22 hyperlipidaemic men (average cholesterol 237 mg/dL) were randomly assigned to one of two groups. After a run-in period of 4 weeks, during which subjects followed their normal diet with the exclusion of dried beans, group A received 110 g/day of extruded dry beans in the form of baked products for 4 weeks, while group B continued with the run-in diet. A wash-out period of 4 weeks followed, after which the experimental intervention was crossed-over. Extruded dry beans did not have significant effects on total serum cholesterol, LDL-cholesterol, apolipoprotein A or B, plasma fibrinogen and plasma viscosity concentrations. HDL-cholesterol concentrations decreased in both the dry bean and control periods.

Study 6 (Fruhebeck *et al.*, 1997) examined instead the effects of a 30 day dietary supplementation with broad bean flour in young men (aged 18–21 years; $n = 40$) with borderline hypercholesterolaemia (average cholesterol 240 mg/dl). All participants (groups A–C) consumed the same basic diet. The control group (A) consumed 90 g control flour daily, whereas the two bean diet groups received either 90 g cooked field bean flour (groups B) or 90 g raw field bean flour (group C) daily. After 30 days, total cholesterol, LDL-C and VLDL-C, triglycerides, glucose, insulin values were lower than initial ones in all subjects, who consumed the diets containing broad bean flour. The legume intake also increased glucagon and HDL-cholesterol levels. Only the results of group B (cooked broad beans) are reported in Table 20.7.

In conclusion a clear reduction in total and LDL-cholesterol is shown only in the three studies involving subjects with initial values above 6.9 mmol/L, whereas studies on borderline hypercholesterolaemic subjects (total cholesterol in the range 6.1–6.9 mmol/L) failed to show any effect. This points out once again the importance of enrolling only subjects affected by a real

hyperlipidaemia, when investigating the effects of a dietary intervention on the cholesterol levels.

Unfortunately, the limited number of studies and the fact that often they used mixtures of pulses or pulses plus oat-bran do not permit the comparison of the pulses and the components responsible for the observed activity cannot be identified. Anderson and Major (2002) proposed this order of importance: soluble dietary fibre, proteins, oligosaccharides, isoflavones, phospholipids, and fatty acids, phytosterols, saponins plus, possibly, other not yet recognised factors. Considering the very low level of isoflavones in pulses, the general impression is that these results above all confirm a minimal, if any role of isoflavones in the hypocholesterolaemic activity of soybean. The similarity of the structure of the vicilins of all grain legumes and the few studies on rats fed legume protein isolates (Lasekan *et al.*, 1995; Rahman *et al.*, 1996; Macarulla *et al.*, 2001; Sirtori *et al.*, 2004) are in favour of a major role of proteins in the hypocholesterolaemic effect exactly as in soybean. A further confirmation of this hypothesis comes from the lack of increased cholesterol clearance via intestinal excretion in the study on pigs (Kingman *et al.*, 1993) that supports a cellular mechanism, i.e. an up-regulation of LDL-receptors, as observed in soybean protein isolates (Lovati *et al.*, 1987).

Pulses are an extraordinary source of many potentially beneficial components and their consumption should be encouraged by physicians and nutritionists as a replacement for animal proteins.

20.6 Future trends

In the past 30 years many investigations have been devoted to the beneficial role of several food components, such as vegetable proteins, unsaturated fatty acids, plant sterols, viscous fibres, nuts, polyphenols, etc. Further research is certainly necessary to single out which are the major beneficial components of pulses and which is their mechanisms of action. The target of these studies will be to provide consumers with new foods and food ingredients for the preparation of a variety of functional foods, possibly with improved sensory characteristics.

However, the future of research in this field is certainly best represented by studies of possible synergies between different diet components. An example in this direction may be found in a very recent paper by Jenkins *et al.* (2003). Forty-six healthy, hypercholesterolaemic adults (25 men and 21 postmenopausal women) with a mean age of 59 years and body mass index of 27.6, were submitted to a randomised controlled trial. Participants were randomly assigned to undergo one of three interventions on an outpatient basis for 1 month: a diet very low in saturated fat, based on milled whole-wheat cereals and low-fat dairy foods (mean initial cholesterol 6.37 mmol/L, $n = 16$; control); the same diet plus lovastatin, 20 mg/day (mean initial cholesterol 6.64 mmol/L, $n = 14$); or a diet high in plant sterols (1.0 g/1000 kcal), soy protein (21.4 g/1000 kcal), viscous fibres (9.8 g/1000 kcal) and almonds (14 g/1000 kcal) (mean initial cholesterol

6.94 mmol/l, $n = 16$; dietary portfolio). The control, lovastatin and dietary portfolio groups had mean decreases in LDL-cholesterol of 8.0, 30.9, and 28.6 per cent, respectively. This experiment has shown that a well-planned dietary portfolio containing vegetable proteins from soy, plant sterols, nuts and viscous fibres may have the same effectiveness of a standard lovastatin treatment in controlling hypercholesterolaemia. This result is of extraordinary importance, because it has definitively demonstrated that a vegetarian diet may be as effective as one of the best hypocholesterolaemic drugs in reducing cardiovascular risk, without any side effects. Indeed we are now facing a completely new era in the prevention of cardiovascular risk by diet.

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21

Food fermentation by lactic acid bacteria for the prevention of cardiovascular disease

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21.1 Introduction to food fermentation

In this chapter we will concentrate on the components that are naturally produced by food micro-organisms and (could) provide protection against cardiovascular diseases. Most attention is focused on the B vitamins, which play a direct role in homocysteine metabolism and low-calorie sugars that can be used in low-carbohydrate, low-calorie diets. All the food components described in this chapter can be produced during food fermentations and, as such, represent a natural way of food enrichment.

Fermentation is one the oldest methods of food processing and can be considered as a desirable microbial activity in foods. This biochemical activity is caused by the enzymes of the fermenting micro-organisms. Fermented food products such as bread, beer, wine, soy sauce, yogurt, and cheese have been known for a long time. Traditionally, fermentation was used as a conservation technique for raw food material. After the fermentation, the fermented product has a much longer shelf-life than the unfermented product. Alternative conservations means were salting or drying. However, some products, e.g. dairy products and (alcoholic) beverages, could only be conserved by fermentation. During the fermentation process by food grade (lactic acid) bacteria or yeasts, food safety can be improved by the production of (lactic) acid or ethanol by these micro-organisms during growth on the raw food material.

Consequently the pH of the food matrix is decreased or the solvent concentration increased, resulting in a micro-environment that prevents or limits the growth of undesirable and/or pathogenic bacteria, yeasts, or molds. Well-known examples are the production of yoghurt or sourdough bread. Another

food preservation property of fermentation occurs through the direct production by the fermenting micro-organisms of compounds with anti-bacterial or anti-fungal properties. For example lactic acid bacteria have the capacity to produce specific proteins or peptides, bacteriocins, that may directly interfere with the cell membrane of other (harmful) micro-organisms. One of such bacteriocins is nisin, which can be produced by *Lactococcus lactis* (Hurst, 1966). Nisin is present in some fermented dairy products, and is also produced and merchandized as a natural additive for the preservation of all kinds of food products (Cleveland *et al.*, 1991).

