Dietary vitamins, polyphenols, selenium and probiotics: biomarkers of exposure and mechanisms of anticarcinogenic action

Edited by
Björn Åkesson, Per Mercke
ECNIS is a Network of Excellence within the European Union’s Sixth Framework Programme, Priority 5: Food Quality and Safety. It brings together some of the best European research groups in a concerted effort to achieve improved understanding of the environmental causes of cancer, of the potential of diet to prevent cancer and of the ways in which heredity can affect individual susceptibility to carcinogens, with the ultimate aim of reducing the cancer burden in Europe.

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The major aim of this review was to summarize the state-of-the-art in use of biomarkers for some anticarcinogenic food components and to identify knowledge gaps, especially those of relevance for the partners of the NoE ECNIS and its contacts. Since this is a vast area, only certain selected topics, as outlined below, are considered in detail. The important links found between use of a substance as a biomarker and its mechanisms of action led to a further aim, that of reviewing the mechanisms of action of some of the most promising anticarcinogenic compounds.

Many compounds contained in the diet have been proposed as having anticarcinogenic properties. These compounds are found in foods of many different types, although plant-based foods appear to be the richest source. The degree to which these compounds are present in plant tissue can vary markedly and be dependent on the plant variety, the growth conditions and other factors. The microbial flora in the gut also play an important role in their action and composition through bioconversion and the release of various bioactive dietary components.

Biomarkers are a key tool for assessing nutritional status and dietary exposure to specific substances. Markers differ considerably in their characteristics, some of them mainly reflecting recent dietary intake and others indicating more the individual’s long-term nutritional status, which to a large extent reflects the kinetics of metabolism and transport, and pool sizes of the substance in question. Also, the confounding factors affecting a given biomarker vary appreciably for different dietary components as well as in terms of the body fluid or tissue considered suitable for sampling. The area of biomarkers has progressed markedly with the advent of new analytical technology and the emergence of data showing that hitherto neglected food components may have important health effects and can be of strong interest. Nutritional biomarkers are essential for studies of the links between dietary intake and the risk of cancer, which is a very complex field indeed in view of the large number of cancer diseases, pathogenetic mechanisms, food components and food preparation methods involved. The ECNIS project aims at addressing important problems in this field and identifying knowledge gaps that exist. Particular attention is directed at identifying dietary factors that modulate environmental and lifestyle factors related to cancer risk. The present review focuses on two major groups of food components, vitamins and selenium on one hand, and bioactive compounds, on the other.

**Vitamins and selenium**

Although the measurement of vitamins in body fluids is a classical area for nutritional biomarker research, particular progress has been made here recently. Current research
on vitamin D has shown its links to a broader spectrum of health matters and diseases, including cancer. The measurement of vitamin D metabolites presents a number of problems including the occurrence of vitamin D in different forms, the variations in measurement between different assays and different laboratories, epidemiologic variations in vitamin D status and its determinants, and the problems in defining the appropriate threshold for vitamin D sufficiency. The determination of vitamin D status is still a challenging task, and since metabolites of vitamin D have important biological effects, strong research efforts in this area should be encouraged. Epidemiologic studies in particular can help advance our knowledge of the association between vitamin D insufficiency and the risk of disease including that of cancer at various sites.

Biomarkers of vitamins A, C and E are an important part of the diet-cancer field. Serum retinol is still the most reliable indicator for evaluating vitamin A deficiency. HPLC methods are those which are most sensitive, but they are not useful as yet for large screenings. Simpler tests using fluorescence or immunological techniques have a high level of accuracy and precision, but there is a need of developing simpler methodologies. Concerning the functions of vitamin A, it appears to have only limited capacity as a direct antioxidant in vivo. Vitamin C, which is water-soluble, can readily be determined in plasma by automated colorimetric assays or by HPLC. The latter has the advantage of possessing lower detection limits, probably higher accuracy as well as the possibility it to determine ascorbate and dehydroascorbate simultaneously, but colorimetric assays have a much higher throughput. The handling and storage of plasma for vitamin C determination has a strong impact on its accuracy. There is limited evidence that plasma ascorbate is a functional antioxidant, as assessed by currently available markers of lipid or protein oxidation in humans, and that the intake of high doses of vitamin C can be used to decrease oxidative stress. In contrast, clear short-term pro-oxidant effects on lipid oxidation have been observed following the infusion of high-dose vitamin C into the bloodstream. No studies are currently available on the relationship between lipid or protein oxidation markers and chronic disease. Regarding vitamin E, both HPLC and GC-MS methodology have high precision and accuracy for its detection in plasma. There is limited evidence that of a protective effect of vitamin E supplementation at levels of 200-2000 IU/d as judged from its effects on markers of lipid oxidation. The effects that have been postulated of high levels of vitamin E supplementation on exercise-induced lipid oxidation, as determined by isoprostanes and by inflammatory markers, are controversial. Vitamin E intervention has not been shown to decrease the oxidation of plasma proteins.

The antioxidant vitamins have been studied very much in relation to risk of cancer, using oxidative DNA damage as a biomarker. Since DNA mutations are a crucial step in carcinogenesis and elevated levels of oxidative DNA lesions have been noted in many tumours, such damage is strongly implicated in the aetiology of cancer. Although it is likely that severe oxidative stress is a consequence rather than a cause of the development of many types of cancer, at present it is impossible to assess the quantitative involvement of oxidative stress in the origin of cancer. Regarding intervention studies using antioxidant supplementation, there are six investigations that have reported beneficial effects
Executive summary

of it on oxidative DNA damage, whereas there are 13 studies that have reported a null effect. There is little support for the notion that ingestion of antioxidant-rich foods is associated with a lower level of spontaneous oxidative DNA damage in leucocytes than produced by the intake of single antioxidants. Most studies have involved healthy individuals, but the few well-controlled studies that have reported realistic appearing levels of oxidative DNA damage in leucocytes isolated from oxidatively stressed subjects do not lend much support to the notion that such a population benefits more from antioxidant supplementation than a normal study population would. In the future greater attention should also be directed at alternative chemopreventive mechanisms such as up-regulation of DNA repair systems and the chemopreventive effects of antioxidants on non-lymphatic tissue.

The relationship of low selenium status and selenium supplementation to cancer disease is another important segment of focus in the diet-cancer field. Several epidemiologic case-control studies have indicated a protective role of selenium in helping prevent the development of cancer. In these studies, use has been made of several biomarkers such as the concentration of total selenium in different body fluids as well as different selenoproteins, primarily glutathione peroxidase. It is important to explore the possibility of using other selenoproteins as biomarkers too. There is also a need of clarifying the mechanistic role of selenium in the etiology of cancer. Experimental studies have shown that selenium compounds affect cell growth, the cell cycle, DNA repair and signal transduction. Both organic and inorganic forms of selenium have been evaluated, methylated selenocompounds having been found to have particularly strong chemoprotective effects. Several large human intervention studies have indicated that selenium supplementation can prevent the development of several different forms of cancer (prostate, lung, colon). Further studies are underway. It is also important to determine whether there is an increased risk of certain forms of cancer after selenium supplementation. The optimal type and dose of selenium supplements needs to also be defined.