Nowadays, food safety and shelf-life can be guaranteed by refrigeration or by direct addition of preservatives to the (processed) food. Nevertheless, there is still a high demand for fermented foods because of their added value in terms of consistency, color, and especially flavor and taste. The current small price difference between natural and chemically produced flavors, combined with the consumers' increasing demand for more natural ('green') products, has increased the interest of the food industry in fermentation for flavor improvement of food products. Good examples are some flavor forming capacities of lactic acid bacteria. Proteases and peptidases, which constitute the proteolytic system of lactic acid bacteria (Kunji *et al.*, 1996) are responsible for the release of amino acids. Subsequent amino acid degradation is important for the formation of flavor compounds such as aldehydes, thiols, and keto acids in fermented dairy products. The degradation routes of amino acids generally involve different reactions, including deamination, transamination, decarboxylation, and cleavage of the amino acid side chain. Aromatic, branched-chain, and sulfurous amino acids, in particular, are precursors of compounds with, respectively, floral, cheesy, and sulfur flavors. Such compounds have been found in various cheeses, including semihard cheeses such as Gouda and Cheddar. Sugar conversion via glycolysis can also lead to the production of flavor compounds such as diacetyl and acetaldehyde, the main flavor components in butter (Hugenholtz *et al.*, 2000) and yoghurt (Chaves *et al.*, 2002), respectively.

Fermentation can also contribute to the nutritional value of the fermented food by increasing the bio-availability of nutrients or by the production of vitamins and other nutritional components by the fermenting micro-organisms. In foods of plant origin, such as cereals, oilseed, roots, and others, antinutritional factors (ANF) may inhibit efficient digestion and absorption processes. This is relevant not only for humans, but also for animals. An example of an ANF is phytic acid that forms complexes with metal ions and in this way limits the availability of these ions for uptake in the gastrointestinal tract. Several lactic acid bacteria, molds and yeast produce the enzyme phytase that degrades phytic acid during fermentation. Other ANF that can be removed from the raw food material by micro-organisms are galacto-oligosaccharides, which may cause flatulence and cramps, protease inhibitors, such as trypsin inhibitors, which limit the degradation of proteins and oligosaccharides, and antinutritional glycosides, for example linamarin, a cyanogenic glycoside present in bitter cassava.

The nutritional value of fermented food products is also greatly enhanced, compared with raw food material, by the production of nutraceuticals, such as antioxidants, vitamins, low-calorie sugars, and oligosaccharides by the fermenting micro-organisms (Hugenholtz *et al.*, 2002). Compounds, like glutathione, or the β-carotenoid lycopene, have antioxidative properties and can prevent oxidative stress that may damage tissue DNA. These compounds cannot be produced by humans, and should be present in the diet in sufficient concentrations.

Vitamins are also essential for human health. Yeast and lactic acid bacteria are a particularly rich source of antioxidants and vitamins, not only because of the uptake and subsequent accumulation of these vitamins in the microbial cells, but also because of the biosynthetic capacity of these micro-organisms to produce such compounds. More recent applications of fermentation is for the production of probiotics, fermented foods that contain living lactic acid bacteria that exert a positive effect in the intestine after consumption. Research has focused on probiotic bacteria such as lactobacilli and bifidobacteria, that can survive the transport through the gastrointestinal tract and that can colonize the small or large intestine. In most cases fermented, probiotic foods are yoghurt-like drinks with a high concentration of these lactic acid bacteria. Some beneficial attributes of probiotics in relation to cardiovascular disease, such as the cholesterol-lowering activities, are briefly discussed towards the end of this chapter. (For a recent overview of the effect of probiotics see the proceedings of the Montreal International Symposium on probiotics and health; Roy, 2002.)

In the remainder of this chapter we will focus on the fermenting capacities of lactic acid bacteria and present an overview of the production of B vitamins, on the production of low-calorie sugars and some probiotic effects related to cardiovascular disease, all present in fermented foods produced by lactic acid bacteria.

21.2 Bioengineering of lactic acid bacteria

Lactic acid bacteria are Gram-positive, non-spore-forming bacteria and are naturally present in raw food material and in the human gastrointestinal tract. They have a long history of use by humans for food production and food preservation. The lactic acid bacteria group include rod-shaped bacteria, such as lactobacilli, and sphere-shaped bacteria, such as streptococci, lactococci, pediococci, and leuconostocs. Lactic acid bacteria are widely used as starter cultures for fermentation in the dairy, meat and other food industries. Their properties have been used to manufacture products such as cheese, yogurts, fermented milk products, beverages, sausage, and olives. Lactic acid bacteria have a relatively simple metabolism and can easily serve as cell factories for the production of flavor compounds (e.g. diacetyl), thickeners (e.g. exopolysacharides), antioxidants (e.g. lycopene), vitamins (e.g. folate), antibiotics (e.g. lantibiotics), oligosacharides, and many more primary or

secondary metabolites. *Lactococcus lactis* is by far the most extensively studied lactic acid bacterium and displays a relatively simple and well-described metabolism where the sugar source is converted mainly to lactic acid. Over the last decades a number of elegant and efficient genetic tools have been developed for this starter bacterium. These tools are of critical importance in metabolic engineering strategies that aim at inactivation of undesired genes and/or (controlled) overexpression of existing or novel ones. In this respect, especially the nisin-controlled expression (NICE) system for controlled heterologous and homologous gene expression in *Lactococcus lactis* has proven to be very valuable (de Ruyter *et al.*, 1996).

The design of a metabolic engineering strategy requires a proper understanding of the pathways that are manipulated and the genes involved, preferably combined with knowledge about fluxes and control factors. Most of the metabolic engineering strategies so far applied in lactic acid bacteria are related to primary metabolism and comprise efficient rerouting of the lactococcal pyruvate metabolism to end products other than lactic acid, including ethanol, diacetyl, and alanine, resulting in high production of both natural and novel end products. Metabolic engineering of more complicated pathways involved in secondary metabolism has only recently begun by the engineering of exopolysaccharide and folate production in *L. lactis* (Boels, 2002; Sybesma, 2003).

21.2.1 Lactic acid bacteria as cell factories for production of B vitamins

Folate (vitamin B9, B10, or B11), cobalamin (vitamin B12), riboflavin (vitamin B2), and pyridoxine (vitamin B6) are present in foods derived from animals, plants (not cobalamin), or in fermented foods. Lactic acid bacteria have the capacity to produce B vitamins that play an important role in one-carbon metabolism (see Box 1 on page 452), the removal of homocysteine and the prevention of vascular diseases. Over the past decade it has emerged that a moderate elevation in plasma concentrations of the amino acid homocysteine (total homocysteine) constitutes a risk factor for atherosclerotic vascular disease in the coronary, cerebral, and peripheral vessels, but the relationship has not been proven to be totally causal. Homocysteine is a sulfur amino acid, which is formed from the essential amino acid methionine. The removal of homocysteine may occur either via transsulfuration to cysteine by two pyridoxal phosphate-dependent reactions or remethylation to methionine. As a result of the ability of lactic acid bacteria to produce B vitamins, levels of these vitamins in fermented (dairy) products are higher compared with the corresponding non-fermented (dairy) products. The natural diversity among dairy starter cultures with respect to their vitamin production can be exploited to design new starter cultures that yield fermented dairy products with elevated vitamin levels. In general, this strategy of natural selection will lead to only limited increase of vitamin levels. Metabolic engineering will be required for more spectacular increase of vitamin levels.

BOX 1: One-carbon metabolism, the link between B vitamins

Folate (vitamin B9, B10, or B11), cobalamin (vitamin B12), riboflavin (vitamin B2), and pyridoxine derivatives (vitamin B6) are B vitamins that cannot be synthesized by humans and that are essential in the prevention of the occurrence of vascular diseases, predominantly because of their involvement in the reduction of the concentration of homocysteine. Folate derivatives are operative in a complex interaction between the donation and reception of carbon groups by various essential cell metabolites that are involved in the synthesis of essential components and prevention of accumulation of undesired components. Besides folate derivatives also other B vitamins such as riboflavin derivatives, cobalamin, and pyridoxal phosphate, are active in the one-carbon metabolism (Fig. 21.1). Serine hydroxymethyl transferase transfers a carbon group from serine to tetrahydrofolate (THF) under formation of glycine and 5, 10-methylene THF. The latter compound can be transformed into different folate derivatives and used in three reactions. Thymidilate synthase uses the carbon group of 5, 10-methylene THF to catalyze the conversion of dUMP to dTMP, generating THF, which can be reused in one-carbon metabolism. Methylenetetrahydrofolate dehydrogenase catalyzes the transformation of 5, 10-methylene THF into 10-formyl THF via formation of 5, 10-methenyl THF. 10-formyl THF is an important cofactor in the biosynthesis of GTP. The intermediate 5, 10-methenyl THF can also be serve as substrate for serine hydroxymethyl transferase yielding 5-formyl THF that possibly regulates the one-carbon metabolism (Stover and Schirch, 1993). The enzyme 5, 10-methylene THF reductase, which uses the riboflavin derivative FAD, catalyzes the transformation of 5, 10-methylene THF into 5-methyl THF.

Methionine synthase is the enzyme that effectively reduces the concentration of the harmful homocysteine by forming methionine and THF from homocysteine and 5-methyl THF respectively. This reaction is catalyzed by cobalamin. The generated THF can be reused in one-carbon metabolism. Methionine can also be formed by the hepatic enzyme betaine-homocysteine methyltransferase, which uses betaine as a methyl donor. Meanwhile the methionine is converted into *S*-adenosyl methionine that is required for other methylation reaction such as the production of methylated lipids, myelin basic protein, 3, 4-dihydrocyphenylalanine, or DNA. During this process methionine is converted into first adenosyl homocysteine and secondly into homocysteine, which should be removed again. An alternative route for removal of homocysteine could occur via the transsulfuration pathway where pyridoxal phosphate catalyzes the enzymatic reactions of cystathionine β-synthase and γ-cystathionase, which yield cysteine and α-aminobutyrate from serine and homocysteine. In summary, the vitamins folate, riboflavin, cobalamin, and pyridoxal phosphate are all involved in the prevention of hyperhomocysteinaemia (Bailey and Gregory, 1999).

In both developing countries and developed countries, many population groups still suffer from deficiency of different B vitamins and have an increased risk for the occurrence of vascular diseases (Ebadi *et al.*, 1982; Carmel, 1996; Brussaard *et al.*, 1997; Baik and Russel, 1999; Rogers *et al.*, 2003; Cunha *et al.*, 2001; Gielchinsky *et al.*, 2001; Herrmann, 2001; Konings *et al.*, 2001; O'Brien *et al.*, 2001; Refsum *et al.*, 2001; Klerk *et al.*, 2002; Powers, 2003; Sunder-Plassmann and Fodinger, 2003). Also, the occurrence of genetic polymorphisms may increase the dependence of certain individuals for higher concentrations of one of these four B vitamins (Hustad *et al.*, 2000; McNulty *et al.*, 2002; Jacques *et al.*, 2002). Several studies have analyzed the protective effect of folate, riboflavin, vitamin B12, and vitamin B6 on lowering homocysteine levels or decreasing the risk for coronary heart disease (Dalery *et al.*, 1995; Verhoef *et al.*, 1996; Folsom *et al.*, 1998; Siri *et al.*, 1998; Lobo *et al.*, 1999; Medrano *et al.*, 2000; Selhub, 2002; Klerk *et al.*, 2002; Quinlivan *et al.*, 2002). There are also studies that conclude that folate is the only B vitamin that is independently, and inversely, related with the total plasma homocysteine concentration (de Bree *et al.*, 2001) and not all studies are totally unambiguous. Conflicting results about the negative effect of homocysteine on cardiovascular diseases also come from prospective studies. Trials which are now in progress may clarify the 'causality' of high homocysteine concentrations and will assess the value of homocysteine-lowering therapy (Herrmann, 2001).

The most important cause of vitamin deficiency is limited dietary intake. Moreover, recent studies have shown that fermented foods are among the 15 most important food items contributing to the folate intake (Konings *et al.*, 2001). Therefore, it is expected that the generation of fermented functional foods with increased levels of all four B vitamins may strongly contribute to a reduction in individuals with a vitamin deficiency.

21.3 Microbial production of folate

Folate usually refers to a family of vitamins with related biological activity. Other terms that are used interchangeably are folic acid and folacin. However, folic acid is actually the name of the chemically synthesized pteroyl glutamic acid. The term 'folic acid' was first used by Mitchell in 1941 which referred to a growth factor found in spinach leaves (which are a good source of this vitamin). Vitamin B9, vitamin B10, and vitamin B11 are other terms used to identify this vitamin. Folate is involved in various essential functions in cell metabolism, such as the synthesis of DNA and RNA. The vitamin is present in various common foods such as orange juice, dark green leafy vegetables, asparagus, strawberries, legumes, meat (liver), and fermented (dairy) products. In these natural products, folates are present with their pteridine ring reduced to 7, 8-dihydrofolate, or 5, 6, 7, 8-tetrahydrofolate (THF). The tetrahydrofolates are present in unsubstituted form or substituted with various one-carbon groups, such as formyl, methenyl, methylene, methyl, and others, that have an active

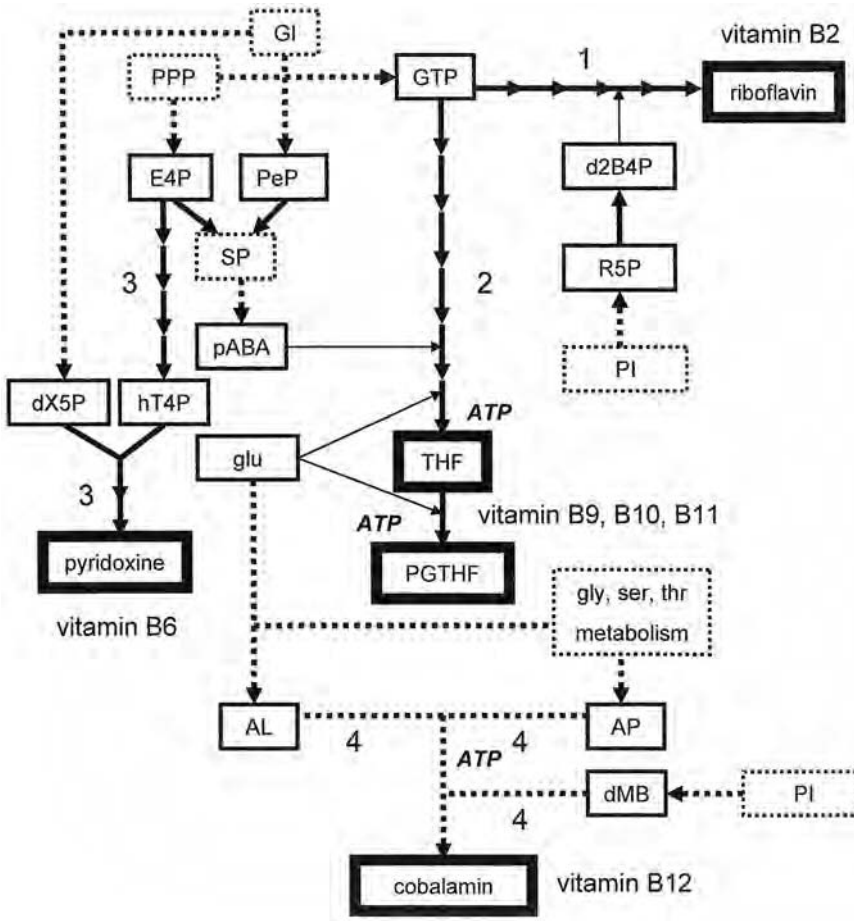

Fig. 21.1 Summary of microbial biosynthetic pathways of B vitamins. AL = 5-amino-levunilate. AP = 1-amino-propan-2-ol. dX5P = 1-deoxy-D-xylulose-5-phosphate. d2B4P = 3,4-dihydroxy-2-butanone 4-phosphate. dMB = Dimethyl benzimidazole. E4P = erythrose-4-phosphate. Glu = glutamate. Gl = glycolyse. GTP = guanosine 5′-triphosphate. hT4P = 4-hydroxy-L-threonine-4-phosphate. pABA = *para*-aminobenzoic acid. PI = pentose interconversion. PPP = pentose phosphate pathway. PeP = phosphoenolpyruvate. PGTHF = polyglutamyl THF. R5P = ribulose-5-phosphate. SP = shikimate pathway. THF = tetrahydrofolate.