Bioactive compounds in foods

Much direction has been directed at the beneficial health effects of flavonoids and other polyphenolic components that occur in the diet. Like other types of dietary chemopreventive agents, flavonoids exhibit a wide range of potentially beneficial activities in terms of cancer prevention. These are usually divided up into blocking and suppressing activities. The blocking activities include antioxidant effects and the modulation of drug-metabolising enzymes and multidrug resistant genes. The suppressing activities include the inhibition of signalling pathways responsible for cell proliferation and survival, and the induction of apoptosis, mainly through intrinsic pathways involving members of the Bcl family, mitochondrial membrane depolarisation, release of cytochrome c and the activation of caspases. Flavonoids can also induce cell cycle arrest by modulating key components of cell cycle regulation, including cyclins, cyclin-dependent kinases and inhibitors. Some recent mechanistic findings on the flavonoids apigenin, epigallo-
catechin gallate, genistein, resveratrol, quercetin, the chalone, xanthohumol and the
novel flavonol tricin have been summarised.

Also, the use of polyphenols as biomarkers of dietary intake has attracted
considerable interest, but their utilisation in this way is hampered by several factors. The
bioavailability of different compounds varies markedly and for many compounds
information on this point is scarce. The half-life of many compounds in plasma is less
than one day and measurements of their plasma levels mainly reflect short-term dietary
intake, which also applies to analyses of these compounds in urine. From an analytical
point of view, the measurement of polyphenols in body fluids is a difficult task.

Hydrolytic and clean-up steps are usually needed, and the sensitivity of detection may be
a limiting factor although the increasing use of detection methods based on mass
spectrometry will solve some of these problems including the identification of analytes.
For some compounds, such as isoflavones and lignans, the availability of immunoassays
is of great value. The more recently developed metabolomic techniques may offer new
possibilities in the future, but further study regarding this will be needed.

Olive oil is one of the foods at which special interest has been directed in the diet-cancer
field because of its containing a special mixture of agents that affect the initiation,
promotion and progression of multistage carcinogenesis. These include tocopherol and caro-
tenoid antioxidants, certain phenolic compounds (tyrosol, hydroxytyrosol, secoiridoids
and lignans), as well as squalene and β-sitosterol. These compounds have a number
of different mechanisms of action. It has also been pointed out that their efficacy
is dependent on their bioavailability. To clarify the matter, a number of recent studies
on the bioavailability of certain minor but important olive oil components (polyphenols,
lignans, squalene and β-sitosterol) are reviewed. An especially interesting matter here is that
intake of olive oil can increase the bioavailability of anticarcinogenic compounds.

The anticancerogenic properties of the glucosinolates have received a great deal
of attention, its becoming increasingly clear that it is their breakdown products that can
influence the initiation and progression of carcinogenesis. They also appear to influence
apoptotic responses to chemotherapeutic agents. A major impediment to our under-
standing of the chemopreventative mechanisms stimulated by glucosinolates is that
relatively little is known regarding the biological effects of glucosinolate breakdown
products other than the isothiocyanates and the indole-containing derivatives. It is
unclear whether the formation of thiocyanates, nitriles, cyano-epithioalkanes and
oxazolidine-2-thiones from glucosinolates, which occurs at the expense of their forming
isothiocyanates, is undesirable in terms of chemoprevention of cancer. It is also unclear
whether the activity of the epithiospecifier protein (ESP), which reduces the formation
of isothiocyanates from glucosinolates, is undesirable. In addition, relatively little
is known of the pharmacokinetic properties of glucosinolate breakdown products
in humans. Without such information, it is difficult to relate responses of cells in culture
to particular concentrations of phytochemicals to the situation in vivo. These are areas
in need of further investigation. Mammalian cells display marked dose responsiveness
to phytochemicals, at low doses of phytochemicals cytoprotective adaptive responses
being activated, whereas at higher doses cell cycle arrest and apoptosis occur. It is presently unclear how these different types of responses are coordinated by the cell and how matters of whether adaptation, growth arrest or apoptosis is to occur are determined. Identification of the mechanisms that control such outcomes would be very useful.

A growing number of in vitro and in vivo studies indicate that combinations of dietary chemopreventive agents can sometimes result in significant levels of activity at concentrations at which any single agent is inactive. This may explain why some food items or diets may show cancer preventive effects that cannot be explained on the basis of the individual bioactive ingredients alone. The development of ideas regarding this has also been stimulated by findings that dietary supplements of only a single compound may have negative effects. Although our understanding of the molecular mechanisms behind the observed combinational effects is still limited, it appears that many combinations of complementary modes of action may be involved. In some systems, combinations of green tea flavonoids are more active than single compounds of this sort, and green tea flavonoids can also show increased activity when present together with other phytochemicals. The synergistic effects of dietary phytochemicals need to be investigated further. This can well contribute to cancer prevention. The development of new dietary supplement regimens and nutraceuticals can also benefit from improved insight into the mechanisms behind the synergistic effects of both natural and synthetic chemopreventive compounds.

Many healthful diet effects are attributed to probiotic bacteria. The strongest evidence for the anticancer effects these can have comes from animal studies, evidence from human studies still being limited. An important goal for the future should be to conduct carefully designed human clinical trials with the aim of corroborating insofar as possible results of the wealth of experimental studies that have been carried out. Several possible mechanisms may explain how lactic acid bacteria can protect against tumour development in the colon. Different strains of the bacteria may possibly target different mechanisms. More work needs to be done to identify the specific strains and strain characteristics responsible for particular antitumour effects and the mediating mechanisms involved. Even with the above reservations and the limited number of human studies available in mind, one can regard the use of lactic cultures for human cancer suppression as interesting, promising and as deserving of closer scrutiny.

As has been indicated here, considerable information of detailed character concerning the mechanisms governing the effects of bioactive food compounds and of nutrients on the cellular processes that relate to carcinogenesis has accumulated. It is a highly challenging task to integrate this knowledge in such a way that useful hypotheses can be formulated that ultimately can be tested in human dietary intervention trials. An understanding of the mechanisms involved is a necessary basis for the development and validation of new biomarkers of nutritional status. It is important here to have insight into recent developments in analytical technology and thorough knowledge of biomarker kinetics. Much hope is attached in this connection to the powerful techniques available today within the field of nutritional genomics.
1. Introduction

1.1. Introductory overview and future prospects

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Dietary assessment methodology and the development and validation of biomarkers of nutritional status are key scientific fields within nutritional science. They have expanded markedly with the advent of new analytical technology and the emergence of scientific findings showing that hitherto neglected food components may have interesting and important health effects. A number of textbooks and reviews are available within the field of nutritional biomarkers [see e.g. 1–6] dealing with the basic theories and methodology, their physiological basis and the use of biomarkers in studies of different types. Various properties of biomarkers are summarised in Table 1.1.