1, *rib* genes. 2, *fol* genes. 3, *pyr* genes. 4, *hem/cob/cbi* genes. Solid lines, enzymatic reaction. Dotted lines, combined enzymatic reactions.

function in C1-metabolism (see Box 1 on page 452 and Fig. 21.1). The recommended daily intake (RDI) for folate for an adult is 400 μg. For pregnant women a daily dose of 600 μg is recommended.

Folate plays a central role in one-carbon metabolism and consequently is directly involved in the continuous removal of the cardiovascular disease risk factor homocysteine (see Box 1). Furthermore, based upon observations in

human studies on folate derivatives, homocysteine, and endothelial function, it was reported that folate supplementation in high doses may have antioxidative effects, may contribute to restoration of impaired NO metabolism and may benefit endothelial function. These effects are independent of the decrease of the homocysteine concentrations (Ashfield-Watt *et al.*, 2001, Stanger and Weger, 2003).

The biosynthetic pathway of folate is composed of several parts responsible for the synthesis of the different moieties of folate:

- The pteridine proportion originates from GTP, that is synthesized in the purine biosynthesis pathway.
- *p*-Aminobenzoic acid originates from chorismate and can be synthesized via the same biosynthesis pathways required for the aromatic amino acids, involving glycolysis, pentose phosphate pathway, and shikimate pathway.
- The third component of a folate molecule is glutamate, which is not synthesized by lactic acid bacteria but is taken up from the cultivation medium.

In a series of reactions that require six enzymes, these three components are modified and coupled to synthesize folate. GTP cyclohydrolase I (*folE*, EC 3.5.4.16), catalyzes the reaction from GTP to dihydroneopterin triphosphate, releasing one formate molecule. The phosphate residues are then removed by the action of one or more phosphatases (probably encoded by *folQ*). Subsequently, dihydroneopterin aldolase (*folB*, EC 4.1.2.25) cleaves the product into glycolaldehyde and 6-hydroxymethyl-7,8-dihydropterin, which is converted to 6-hydroxymethyl-7, 8-dihydropterin pyrophosphate by 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase (*folK*, EC 2.5.1.15). Dihydropteroate synthase (*folP*, EC 2.7.6.3) couples *p*-aminobenzoate to produce 7, 8-dihydropteroate. Addition of glutamate to the carboxy-end of *p*-aminobenzoate by the bifunctional protein folate synthetase/polyglutamyl folate synthetase (*folC*, EC 6.3.2.12/17) produces dihydrofolate. After further reduction to tetrahydrofolate, by dihydrofolate reductase (*folA*, EC 1.5.1.3), polyglutamyl folate is produced by subsequent addition of glutamate molecules to the glutamyl residue of folate by the activity of polyglutamyl folate synthetase (*folC*, EC 6.3.2.17). Different substituted folate derivatives are synthesized in a number of enzymatic steps involved in C1-metabolism and used for specific metabolic activities.

The partial sequencing of *L. lactis* subsp. *cremoris* MG1363 has revealed the existence of a folate gene cluster containing all genes encoding the folate biosynthesis pathway. Recently, metabolic engineering of the folate biosynthesis in *L. lactis* has shown that the folate production can be increased more than 50-fold by the overexpression of the complete folate gene cluster (Sybesma, 2003). When this strain would be used for the production of a fermented milk product the consumption of 50 ml of such a fermented product, instead of 2 liters of normal fermented milk, would be enough to obtain the RDI for folate (400 μg). Moreover, the control of the polyglutamyl folate tail length could also benefit

the folate accessibility and bioavailability upon the consumption of natural folates (Sybesma *et al.*, 2003b). Alternatively, it was shown that by optimization of the fermentation conditions for *L. lactis* or *Streptococcus thermophilus* folate, production levels could be significantly increased by pH control, by the addition of *p*-aminobenzoic acid, by reducing the concentration of aromatic amino acids, and by limitation of the growth rate. Higher folate production can be explained by higher production of cell material and by specific stimulation of the folate biosynthesis (Sybesma *et al.*, 2003a).

One clear advantage of consumption of natural folates over the chemically produced pteroyl glutamic acid (oxidized form of dihydrofolate) is related to early detection of cobalamin deficiency. The chemically synthesized folate is transformed into 5-methyl THF during transit through the gut mucosa. However, under circumstances of cobalamin deficiency, the methionine synthase may have a reduced activity and other folate derivatives no longer produced in the blocked one carbon metabolism. This creates a pseudo folate deficiency and may hide the cobalamin deficiency (Scott, 1999). The consumption of other natural folate derivatives than 5-methyl THF is assumed to result in a better continuation of the one-carbon metabolism and cobalamin deficiency may be earlier diagnosed.

21.4 Microbial production of riboflavin

Riboflavin is a precursor of the coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) that are required in reactions such as the enzymatic oxidation of carbohydrates. Riboflavin and its derivatives are found in a wide variety of foods, although milk and milk products make a particularly important contribution to the riboflavin intake of populations in Western countries. This vitamin can also be obtained in the diet from meat (liver), vegetables, fermented (dairy) products, eggs, and fortified foods such as bread and cereal products. The daily recommended intake of dietary riboflavin for an adult is 1.6 mg.

Riboflavin is also involved in one carbon metabolism via the enzyme 5, 10-methylene tetrahydrofolate reductase, which uses the riboflavin derivative FAD for the catalysis of 5, 10-methylene THF into 5-methyl THF (see Box 1 on page 452 and Fig. 21.1). Consequently, riboflavin is necessary for the removal of homocysteine from the blood. The importance of riboflavin in the prevention of the occurrence of vascular diseases was mainly seen among individuals that have a mutation in the gene encoding methylene tetrahydrofolate reductase (Hustad *et al.*, 2000; McNulty *et al.*, 2002; Jacques *et al.*, 2002), but also among healthy subjects (Moat *et al.*, 2003). Further evidence comes from a report of elevated homocysteine in the skin of riboflavin-deficient rats (Lakshmi *et al.*, 1990). Moreover, dihydroriboflavin, produced from riboflavin by NADPH-dependent flavine reductase, has been shown to be an efficient reducing agent for heme proteins containing ferric iron and therefore a potential antioxidant that could protect tissues from oxidative injury (reviewed by Powers, 2003).

Riboflavin was traditionally manufactured using chemical processes, but in recent years biotechnological processes have become increasingly popular using organisms such as *Bacillus subtilis, Ashbya gossypii* and *Candida famata* (Stahmann *et al.*, 2000). Riboflavin can also be produced by several lactic acid bacteria and its biosynthetic pathway has been elucidated. In *L. lactis* the corresponding biosynthetic genes have been identified, as well. Also for this B vitamin, GTP is the main precursor. GTP cyclohydrolase II/3, 4-dihydroxy-2-butanone 4-phosphate synthase (*ribA*, EC 3.5.4.25) catalyzes the formation of the riboflavin intermediate product 2, 5-diamino-6-hydroxy-4-(5-phospho-ribosylamino) pyrimidine. In a subsequent reaction diaminohydroxyphospho-ribosylaminopyrimidine deaminase/5-amino-6-(5-phosphoribosyl-amino) uracil reductase (*ribG*, EC 3.5.4.26) forms 5-amino-6-(5-phosphoribosylamino)uracil which is reduced by 5-amino-6-(5-phosphoribosylamino)uracil reductase (*ribG*, EC.1.1.1.93). Finally, riboflavin is formed by the combined activity of the beta (*ribH*, EC 2.5.1.9), and alpha chain (*ribB*, EC 2.5.1.9) of riboflavin synthase.