<table>
<thead>
<tr>
<th>Table 1.1. Some major issues regarding nutritional biomarkers</th>
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<tr>
<td>Markers of dietary intake vs. markers of nutritional status</td>
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<td>Markers of differing kinetics (response times)</td>
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<tr>
<td>Markers of nutrients vs. markers of non-nutrient dietary components</td>
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<tr>
<td>Types of confounding factors and errors affecting the assessment of dietary intake and nutritional status</td>
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<tr>
<td>Choice of body fluids or tissues for sampling and the handling of samples</td>
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<tr>
<td>Analytical methodology and throughput</td>
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A major area of use of nutritional biomarkers is in studying the risk of different types of cancer diseases in relation to dietary intake and nutritional status. This is a very complex field in view of the large number of different types of cancer, the pathogenetic mechanisms involved, that role that various food components and food preparation methods play in connection with this. The ECNIS project aims at addressing important problems in this field and identifying various knowledge gaps. A main concern is to identify dietary factors that modulate environmental and lifestyle factors that can affect risk of cancer and which in the long run can also provide support for the development of functional foods.

The major aim of this review is to summarize the state-of-the-art of biomarkers that can be applied to various anticarcinogenic food components and to highlight different knowledge gaps. A further aim is to examine and explore the mechanisms responsible for the action for different promising anticarcinogenic compounds. A brief overview of food components that appear to have an anticarcinogenic effect is provided in Appendix 1.2.
Dietary components as biomarkers

Many biomarkers involve the measurement of a nutrient or some other dietary component in the blood, plasma, urine or hair. The selection of the type of sampling to carry out depends very much on the components to be studied and the sampling facilities available. Some methods, such as the analysis of cobalamine and folate in plasma, are in routine daily use in clinical laboratories, whereas others are performed only in a few specialised research laboratories. Biomarkers show temporal variation, and some of them reflect recent dietary intake strongly such as the amounts of various components consumed a short time before that occur in the urine. Various diet-related compounds in the plasma also differ considerably in turnover times. The amount of a substance occurring in the erythrocytes, often reflects much more the long-term intake if it than the plasma level does, since the erythrocyte cells have a lifetime of about 120 days [3]. A large number of confounding factors such as hormones, the gut flora, diurnal variations, interactions between dietary or endogenous components or xenobiotics, variations in metabolism or in excretory pathways, and inflammatory and other diseases can influence the levels of different biomarkers [3]. The level of a biomarker often varies as well with age, gender and gene polymorphism (as outlined below).

Functional biomarkers of nutrients

Since many nutrients are essential for the activity of enzymes (as coenzymes for example) the levels of activity involved are sometimes used as nutritional biomarkers such as in activation assays for thiamine and riboflavin [3]. Also, trace elements are coupled to the levels of enzyme activities, for example zinc and copper to superoxide dismutase activity and selenium to the activity of glutathione peroxidase and other selenoproteins. As reviewed in this volume there is a strong link between selenium content and glutathione peroxidase activity since it is covalently bound in the peptide chain. Also proteins with a transport function as well as other functions are used as biomarkers, such as the retinol-binding protein and ceruloplasmin. In addition, different nutrient-dependent physiological tests can be employed [2].

Use of nutritional biomarkers

It is often the case that a biomarker does not respond to changes in the status of a nutrient equally well over the entire range of nutritional status, the main function of some markers being that of serving as indicators of malnutrition, and they may not respond to overexposure of nutrients at all. A classification of feasibility (predictive power) and of degree of responsiveness to different ranges of intake for various biomarkers is provided by Bates et al. [3]. In some cases a biomarker or a set of biomarkers may reflect the intake of a specific food containing a specific pattern of particular substances. One such example is olive oil discussed in this volume by Sotiroudis and Kyrtopoulos.
Although several of the biomarkers mentioned here are widely used, it is known generally that the levels of nutrients in extracellular fluids do not necessarily reflect their level or saturation at crucial cellular sites or in storage tissues. For this reason, methods for measuring nutrients or nutrient-dependent variables in cells in the blood (mainly leucocytes), buccal mucosa, urine, faeces, adipose tissue or biopies from other organs have been developed. Some of these samplings require much skill and effort and can only be used in small studies. Their use may increase, however, if the analytical methodology improves so that smaller amounts of sample suffice.

Also, in the case of some analyses carried out on samples of the plasma the main bottlenecks are linked with the precision or the throughput of the analytical methods involved, as exemplified in the present volume by Linseisen and his colleagues in connection with vitamin D and polyphenols. There are particularly good reasons for using the 25-hydroxy vitamin D as a biomarker in extended studies in the future. The two contributions mentioned also illustrate the difficulty in defining valid cut-off points.

The assessment of dietary intake in epidemiological studies has been a major use of biochemical markers of nutritional status. An overview of this matter is provided in [7]. This topic is only taken up briefly in the present volume, although epidemiological findings on the association between the selenium level and cancer are reviewed by Gromadzińska et al. Biomarkers in studies of that type have been used either alone, together with an assessment of dietary intake by a food frequency questionnaire or by some other methods, or for the calibration of the dietary assessment methods [8]. Although the use of the biomarker concept for these purposes is very attractive, it should be borne in mind that there are very few (if any) “ideal” biomarkers that have been validated for studies of different types. A number of statistical methods for the correction and evaluation of such data are available [8].

**Nutrient biomarkers in relation to genetic polymorphism**

It is generally assumed that genetic polymorphism influences the response of biomarkers in different individuals but for most biomarkers this has not yet been much studied. As summarised by Hunter [9], information on the interactions between dietary intake and gene polymorphisms “can serve to 1) define susceptible subpopulations, thus strengthening dietary associations; 2) help establish causality of food and nutrient associations in epidemiology studies; 3) aid in distinguishing causal components of complex dietary mixtures; and 4) eventually provide the basis for gene-based screening tests”. Folate has served as a prototype here for demonstrating how genetic make-up can influence the nutrient status of an individual. The influence that the C677T polymorphism found in methylene tetrahydrofolate reductase (MTHFR) has on folate physiology and the risk of disease is a much studied example of this [10–12]. There are also very interesting hypotheses regarding the role of folate and other dietary components involved in methyl transfer in the epigenetic regulation of methylation status and its association with the risk of disease [11,12]. These matters are outside the scope of the present review.
Biomarkers and response to dietary supplements

An important use of biomarkers is to aid in the assessment of compliance in meal studies and dietary intervention studies. The content or profile in the blood of β-carotene, α-tocopherol, selenium and polyenoic fatty acids for example, usually responds to an increase in the supply of them, although for other nutrients and their biomarkers there may be little or no response due to homeostatic regulation of their plasma levels, the existence of a renal threshold or other factors.