Similarly as described for the increased production of folate, a hyper riboflavin-producing *L. lactis* strain was generated. By overexpression of all the genes involved in riboflavin biosynthesis *L. lactis* was changed from riboflavin consumer into riboflavin producer and almost 9 mg/L riboflavin was produced, which equals approximately nine times the RDI for riboflavin (Burgess *et al.*, 2003). As an alternative for metabolic engineering, a natural, riboflavin-overproducing strain was isolated by exposing the bacterium to the riboflavin analogue roseoflavin. In the roseoflavin-resistant variants, riboflavin production was observed (1 mg/L). This mutant contained a single base change in the regulatory region upstream of the riboflavin biosynthetic genes (Sybesma *et al.*, 2004).

21.5 Microbial production of vitamin B12 (cobalamin) and vitamin B6 (pyridoxine)

21.5.1 Cobalamin

Cobalamin, vitamin B12, belongs to the corrinoids, which are compounds having a corrin nucleus in common. The complicated biosynthetic pathway of cobalamins occurs only in bacteria and archaea (Schneider and Stroinski, 1987; Martens *et al.*, 2002) and in some algae. Usual dietary sources of B12 are animal food products (meat, milk, eggs, and shellfish) (Ball, 1998) or fermented food products. Another source of B12 for humans is the vitamin B12 synthesis by human small intestinal bacteria (Albert *et al.*, 1980). In humans, cobalamin is required in trace amounts to assist the actions of only two enzymes, methionine synthase and (*R*)-methylmalonyl-CoA mutase. The daily recommended intake for vitamin B12 is $1-2\,\mu$g. Cobalamin is also involved in one carbon metabolism and catalyses the conversion of homocysteine into methionine by methionine synthase (see Box 1 on page 452 and Fig. 21.1).

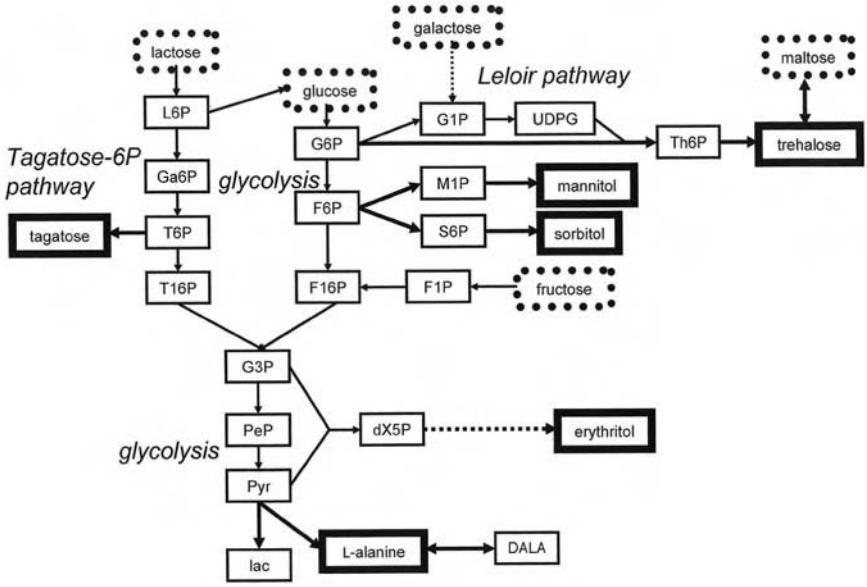

Fig. 21.2 Metabolic pathways for conversion of high-calorie sugars lactose, glucose, fructose, galactose and manose into low-calorie sugars L-alanine, sorbitol, manitol, erythritol, threhalose and tagatose in micro-organisms. Solid lines, single enzymatic reaction. Dotted lines, combined enzymatic reactions. Thin lines represent common metabolic reactions in micro-organisms. Bold lines represent enzymatic reactions that should be introduced or changed by metabolic engineering in most micro-organisms. dAla = D-alanine. dX5P = 1-deoxy-D-xylulose-5-phosphate. F16P = fructose-1,6-diphosphate. F1P = fructose-1-phosphate. F6P = fructose-6-phosphate. Ga6P = galactose-6-phosphate. G1P = glucose-1-phosphate. G6P = glucose-6-phosphate. G3P = glyceraldehyde-3-phosphate. Lac = lactate. L6P = lactose-6-phosphate. M1P = mannitol-1-phosphate. Pep = phosphoenolpyruvate. Pyr = pyruvate. S6P = sorbitol-6-phosphate. T16P = tagatose-1,6-biphosphate. T6P = tagatose-6-phosphate. Th6P = trehalose-6-phosphate. UDPG = UDP-glucose.

Cobalamins are synthesized by certain micro-organisms from the following substrates: aminolevulinic acid, *S*-adenosyl-L-methionine, cobalt, glutamine, (*R*)-1-amino-2-propanol, and 5, 6-dimethylbenzimidazole (Fig. 21.2). Two pathways for formation of the corrin ring have been found: (1) an aerobic pathway (in *Pseudomonas denitrificans*), and (2) an anaerobic pathway (in *Propionobacterium shermanii* and *Salmonella typhimurium*), which differ in the mechanism of cobalt insertion (Roth *et al.*, 1996). The aerobic process utilizes two enzymes and is dependent on molecular oxygen, in stark contrast to the anaerobic mechanism which is controlled by cobalt and requires only one enzyme (Roessner *et al.*, 2001). Most of the steps in the pathway for biosynthesis of vitamin B12 have been characterized in *Pseudomonas denitrificans* (Debussche *et al.*, 1993). No less than 22 *cob* genes involved in cobalamin biosynthesis have been isolated and the functions of the majority of the polypeptides encoded by these genes have been identified. The biosynthetic route to adenosylcobalamin from its five-carbon precursor, 5-aminolevulinic

acid, can be divided into three sections: (1) the biosynthesis of uroporphyrinogen III from 5-aminolevulinic acid, which is common to both pathways; (2) the conversion of uroporphyrinogen III into the ring-contracted, deacylated intermediate precorrin 6 or cobalt-precorrin 6, which includes the primary differences between the two pathways; and (3) the transformation of this intermediate to form adenosylcobalamin. A detailed description of this pathway in *Pseudomonas denitrificans* is exemplified by Scott and Roessner (2002).

Vitamin B12 for human and animal nutrition, is basically produced by fermentation using large-volume cultures of the bacteria *Pseudomonas denitrificans*, *Propionobacterium shermanii* and *Propionobacterium freudenreichii* (Hugenholtz *et al.*, 2001; US6156545, 2000). Until recently cobalamin production was not known to be produced by lactic acid bacteria that could be applied for enrichment of fermented foods. However, Taranto *et al.*, (2003) described the biosynthesis of cobalamin by a *Lactobacillus reuteri* strain. This bacterium, which also has certain probiotic properties, may be used to increase the cobalamin levels in fermented foods. Sequence analysis of the genome of this strain has identified the presence of almost all genes necessary for the cobalamin biosynthesis (Santos and Vera, personal communications).