Biomarkers are also used in long-term human intervention studies in which there are clinical endpoints. Studies in which nutrient antioxidants or combinations of those are employed are of special relevance for various topics taken up in the present volume. To the surprise of the scientific community it was found that some antioxidants, especially β-carotene, had negative effects, a meta-analysis showing there to be an increase in mortality in studies of antioxidant supplementation by β-carotene, vitamin A and/or vitamin E [13]. It was also stated that “in four trials (three with unclear/inadequate methodology), selenium showed significant beneficial effect on the incidence of gastrointestinal cancer. The potential preventive effect of selenium should be studied in adequate randomised trials” [13]. The results of the first generation of nutritional intervention studies concerned with prevention of cancer were summarized recently [14], the criteria to be employed for the evaluation of efficacy in the future being proposed [14,15].

The finding of negative effects after long-term dietary supplementation of various antioxidant nutrients has led to the increased study instead of the effects and mechanisms of action of non-nutrient antioxidants or bioactive components, work that is as reviewed by Manson, Hayes, de Kok and their colleagues in this volume.

Biomarkers for the assessment of dietary effects on carcinogenesis

Biomarkers used as a surrogate endpoint in studies of the effect that dietary manipulation has at different stages of carcinogenesis are another type of biomarkers of importance in the diet-cancer field. A possible further use to which such biomarkers could be put would be to substantiate ‘anticancer’ claims regarding various food components, a matter reviewed recently [16,17]. Biomarkers of this category include tumours and the mortality in animal models, as well as precancerous lesions, adenomatous polyps, cell proliferation and differentiation, apoptosis, such enzymes as cyclo-oxygenase 2, gut-lumen enzymes and carcinogen-metabolising enzymes, certain metabolites and various effects on DNA and its metabolism [16]. In the present volume Loft et al. review the evidence that individual antioxidants as well as for antioxidant-rich diets affecting oxidative DNA damage, and Dragsted summarises the effects of vitamins A, C and E on biomarkers of oxidative stress. Other aspects of cancer-related biomarkers have been the subject of a previous review conducted within the ECNIS project [18].
Application of omics technologies

Emerging “omics” technologies — transcriptomics, proteomics, genomics and metabolomics — will probably have a strong influence on research on relations between nutrition and cancer [12,19]. The use of transcriptomics opens up the possibility of monitoring the changes in global gene expression caused by a wide array of dietary components in different experimental systems. Proteomics, in turn, makes it possible for the same type of experiments to be carried out at the protein level, providing unique and possibly more detailed information. Transcriptomics and proteomics can both be expected to become major tools in gaining a better understanding of the cancer-protective effects of different nutrients. The metabolomics approach involves the multiparametric measurement of metabolites and provides a comprehensive account of the metabolic profile of different biological systems, such as various body fluids [20,21]. Genomics, finally, includes techniques for the global genotypic characterization of individuals with the aim of discovering polymorphisms associated with susceptibility [9]. SNP arrays are one powerful genomic tool enabling whole-genome association studies of up to 500,000 SNPs (single nucleotide polymorphisms) to be carried out in a single run. The “omics” technologies as a whole will surely play a key role in future nutritional research regarding the protective role of diet in connection with cancer. The use of results obtained by various of these techniques involves complex ethical considerations [22].

Concluding remarks

The contributions the present volume contains clearly indicate that a deeper understanding is needed of the mechanisms underlying the protective effects that various food components can have in preventing or counteracting carcinogenesis. Considering the links to the susceptibility the individual has inherited is becoming increasingly important too. At the same time it is often difficult to single out the mediating components in the protective food patterns emerging from epidemiological studies diet-cancer relationships. It is important, therefore, to identify the compounds that are most active in different experimental systems, and to study variations in their activity when they are ingested as a part of different foods, together with the combinatorial effects of phytochemicals and other food components. There is also much uncertainty regarding the relevance of observed in vitro or short-term effects in humans in relation to the long-term effects documented in chemopreventative human studies [7,23].

The newly obtained findings in the molecular nutrition area can be used to make more rapid progress in the development and validation of biomarkers. Such biomarkers should reflect not only exposure to food components but also damage to biological components (DNA, protein and lipids) and other targets of the diet-carcinogenesis interaction [18].
References

Appendix 1.2. Overview of possible anticarcinogenic food components

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There is a vast range of dietary compounds proclaimed to have anti-carcinogenic properties as investigated by the scientific community. These protective compounds are found basically in all types of food, but plant-based foods appear to be their richest source. Different plant families harbor different mixtures of compounds of this sort. Plant tissues can also vary enormously in the store of such compounds, depending on the plant variety and the growth conditions, for example. The microbial flora in the gut has also been shown to play an important role in the modification of the composition and bioavailability of ingested compounds by mediating bioconversion and release of different dietary components.

To provide an overview of the cancer-protective compounds at which special attention is directed in the literature and assemble various criteria used in selecting such compounds, a list of putative anticarcinogenic food components has been generated (Table 1.3.). This information was collected with use of a PubMed search conducted in 2005.

Table 1.2. A step-wise search in the database PubMed for reviews linking biomarkers of nutrition and diet to cancer

<table>
<thead>
<tr>
<th>PubMed search</th>
<th>No. of refs</th>
</tr>
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<tbody>
<tr>
<td>Biomarker</td>
<td>305256</td>
</tr>
<tr>
<td>Biomarker AND diet</td>
<td>3351</td>
</tr>
<tr>
<td>Biomarker AND (diet OR nutrition)</td>
<td>4173</td>
</tr>
<tr>
<td>Biomarker AND (diet OR nutrition) AND cancer</td>
<td>869</td>
</tr>
<tr>
<td>Biomarker AND (diet OR nutrition) AND cancer /reviews/</td>
<td>181</td>
</tr>
<tr>
<td>Biomarker AND (diet OR nutrition) AND cancer /reviews/last 10 years</td>
<td>146</td>
</tr>
</tbody>
</table>

The forty-seven reviews listed below were selected from among the 146 reviews arrived at last in this search. The criteria used for the selection were their relevance as adjudged from their title, emphasis on recent reviews, and avoiding of similar articles by any given author.
### Table 1.3. An overview of putative anticarcinogenic food components as assembled from 33 out of the 47 selected reviews that were selected

<table>
<thead>
<tr>
<th>Class of compound</th>
<th>Example of bioactive substance</th>
<th>Food source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acids</td>
<td>Omega-3/omega-6 Vegetable oils</td>
<td>Bartsch et al. [1], Branca et al. [2], Ferguson [3], Greenwald [4], Turini and DuBois [5]</td>
<td></td>
</tr>
<tr>
<td>Starch and resistant starch</td>
<td></td>
<td>Branca et al. [2], Ferguson [3]</td>
<td></td>
</tr>
<tr>
<td>Fibre/non-starch polysaccaride</td>
<td>Vegetables</td>
<td>Branca et al. [2], Ferguson [3], Gill and Rowland [6], Manson et al. [7], Rafter et al. [8], Sanderson et al. [9], Turini and DuBois [5]</td>
<td></td>
</tr>
<tr>
<td>Pre- and probiotics</td>
<td>Butyrate</td>
<td>Branca et al. [2], Gill and Rowland [6], Milner [10], Rafter [11], Sanderson et al. [9]</td>
<td></td>
</tr>
<tr>
<td>Vitamins</td>
<td>Vitamin A including retinol and retinoic acids</td>
<td>Branca et al. [2], Ferguson [3], Kelloff et al. [16], Manson et al. [7], Milner [10], Rafter et al. [8], Sanderson et al. [9], Vlastos et al. [12]</td>
<td></td>
</tr>
<tr>
<td>Carotenoids</td>
<td>α- and β-Carotene Orange vegetables</td>
<td>Branca et al. [2], Bowen et al. [13], Crews et al. [14], Ferguson [3], Granado et al. [15], Kelloff et al. [16], Loft and Paulsen [17], Manson [18], Maruvada and Srivastava [19], Mayne [20], Milner [10], Rafter et al. [8], Seifried et al. [21], Sharma and Farmer [22], Vlastos et al. [12], Wild et al. [23]</td>
<td></td>
</tr>
<tr>
<td>Carotenoids</td>
<td>Lycopene Tomatoes</td>
<td>Crews et al. [14], Ferguson [3], Granado et al. [15], Kelloff et al. [16], Loft and Paulsen [17], Manson et al. [7], Mayne [20], Sharma and Farmer [22], Vlastos et al. [12], Wild et al. [23]</td>
<td></td>
</tr>
<tr>
<td>Carotenoids</td>
<td>Lutein Green vegetables</td>
<td>Branca et al. [2], Gerber et al. [26], Kelloff et al. [16], Loft and Paulsen [17], Manson et al. [7], Mayne [20], Sharma and Farmer [22], Vlastos et al. [12], Wild et al. [23]</td>
<td></td>
</tr>
<tr>
<td>Carotenoids</td>
<td>Zeaxanthin</td>
<td>Manson [18], Maruvada and Srivastava [19], Mayne [20], Milner [10], Rafter et al. [8], Seifried et al. [21], Sharma and Farmer [22], Vlastos et al. [12], Wild et al. [23]</td>
<td></td>
</tr>
<tr>
<td>Carotenoids</td>
<td>β-Cryptoxanthin</td>
<td>Mayne [20], Milner [10], Rafter et al. [8], Seifried et al. [21], Sharma and Farmer [22], Vlastos et al. [12], Wild et al. [23]</td>
<td></td>
</tr>
<tr>
<td>Vitamin E</td>
<td></td>
<td>Branca et al. [2], Ferguson [3], Greenwald [4], Seifried et al. [21], Kelloff et al. [16], Loft and Paulsen [17], Manson et al. [7], Mayne [20], Sharma and Farmer [22], Vlastos et al. [12], Wagner et al. [24], Wild et al. [23]</td>
<td></td>
</tr>
<tr>
<td>Vitamin D</td>
<td></td>
<td>Branca et al. [2], Ferguson [3], Kelloff et al. [16], Konety and Getzenberg [25], Milner [10], Wild et al. [23]</td>
<td></td>
</tr>
<tr>
<td>Vitamin C</td>
<td></td>
<td>Branca et al. [2], Ferguson [3], Loft and Paulsen [17], Mayne [20], Seifried et al. [21], Sharma and Farmer [22], Vlastos et al. [12]</td>
<td></td>
</tr>
<tr>
<td>B vitamins (Folic acid)</td>
<td></td>
<td>Branca et al. [2], Crews et al. [14], Ferguson [3], Gill and Rowland [6], Greenwald [4], Kelloff et al. [16], Maruvada and Srivastava [19], Mason [26], Milner [10], Rafter et al. [8], Sanderson et al. [9], Turini and DuBois [5], Vlastos et al. [12], Wild et al. [23],</td>
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</table>
Table 1.3. An overview of putative anticarcinogenic food components as assembled from 33 out of the 47 selected reviews that were selected — cont.