21.5.2 Pyridoxine

Vitamin B6 (pyridoxine) is a group name for pyridoxal, pyridoxamine, and pyridoxal-5-phosphate and derivatives. Good sources of pyridoxine and derivatives are eggs, chicken, carrots, fish, liver, kidneys, peas, wheat germ, walnuts, and fermented food products. This vitamin is necessary for proper absorption of vitamin B12 and also assists in the removal of homocysteine via the transsulfuration pathway (see Box 1 on page 452 and Fig. 21.1), where pyridoxal phosphate catalyzes the enzymatic reactions of cystathionine β-synthase and γ-cysthationase, which yield cysteine and α-aminobutyrate from homocysteine and serine. The RDI of dietary pyridoxine or pyridoxine derivatives for an adult is 1.6 mg. Pyridoxine and pyridoxine 5′-phosphate are manufactured chemically, but the active pyridoxal′ phosphate is produced biochemically by pyridoxamine oxidase.

Biochemistry, enzymology, and genetics of *de novo* vitamin B6 biosynthesis have been primarily investigated in *Escherichia coli* (Laber *et al.*, 1999). In *E. coli* the coenzyme pyridoxal 5′-phosphate is synthesized *de novo* by a pathway that is thought to involve the condensation of 4-(phosphohydroxy)-L-threonine and 1-deoxy-D-xylulose(-phosphate). Both precursors are ultimately derived from glucose. Only five pdx genes, *pdxB, serC* (*pdxF*), *pdxA, pdxJ,* and *pdxH,* have been identified in *E. coli*. *pdxB* codes for the enzyme erythronate-4-phosphate dehydrogenase that oxidizes D-erythroic acid 4-phosphate to 2-oxo-D-erythroic acid 4-phosphate (EC 1.1.1.-). The latter compound is transaminated to 4-hydroxy-L-threonine 4-phosphate by 3-phosphoserine transaminase (*serC/pdxF* EC 2.6.1.62). The protein products of *pdxA,* annotated as

4-hydroxythreonine-4-phosphate dehydrogenase, and *pdxJ,* annotated as pyridoxal phosphate biosynthetic protein, play a role in the condensation reaction that generates pyridoxine or pyridoxine 5′-phosphate from the two intermediates, 4-hydroxy-L-threonine(-5-phosphate) and 1-deoxy-D-xylulose(-5-phosphate). The protein product of pdxH is pyridoxol 5-oxidase (EC 1.1.3.13), which converts pyridoxol phosphate into pyridoxal phosphate. The precursor 1-deoxy-D-xylulose(-phosphate) is formed by the condensation of D-glyceraldehyde 3-phosphate and pyruvic acid by the enzyme pyruvate dehydrogenase accompanied by decarboxylation (Hill *et al.*, 1996; Laber *et al.*, 1999).

Knowledge of vitamin B6 biosynthesis in organisms other than *E. coli* is sparse (Drewke and Leistner, 2001). Database searches revealed that the key enzymes involved in ring closure of the aromatic pyridoxin ring (PdxA; PdxJ) are present mainly in genomes of bacteria constituting the gamma subdivision of proteobacteria. However, the Gram-positive *Bacillus subtilus* subjected to random mutagenesis was also reported to produce vitamin B6 (Pflug and Lingens, 1978). The biosynthesis of pyridoxine in food grade *Saccharomyces cerevisiae* is also known (Ishida and Yamada, 2002) and high concentrations of pyridoxine were also detected in food yeast *Kluyveromyces fragilis* (Paul *et al.*, 2002). To our knowledge, no lactic acid bacteria are known that are able to produce vitamin B6.

The application of metabolic engineering of food grade lactic acid bacteria for the *in situ* production of vitamin B6 has not yet begun. Analysis of the genome of *Lactobacillus plantarum* (Kleerebezem *et al.*, 2003) and *Lactococcus lactis* (Bolotin *et al.*, 2001) reveals that the biosynthesis of the pyridoxine precursor 1-deoxy-D-xylulose 5-phosphate, if it occurs via the condensation of D-glyceraldehyde 3-phosphate and pyruvic acid by the enzyme pyruvate dehydrogenase accompanied by decarboxylation, may be in these food grade lactic acid bacteria. However, the biosynthesis of the other precursor 4-hydroxy-L-threonine from erythrose-4-phosphate is probably not possible, because of the absence of *pdxB* encoding erythronate-4-phosphate dehydrogenase. Moreover, the genes encoding for the enzymes responsible for the union of 1-deoxy-D-xylulose 5-phosphate and 4-hydroxy-L-threonine *pdxA* and *pdxJ* are also absent in the genome of these sequenced lactic acid bacteria. The genome analysis suggest that only *serC/pdxF,* encoding 3-phosphoserine aminotransferase, is present in these organisms. Therefore, the expression of *pdxB*, *pdxA*, and *pdxJ* in, for instance, the model organism *L. lactis* would be an easy way to confirm if the requirement for pyridoxine can be overcome. The expression of *pdxH* could confirm whether pyridoxal-phosphate could be transformed into different pyridoxal derivatives in a lactic acid bacterium.

21.5.3 Conclusions about bacterial vitamin production

The development of fermented foods, enriched in vitamins by using vitamin-producing starter cultures, could compensate for the general occurrence of B vitamin deficiencies. Folate and riboflavin can be produced by a large number of

lactic acid bacteria. The cobalamin production was also recently detected in lactic acid bacteria and only the production of pyridoxine has not yet been observed in lactic bacteria. However, it is expected that in the near future metabolic engineering will generate starter cultures that will result in the in situ production of all of these four B vitamins. Recently, a *Lactococcus lactis* strain was developed that overproduced both folate and riboflavin by combining metabolic engineering and natural selection (Sybesma *et al.*, 2004).

21.6 Low-calorie substitutes for sugar: polyols and other sweeteners

Obesity is a rapidly spreading disease among the human population. It is no longer a freak phenomenon observed in lower-educated but rich families in both Western and Asian societies, but it is now appearing in families all over the world, including the well educated. Many current health problems are supposedly related to obesity and it has been estimated that over the past 5 years more than 10 per cent of the health-care related expenses in Europe have been spent on controlling overweight and on obesity-related health problems. Cardiovascular disease is clearly associated with overweight and, for several decades now, one diet after another has been developed to control or, preferably, lose body weight. For the last 10–15 years, low-fat diets have been heavily promoted and also proven to be successful (Brehm *et al.*, 2003). Recent developments, however, indicate that (very) low-carbohydrate diets (VLCD), even when containing high-fat and protein such as the Atkins diet, are much more effective in reducing body weight. These diets were originally discouraged by physicians because they were thought to be too stressful for obese patients (Folsom *et al.*, 1998), but recent publications on this aspect clearly show that VLCDs are not only much more effective for losing body weight than, for instance low-fat diets, but do not have any detrimental effect on obese patients (Samaha *et al.*, 2003).

One strategy to obtain low-calorie foods is by replacing the normal, high-calorie sugars by low-calorie substitutes. The most prominent example is the use of Nutrasweet, aspartame, as a replacement for saccharose in sweets, in soft drinks, in cakes, etc. This sugar-replacer is made by chemical synthesis and can only be used in reconstituted foods. In this chapter we will describe production of and/or replacement of high-calorie sugars by naturally occurring low-calorie sugars through fermentation (see Box 2). The micro-organisms that can be used are basically the same as described above for vitamin production, the lactic acid bacteria, the propionibacteria, the bifidobacteria and also, occasionally, some (food-grade) yeasts or fungi. The low-calorie sugars or sweeteners that will be discussed are the polyols mannitol, sorbitol, and erythritol, the amino acid L-alanine, the hexose tagatose and the disaccharide trehalose.