<table>
<thead>
<tr>
<th>Class of compound</th>
<th>Example of bioactive substance</th>
<th>Food source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minerals and trace elements</td>
<td>Selenium (including selenomethionine and other selenium compounds)</td>
<td>Branca et al. [2], Crews et al. [14], Ferguson [3], Greenwald [4], Seifried et al. [21], Kelloff et al. [16], Mayne [20], Milner [10], Rafter et al. [8], Sharma and Farmer [22]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Calcium</td>
<td>Branca et al. [2], Gill and Rowland [6], Greenwald [4], Kelloff et al. [16], Maruvada and Srivastava [19], Milner [10], Turini and Dubois [5]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Iron</td>
<td>Maruvada and Srivastava [19], Wild et al. [23]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Iodine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isothiocyanates</td>
<td>Benzyl isothiocyanate</td>
<td>Cruciferous vegetables</td>
<td>Bianchini and Vainio [27], Branca et al. [2], Ferguson [3], Manson [18], Sanderson et al. [9], Sharma and Farmer [22], Zhang [28]</td>
</tr>
<tr>
<td></td>
<td>Phenethyl isothiocyanate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sulforaphane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulphones</td>
<td>Oltipraz (synthetic)</td>
<td>Cruciferous vegetables</td>
<td>Ferguson [3], Manson [18]</td>
</tr>
<tr>
<td>Dithiolthiones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucosinolates</td>
<td>Indole-3-carbinol</td>
<td>Cruciferous vegetables</td>
<td>Bianchini and Vainio [27], Ferguson [3], Kelloff et al. [16], Manson [18], Manson et al. [7]</td>
</tr>
<tr>
<td></td>
<td>3,3'-Diindolylmethane</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Indole-3-acetonitrile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allium compounds</td>
<td>Diallyl sulphide</td>
<td>Onions, garlic, scallions, chives</td>
<td>Branca et al. [2], Ferguson [3], Kelloff et al. [16], Manson [18]</td>
</tr>
<tr>
<td></td>
<td>Allylmethyl trisulphide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavonoid polyphenolics</td>
<td>Tangeretin</td>
<td>Citrus fruits, berries, tomatoes, potatoes, broad beans, broccoli, squash, onion</td>
<td>Crews et al. [14], Duthie and Crozier [29], Manson [18], Manson et al. [7], Milner [10], Rafter et al. [8], Sanderson et al. [9], Seifried et al. [21], Sharma and Farmer [22]</td>
</tr>
<tr>
<td></td>
<td>Apigenin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nobiletin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rutin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kaempferol</td>
<td>Radish, horse-radish, kale, endive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Taxifolin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catechins</td>
<td>Catechin</td>
<td>Tea, chocolate</td>
<td>Branca et al. [2], Ferguson [3], Kelloff et al. [16], Manson [18], Manson et al. [7], Rafter et al. [8], Saleem et al. [30], Sanderson et al. [9], Seifried et al. [21]</td>
</tr>
<tr>
<td></td>
<td>Epicatechin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epigallocatechin (EGC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epigallocatechin gallate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenolic acids</td>
<td>Caffeic acid</td>
<td></td>
<td>Ferguson [3], Manson [18]</td>
</tr>
<tr>
<td></td>
<td>Ferulic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ellagic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoflavones</td>
<td>Genistein</td>
<td>Cereals, pulses (millet, sorghum, soybeans)</td>
<td>Adlercreutz [31], Atkinson and Bingham [32], Branca et al. [2], Ferguson [3], Gill and Rowland [6], Kelloff et al. [16], Manson [18], Manson et al. [7], Rafter et al. [8], Sanderson et al. [9], Seifried et al. [21], Wild et al. [23], Wiseman [33]</td>
</tr>
</tbody>
</table>
Table 1.3. An overview of putative anticarcinogenic food components as assembled from 38 out of the 47 selected reviews that were selected — cont.

<table>
<thead>
<tr>
<th>Class of compound</th>
<th>Example of bioactive substance</th>
<th>Food source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylxanthines</td>
<td>Caffeine</td>
<td>Tea, coffee, cola, cacao</td>
<td>Manson [18]</td>
</tr>
<tr>
<td></td>
<td>Theophylline</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Theobromine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoterpenes</td>
<td>Limonene</td>
<td>Citrus fruits (peel)</td>
<td>Ferguson [3], Kelloff et al. [16], Manson [18], Rafter et al. [8]</td>
</tr>
<tr>
<td></td>
<td>Perillyl alcohol</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Geraniol</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Menthol</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Carvone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other non-flavonoid phenolics</td>
<td>Hydroxytyrosol</td>
<td>Olive oil</td>
<td>Adlercreutz [31], Bartsch et al. [1], Branca et al. [2], Wiseman [33]</td>
</tr>
<tr>
<td></td>
<td>Curcumin</td>
<td>Turmeric</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Resveratrol</td>
<td>Grapes, strawberries, raspberries, black-berries, walnuts, pecans.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lignans</td>
<td>Cereals, olive oil</td>
<td></td>
</tr>
</tbody>
</table>

References (selected from the PubMed search described above)

2. Vitamins and selenium

2.1. Biomarkers of exposure to and effects of vitamins A, C and E

Lars O. Dragsted
University of Copenhagen, Copenhagen, Denmark

Since antioxidant vitamins can affect an organism’s capacity for defence against reactive oxygen species, biological markers of the dietary exposure to these vitamins is of importance. There is also a need of effect biomarkers for determining the ability of these and other antioxidants to increase the overall antioxidant capacity and decrease the oxidative damage occurring in biological samples. This chapter is concerned with such markers, except for markers of DNA damage, which are dealt with elsewhere in this volume.

Vitamin A

Biomarkers for vitamin A in body fluids

The vitamin A (all-trans-retinol and its esters) level was originally determined by bioefficiency assay, a technique that was later superseded by various chromatographic and fluorescent techniques. Due to worldwide concern for vitamin A deficiency (VAD), the development of fast and simple methods for the determination of vitamin A status has long been given a high priority. Direct fluorescence methods for assessing the retinol level in plasma or in dried blood are feasible because of the high intensity of retinol fluorescence when it is bound to its transporter, the retinol binding protein (RBP) [1,2]. With the advent of high performance liquid chromatography (HPLC), these techniques took over and today retinol can be determined in serum routinely by direct- [3,4] or reversed-phase [5–7] liquid chromatography. The reversed-phase techniques are faster and smaller sample volumes suffice but they are generally unable to discriminate between the various isomers of vitamin A to the same extent as the direct-phase methods can, although reversed-phase methods able to separate certain of the retinol isomers have been published [8]. The direct-phase methods can also typically measure a large number of other lipid-soluble vitamin isomers in the same run, such as pro-vitamin A carotenoids, xanthophylls, tocopherols and tocotrienols, menadione and phylloquinones. The structure of vitamin A and pro-vitamin A carotenoids is shown in Figure 2.1. The observation that RBP occurs in plasma in a virtually 1:1 ratio to retinol has prompted the development of radial diffusion assays and enzyme immunoassays for RBP as a surrogate marker for plasma or serum retinol [9–11]. The possibility of using dried blood spots, which is advantageous from a collection standpoint, has also been demonstrated [1].
A comparison of the various tests in terms of price, speed of performance and coefficient of intraindividual variability is shown in Table 2.1. Although the accuracy for each of the methods taken up in Table 2.1. is good (less than 5% deviation from the standards) and the interday variability is low, the correlation coefficient between the HPLC methods and ELISA is only around 0.8, probably due to differences in linearity. Since the latter methods are less demanding in terms of equipment they have considerable potential for screening purposes in the less developed countries where VAD is still causing blindness, growth retardation and decreased resistance to infections in large numbers of children.

![Fig. 2.1. Structures of retinol (vitamin A) and of the most important pro-vitamin A carotenoids.](image)

Table 2.1. The performance of different methods for determining serum vitamin A

<table>
<thead>
<tr>
<th>Test method</th>
<th>CV%</th>
<th>Cost/sample (relative)</th>
<th>Speed (samples/day)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reversed-phase HPLC</td>
<td>4</td>
<td>20</td>
<td>25</td>
<td>[7]</td>
</tr>
<tr>
<td>Direct-phase HPLC</td>
<td>4</td>
<td>30*</td>
<td>20</td>
<td>[4]</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>&lt;10</td>
<td>2a</td>
<td>50–100*</td>
<td>[1]</td>
</tr>
<tr>
<td>ELISA (RBP)</td>
<td>9</td>
<td>1</td>
<td>150</td>
<td>[9]</td>
</tr>
</tbody>
</table>

\* Estimates from the author’s lab. This may change with new faster LC techniques.
Biomarkers of vitamin A related effects in the eye

VAD leads to dryness of the conjunctiva of the eye and moderate deficiency leads to decreased night vision due to interruption of light-sensitive chemical processes in the eye. Permanent blindness may ensue in severe cases. The WHO has compared the sensitivity of different methods for determining VAD (as modified in Table 2.2.). Biological effects on the eye are still the only reliable means of detecting moderate to severe VAD, whereas the biochemical detection of retinol in blood samples is needed to identify mild cases and to be able to intervene at an early stage [12]. As already indicated, simple yet sensitive assays to determine the presence of this condition are thus still in demand. Histological markers that are employed include corneal cytology of the eye by direct visual inspection and by sampling a specimen of the conjunctiva for staining and microscopy. Functional markers include dark adaptation and the ability to see contrasts. The direct visual tests include staining with rose Bengal to visualize dry areas or to detect inflammation, but tests of this sort have been shown to be less reliable [13]. The standard today is the conjunctival impression cytology test which makes use of a vacuum pump to lift a small portion of the epithelium from the inferior temporal conjunctiva onto a filter paper disc, fix it in glacial acetic acid and stain it with periodic acid Schiff and haematoxylin for histological examination [14,15].

Table 2.2. Biological indicators of subclinical VAD*

<table>
<thead>
<tr>
<th>Indicator (cutoff)</th>
<th>Prevalence cutoffs for defining a public health problem and assessing its level of importance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mild</td>
</tr>
<tr>
<td>Functional tests</td>
<td></td>
</tr>
<tr>
<td>Night blindness (age-specific)</td>
<td>&gt; 0 to &lt; 1%</td>
</tr>
<tr>
<td>Biochemical markers</td>
<td></td>
</tr>
<tr>
<td>Serum retinol (≤ 0.70 Kmol/l)</td>
<td>≥ 2 to &lt; 10%</td>
</tr>
<tr>
<td>Breast-milk retinal (≤ 1.05 Kmol/l)</td>
<td>&lt; 10%</td>
</tr>
<tr>
<td>Histological markers</td>
<td></td>
</tr>
<tr>
<td>Abnormal conjunctival impression cytology</td>
<td>&lt; 20%</td>
</tr>
</tbody>
</table>

* Common biological indicators of subclinical VAD in children 6-71 mo of age. A public health problem is considered to exist when the prevalence criteria of at least two of the above indicators of VA status are met (adapted from [1,12]).

The dark adaptation tests measure the time needed to adapt to a defined level of limited illumination. A fast adaptation procedure involving the ability to discriminate between red and blue objects for field and for screening studies has been described [16]. When the eye shifts from cone-mediated day vision to rod-mediated night vision, the Purkinje shift occurs, the retinal peak light sensitivity shifting from red towards blue, blue objects being perceived then as lighter shades of grey than for red objects. Patients have to be taught use of the test, which must be repeated afterwards several times. In some studies the test results have been found to be more closely related to vitamin A intake by dietary assessment than to the plasma retinol level [13].
Biomarkers of oxidative stress after supplementation with vitamin A

There seem to be no studies reporting on markers of oxidative stress or oxidative status following intervention with use of increased doses of vitamin A. Neither retinol nor beta-carotene supplements to blood samples *in vitro* have been found able to affect a number of markers for antioxidant stability of the plasma and erythrocytes [17], which indicates that this vitamin may have a limited capacity to act as an antioxidant *in vivo*.

Conclusions

Serum retinol is still the most reliable indicator of vitamin A deficiency. HPLC methods are the most sensitive, but they are not useful for large screenings or for field studies in poor areas of the world where deficiency is a common problem. Although simpler tests using fluorescence, as well as immunological techniques possessing good accuracy and high precision exist, there is still a need of methodology which is simpler, faster and cheaper yet and requiring no complicated sample treatment or use of complex equipment. Vitamin A appears to have only limited capacity as a direct antioxidant *in vivo*.

Vitamin C

Biomarkers of vitamin C in body fluids

Vitamin C (ascorbate and dihydroascorbate, Figure 2.2.) levels have been determined in plasma, serum, dialysates and other body fluids by colorimetric and fluorimetric techniques, by enzymatically based assays and by HPLC with or without post-column derivatisation. Since ascorbic acid is easily oxidised to dehydroascorbate, which can subsequently be degraded to diketogulonic acid, initial treatment by stabilising acids such as metaphosphoric and perchloric acids has to be performed quickly after isolation of a sample for analysis. The effects of the anticoagulants used during sample collection has been compared in one study, heparin being found to result in only a minimal loss, EDTA in contrast giving rise to a significant loss of vitamin C within a 30 min period. Also, oxalate and citrate were found to be less efficient in stabilizing ascorbate than other anticoagulants were [18]. Storage time of the sample and storage conditions are important factors determining the stability of vitamin C. In one early study, the concentrations of ascorbate and dehydroascorbate were found to be unaffected in samples stored in the laboratory at a temperature of 12°C for up to 6 hours [19], but in other studies considerable time- and temperature-dependent losses were found already from the first hour of storage onwards even after optimization of the collection conditions [18]. In another study the storage time and temperature were found to have no effect on loss of vitamin C during 2–14 day storage at either –25°C or –75°C, but a 3.5% loss due to freezing was observed [20]. In a third study, pre-treatment with metaphosphoric acid was compared with treatment by dithiotreitol, a commonly used laboratory antioxidant. The latter performed slightly better than the former since no loss of vitamin C was evident after storage at –80°C for 6 years, whereas the standard procedure involving the addition of metaphosphoric acid led to a small but significant mean loss of < 1% per year [21].
However, since treatment with dithiotreitol is known to reduce dehydroascorbate to ascorbate, this procedure cannot be recommended if both compounds are to be determined separately. The normal range of plasma concentrations of dehydroascorbate is controversial and the observation of this compound in plasma may be a result of metal-catalysed oxidation following acidification [22]. If dehydroascorbate is physiologically present, its true concentration seems to be very low, around 0.1% of the total plasma vitamin C in non-smokers when the sample has been handled carefully, and about 1.8% under the same conditions in the case of smokers [23], possibly reflecting higher leakage of haem in this group. There was a significant increase in the concentration of dehydroascorbate over time at low total vitamin C concentrations [23].