BOX 2: Production of alternative, low-calorie, sugars by lactic acid bacteria

Lactic acid bacteria have the potential to produce a wide range of metabolites, some with similar sweetening power as glucose or even sucrose. If they are carbohydrates and not, or poorly, degraded by the human body, such as the polyols mannitol, sorbitol, and erythritol, the hexose tagatose and the disaccharide trehalose, they can be considered low-calorie sugars. If they are compounds with similar sweetness as glucose, but just fewer calories such, as L-alanine, they can be considerd low-calorie sweeteners. The biosynthesis pathways, in lactic acid bacteria, leading to the production of these sweeteners is shown in Fig. 21.2. Metabolic intermediates such as fructose-1-P, galactose-P, erythrose-P, and glucose-6-P serve as direct precursors for the production of the low-calorie sugars. L-Alanine is the only component which is not naturally produced by the lactic acid bacteria but is produced as a result of metabolic engineering, through introduction of alanine dehydrogenase from *Bacillus subtilis*.

21.6.1 Production of polyols

Polyols, such as mannitol and sorbitol, are low-calorie sugars that could replace sucrose, lactose, glucose or fructose in food products as they display equivalent sweetness and taste (Wisselink *et al.*, 2002). They can also serve as antioxidant in biological cells (Shen *et al.*, 1997) as shown by increased survival of cells during freezing and/or drying in the presence of mannitol (Efiuvwevwere *et al.*, 1999).

Sorbitol production by lactic acid bacteria

Sorbitol is a popular low-calorie sugar which is, currently, produced by chemical conversion/reduction (Silveira and Jonas, 2002). Many lactic acid bacteria, however, have the potential to metabolize sorbitol and, in special circumstances, to use this potential to produce sorbitol. Recently, it was demonstrated in *Lactobacillus plantarum*, that it could be transformed into a sorbitol-producer by disrupting the lactate dehydrogenase enzymes and overexpressing the sorbitol dehydrogenase (http:// www.nutracell.com). Substantial conversion of the high-calorie sugars sucrose, lactose, and glucose into sorbitol could be observed using these constructed strains. This type of strain opens possibilities to produce fermented foods, such as olives, pickles, and sauerkraut but also fermented dairy products, with much lower caloric value.

Mannitol

Some heterofermentative lactic acid bacteria, such as *Leuconostoc mesenteroides* (Soetaert, 1991) and *Lactobacillus intermedius* (Saha and Nakamura, 2003), are able to produce large amounts of mannitol from fructose or fructose-containing sugars such as sucrose. They contain a mannitol dehydrogenase enzyme which directly reduces fructose to mannitol using the

reducing equivalents that would otherwise be used for the production of ethanol. Using these lactic acid bacteria, typically 70 per cent or more of the fructose can be converted into mannitol and production levels up to 300 g/l have been reported. These micro-organisms are clearly suited for production of mannitol as sugar-replacing ingredient, however, the current production process for mannitol – by catalytic hydrogenation – is still a much cheaper process.

Production of mannitol by these lactic acid bacteria can be effectively used in the production of fermented foods. Unfortunately, the two heterofermentative lactic acid bacteria mentioned above, are not widely used in food fermentations. Lactic acid bacteria such as *Lactococcus lactis* and *Lactobacillus plantarum* are much more suited for this purpose. In view of this, mannitol production was induced in these two lactic acid bacteria, using the technique of metabolic engineering similarly as described above for sorbitol. In *Lb. plantarum* high production of mannitol could already be reached by disrupting the lactate dehydrogenase activity (Ferain *et al.*, 1996). In *L. lactis*, disruption of LDH only lead to transient mannitol production (Neves *et al.*, 2000), but combination with overexpression of the mannitol phosphate dehydrogenase resulted in substantial mannitol production (Wisselink *et al.*, 2004). A similar approach reported for *Lactobacillus fermentum* (Aarnikunnas *et al.*, 2003) was much less effective. If production of mannitol can be further increased, these novel strains could have great potential for the use of fermentation to convert high-calorie sugars, as present in plant materials and milk, into the low-calorie sugar, mannitol.

Erythritol

Erythritol holds promise as a low-calorie sugar substitute. Erythritol is a sugar alcohol (polyol) which is not metabolized in the human body, although it is absorbed in substantial amounts from the small intestine, and therefore excreted in the urine. Human tolerance to repeated oral doses of erythritol has been examined in double-blind, crossover, studies and it was concluded that the repeated ingestion of erythritol at daily doses of 1 g/kg body weight was well tolerated by humans (Tetzloff *et al.*, 1996). Erythritol is produced in high amounts by specific bacteria such as the wine bacterium *Oenococcus oeni* (Veiga da Cunha *et al.*, 1993), formerly known as *Leuconostoc oenos*, and by the filamentous fungus *Aspergillus niger* (van der Veen *et al.*, 1995).

21.6.2 Other, natural, low-calorie sweeteners

Tagatose

Tagatose is a hexose sugar that is poorly degraded by most living cells including the cells in the human body. It has recently attained GRAS (generally recognized as safe) status under US Food and Drug Administration (FDA) regulations, thereby permitting its use as a sweetener in foods and beverages (Levin *et al.*, 1995). For this reason, it has large potential as a low-calorie sugar since it has similar sweetening power as sucrose and can be used, effectively, in

products that depend on the bulking effect of sugars such as sucrose. It has been included in the FDA-approved health claim stating that some sugar alcohols provide protection against dental caries, since the micro-organisms involved in caries, such as *Streptococcus mutans*, are not able to utilize this sugar (Zehner, 1988; Bertelsen *et al.*, 1999).

Tagatose, as a free sugar, is found only rarely in nature. In the phosphorylated form, however, it is found in some micro-organisms that are able to convert the milk sugar, lactose. These micro-organisms, such as *Lactococcus lactis*, use a tagatose pathway (Fig. 21.2) for converting the galactose-moiety of lactose and produce tagatose-P and tagatose-1, 6-diphosphate as intermediates in metabolism (van Rooijen *et al.*, 1991). Using this metabolic potential, engineered strains of *L. lactis* have been constructed that accumulate high amounts of the phosphorylated, and also free, tagatose, by blocking the further conversion of the phosphorylated tagatose (Hugenholtz *et al.*, 2002). Using these tagatose-producing strains, it could become possible to produce low-lactose and high-tagatose-containing dairy products.

L-Alanine

Alanine is an industrially relevant food and pharmaceutical ingredient. It is, in the form of L-alanine, not only a major component of amino acid-rich medical supplements, but also a substrate for many (bio)chemical synthesis reactions in industry. In addition, alanine has a comparable sweetening power as the carbohydrates glucose and fructose as a supplement and can serve as a low-calorie sweetener in foods as a result of its much lower caloric value. Currently, *Corynebacterium glutamicum* is used for the commercial production of this amino acid. However, for alanine-formation in fermented foods it would be highly desirable to obtain alanine-producing lactic acid bacteria. Using metabolic engineering on the level of pyruvate metabolism, high production of alanine was achieved in both *Lactococcus lactis* and in *Lactobacillus plantarum*. The (heterologous) *Bacillus sphaericus alaD* gene, encoding for alanine dehydrogenase enzyme, was cloned in *L. lactis*, using the NICE system (de Ruyter *et al.*, 1996). The alanine dehydrogenase enzyme converts pyruvate to L-alanine in the presence of ammonium. Upon introduction of this system in lactate dehydrogenase-deficient lactococcal cells, a complete conversion of the pyruvate pool to alanine was obtained when cells were incubated under the appropriate, high ammonium, conditions (Hols *et al.*, 1999). The alanine produced by these cells consisted of a mixture of both stereo-isomers (D- and L-alanine) as a consequence of the endogenous alanine racemase activity of *L. lactis* (encoded by the *alr* gene). To obtain a stereo-specific L-alanine producing bio-reactor, the *alr* gene was functionally disrupted in the lactate dehydrogenase-deficient lactococcal strain. High-level expression of alanine dehydrogenase in the resulting cells indeed led to stereo-specific L-alanine production as the only end product of fermentation (Hols *et al.*, 1999).

Trehalose

Trehalose is a well-known non-reducing disaccharide synthesized by a wide variety of organisms. It is only partially digested in humans, and therefore it is considered a dietetic sugar. It is also poorly metabolized by many other organisms, including lactic acid bacteria. The oral bacteria *Streptococcus mutans* and *Streptococcus salivarius*, for instance, are not able to perform acidification with trehalose as the only carbohydrate (Neta *et al.*, 2000). In comparative studies with human volunteers, mouth rinses with trehalose solutions led to significantly reduced acidification in plaques compared with rinsing with sucrose solutions. By feeding rats with trehalose-containing diets, instead of sucrose, almost complete suppression of dental caries was observed (Neta *et al.*, 2000).

Other (biological) activities that can be attributed to trehalose are protection of proteins and whole cells against denaturation under different stress conditions such as heating, drying and freezing (Felix *et al.*, 1999; Guo *et al.*, 2000). The preserving properties of trehalose on different biological systems have been widely demonstrated with different enzymes (Singer and Lindquist, 1998), membranes (Felix *et al.*, 1999), cells (Guo *et al.*, 2000), tissues and organs (De Carlo *et al.*, 1999; Wu *et al.*, 1999). Especially relevant in the context of this overview are the applications of trehalose for preserving different kinds of food stuffs, keeping the freshness and taste of the product even after prolonged storage. More recently, it has been shown that trehalose has important stabilizing effects on human proteins, preventing protein aggregation as well as formation of pathological conformational forms, and its application against illnesses, such as the Creutzfeld–Jakob disease has been disclosed (De Carlo *et al.*, 1999). Given the beneficial properties of trehalose, the development of food products in which trehalose is produced *in situ* at the expense of other sugars, such as lactose, fructose or glucose, is highly desirable.

As with many other micro-organisms (Welsh *et al.*, 1991), in *Propionibacterium* trehalose accumulation has been shown to occur as a result of different stress conditions, such as low temperature, high osmolarity and other adverse growth conditions (Cardoso *et al.*, 2001). Trehalose is formed from the glycolytic intermediate glucose 6-phosphate via glucose 1-phosphate, UDP-glucose, trehalose 6-phosphate to trehalose (Deborde *et al.*, 1996). The enzymes that are specific for trehalose production are trehalose-6-phosphate synthase and trehalose 6-phosphate phosphatase. The intracellular concentrations that are reached vary enormously among different strains and are also strongly dependent on the growth conditions applied. At low pH, in the presence of oxygen and high salt, intracellular trehalose concentrations exceeding 1 M have been observed in propionibacteria. Effectively, under the described conditions, almost 50 per cent of the bacterial dry weight consists of trehalose. Also in some food fermentations using propionibacteria, high amounts of trehalose were found (http:// www.nutracells.com). This demonstrates the actual potential of the use of propionibacteria for production of the low-calorie sugar trehalose in (fermented) foods.

21.6.3 Conclusions about low-calorie sugar production

Many potentially interesting low-calorie sugars can be, and are, made by lactic acid bacteria and other food-grade micro-organisms such as propionibacteria. Most of these sugars are produced as intracellular components, only, and thus limiting the overall production level. For practical use, an efficient export system for these sugars would definitely promote high production levels. This is clearly evident in the case of L-alanine production where nearly 100 per cent conversion of the high-calorie sugars glucose and lactose into L-alanine has been reported.

21.7 Lactic acid bacteria, cholesterol control, lipase activity, and antioxidant production

In this last part of the chapter, several activities of lactic acid bacteria will be discussed, which range from cholesterol conversion to production of antioxidants. These activities are described only briefly since these activities only have suggestive effects in relation to cardiovascular disease or some effects have been observed but no underlying mechanism has been described, as is the case for some probiotics.

21.7.1 Cholesterol control

Probiotics

Probiotics are basically fermented food products that contain live (lactic acid) bacteria with some sort of health-promoting activity. Many of these products have been launched on the market over the last 10 years with claimed or suggested activities in promoting the digestive system of the consumer, in protecting against bacterial and viral infections and even in controlling life-threatening illnesses such as (colon) cancer. In relation to cardiovascular disease, cholesterol-lowering activities have been described for some probiotics (Usman and Hosono, 2001; Naruszewicz *et al.*, 2002). The exact mechanism of this action is either unknown or assumed to be related to bile-salt deconjugation and/or to production of short-chain fatty acids that both interfere with cholesterol-synthesis and recovery (Pereira and Gibson, 2002a, b).

Cholesterol-oxidase

Direct metabolism of cholesterol has also been reported in several micro-organisms, including food-grade bacteria (Fujishiro *et al.*, 2002). Several attempts have been made to introduce this enzyme activity in some lactic acid bacteria (Brigidi *et al.*, 1993), but although cloning of the genes presents no problems, successful expression of enzyme activity has not yet been reported in these bacteria. Even if the concept would work, it remains questionable if the cloned enzyme would operate *in situ* in the human body, since oxygen is required for enzymatic activity.

21.7.2 Lipase activities

An interesting concept in relation to low-calorie diets is the use of lipase activities to reduce the fat content in foods. Although lactic acid bacteria are not renowned for their lipolytic activity, they can be used very effectively as a carrier for enzymes. This carrier property was used to deliver a lipase from *Staphylococcus hyicus*, cloned into *Lactococcus lactis*, into to pigs and proven successful in stimulating fat consumption (Drouault *et al.*, 2002). Of course, it remains to be seen if this concept can be applied efficiently, for reduction of body fat, in humans suffering from obesity.

21.7.3 Anti-oxidant production

It is becoming more and more evident that antioxidants can provide protection against cardiovascular diseases. Reduced levels of antioxidants such as vitamins A, C and E, are found in patients suffering from these diseases (Cunha *et al.*, 2001; Yardim-Akaydin *et al.*, 2003) and these antioxidants are thought to reduce lipid peroxidation and, thus, atherogenesis (Rimm and Stampfer, 1997). Several food products, with increased levels of antioxidants, are currently marketed as health-promoting foods and several examples are discussed in other chapters of this book. Also thiols, such as thioredoxin, are claimed to provide similar protection (Yamawaki *et al.*, 2003). In many different micro-organisms, including lactic acid bacteria, thiols such as glutathione, thioredoxin, and rubredoxin (Carmel-Harel and Storz, 2000), not only supply protection against (oxidative) stress (Smirnova *et al.*, 2001) but also serve as electron acceptors in several metabolic reactions. Glutathione is present in some lactic acid bacteria (Fernandes and Steele, 1993) such as *Lactococcus lactis* (Wiederholt and Steele, 1994), while conspicuously absent in many others (Fahey *et al.*, 1978) including *Lactobacillus plantarum* (Li and Molenaar, personal communication). In the latter micro-organism other thiols, such as thioredoxin, presumably serve in the redox reactions for electron transfer. With the full knowledge of the genome sequence, it is now possible to increase the production and/or accumulation levels of these thiol-compounds in the cells and in the fermented products. This provides extremely useful study material for establishing once and for all whether these antioxidants have any real nutritional effects. Also the above-mentioned low-calorie sugars mannitol and trehalose are reported to react with oxygen radicals and, as such, have antioxidant activity. However, until now, there is no evidence that increased uptake of these metabolites actually raises the antioxidant level in the human blood or in human cells. So apart from protecting food constituents (protein, flavor) against oxidation, the nutraceutical activity of these antioxidants is still very much in doubt.

21.8 Conclusions

Fermentation of food materials by lactic acid bacteria and several other food-grade micro-organisms can result in foods providing increased protection

against cardiovascular diseases. The bacteria produce beneficial components during fermentation such as B vitamins and antioxidants, but also have metabolic activity that convert high-calorie into low-calorie sugars and that can degrade lipids and cholesterol. All-in-all, fermented foods can be seen as naturally produced, and thus extremely attractive, alternatives for the artificially prepared diet-foods and additives that are currently crowding the health market.

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