![Diagram of ascorbate, the ascorbyl radical and dihydroascorbate](image)

**Fig. 2.2.** Structures of ascorbate, the ascorbyl radical and dihydroascorbate, together constituting vitamin C.

The colorimetric and fluorometric methods are generally based on redox-reactions, with ascorbate and dihydroascorbate leading to the formation of a chromophore or a fluorophore, which can be photometrically measured by use of manual or automated equipment. Most of these methods are quite unspecific [24], but a few of them use assay blanks produced by adding ascorbate oxidase to the sample, creating greater sensitivity with retention of speed. Some of these methodologies are very fast, allowing high sample handling rates to be achieved through automation [25]. Results in determining plasma ascorbate in this way correlate well with those obtained by use of chromatographic methods [18]. Dehydroascorbate is not readily determined by use of this approach.

The HPLC methods for detecting plasma ascorbate electrochemically give results similar to those using UV detection [26]. Postcolumn derivatization can be used to reduce dihydroascorbate so that it can be determined by use of an electrochemical detector; the stereoisomer of ascorbate, erythorbate, can be determined simultaneously [25,27–28]. A method for the simultaneous detection of ascorbate and uric acid by means of capillary zone electrophoresis (CZE) has also been described [29]. Recovery is better than 98% with
use of the HPLC and CZE methods and good linearity is obtained even at low ascorbate concentrations.

In interlaboratory comparisons, quite disparate results have been obtained with use of these techniques. In a European study of laboratories carrying out population surveillance, a 13–20% interlaboratory variation was found using plasma samples in the ‘normal’ range of 36–94 µmol/l in a second round after corrections had been instituted at several laboratories [30]. In another study an interlaboratory difference of about 15% was observed, whereas the intralaboratory variation was about 2 µmol/l, irrespective of the concentration, which led to relatively larger relative errors being registered at low concentrations [21]. The performance of different methods for the measurement of vitamin C in plasma is summarized in Table 2.3.

<table>
<thead>
<tr>
<th>Test method</th>
<th>CV% AA interday</th>
<th>CV% DHAA interday</th>
<th>Limit of detection</th>
<th>Speed (samples/day)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reversed phase HPLC</td>
<td>&lt; 4%</td>
<td>&lt; 6%</td>
<td>0.1 mg/l</td>
<td>40–50</td>
<td>[36]</td>
</tr>
<tr>
<td>Capillary electrophoresis</td>
<td>1.3%</td>
<td>ND</td>
<td>0.5 mg/l</td>
<td>40–50</td>
<td>[37]</td>
</tr>
<tr>
<td>Automated colorimetry</td>
<td>&lt; 5%</td>
<td>ND</td>
<td>3 mg/l</td>
<td>500</td>
<td>[38,39]</td>
</tr>
</tbody>
</table>

ND – not determined.

Vitamin C and lipid oxidation markers

Many different biomarkers of radical mediated lipid oxidation exist but for the purpose of this review the more commonly used assays appear adequate for comparing studies in this area. The assays employed to this end here are the following: plasma thiobarbituric acid reactive compounds (TBARS), ex vivo LDL oxidation, antioxidant capacity markers, plasma lipid hydroperoxides, and plasma or urinary isoprostanes. Only randomised study designs are included in this review.

The most commonly used marker is determination of TBARS, with or without calibration, to detect malondialdehyde. The method is based on the liberation of aldehydes from amino groups by acid or alkaline hydrolysis followed by a colour reaction involving use of thiobarbituric acid. The product can be determined spectrophotometrically, either directly or online, following HPLC separation. This marker is highly controversial since it is variable both within and between laboratories, since it may partially measure aldehydes deriving from endogenous metabolism, and since there is no generally accepted assay procedure [31]. These flaws have caused some journals to generally regard the method as being invalid as a lipid oxidation biomarker [32]. The interday coefficient of variation (CV) for TBARS with use of HPLC is in the order of 10–20 %, depending on the method employed for hydrolysis, but as already mentioned this relatively simple method has an odd tendency of sometimes giving spurious results and of varying from one analytical series to another, also within the same laboratory.
In a randomised 2-months intervention study of 59 healthy smoking males MDA was found to increase significantly following daily doses of 250 mg ascorbate combined with 200 IU vitamin E, 30 mg beta-carotene and 100 µg organic selenium, given in a normal formulation, but MDA to be unaffected by a slow-release formulation as compared with placebo treatment [33]. In another, randomised double-blind crossover study the effect of vitamin C supplementation (six weeks, 250 mg/day) was determined in 20 subjects each showing normal (67 µmol/l) or below average (32 µmol/l) plasma vitamin C concentration at baseline. No differences between groups in plasma malondialdehyde concentrations were observed either before or after supplementation [34]. In another counterbalanced design, 25 males were exposed to vitamin C (500 or 1000 mg/d) and/or to exercise. No effect of either treatment on plasma MDA was observed [35]. In a study comparing 8 smoking women with 8 controls during a 14-day period in which 1 g ascorbate was administered daily plasma TBARS was found to not be affected [36]. In a larger study involving 56 smokers, the intake of 500 mg/d of vitamin C was found not to affect MDA as determined by HPLC [37]. In a third study of this sort, giving a combination of vitamin C (272 mg/d) and vitamin E (800 IU/d) compared to placebo was found to not affect plasma MDA in 77 smokers treated for 90 d [38]. Oxidative stress in 10 volunteers as determined by increased plasma MDA induced by infusion of free fatty acids was also found to be unaffected by high-dosage vitamin C infusion [39]. Infusion of large doses of vitamin C (5g) in combination with EDTA treatment resulted initially in a marked increase in plasma MDA but in an overall decrease in this parameter after 16 repeated sessions [40]. Oxidative stress induced by Zn deficiency was found to respond to 250 mg/d vitamin C, given for 3 months, as compared with placebo treatment, by a decrease in plasma MDA concentration [41]. Overall it appears that the intake of vitamin C does not consistently affect plasma MDA but that significant increases may be observed following the infusion of large, acute doses.

Another controversial marker used by many laboratories is the ex vivo LDL oxidation assay, in which isolated LDL is exposed to copper chloride or to a semistable radical such as AAPH, the oxidative formation of conjugated dienes being followed spectrophotometrically at 234 nm [42]. The lag-time to oxidation and/or the slope of the oxidation curve are used as end points. The method is disputed because the outcome depends on the antioxidants present in the LDL and these may be lost during LDL isolation. A faster method applicable directly to a plasma or serum sample overcomes this problem by using a peroxide-sensitive fluorescent probe with high affinity for LDL [43]. The inter-day CV for this latter assay is less than 10%.

In a group of 48 middle-aged male and female participants in a 36-month intervention study receiving 500mg/d of either vitamin C, vitamin C plus 182 mg/d dl-α-tocopherol, 182 mg/d dl-α-tocopherol alone, or a placebo in a parallel design, no effect was observed at 12 or at 36 months in the vitamin C group in terms of susceptibility of isolated LDL or VLDL to oxidation ex vivo. In addition, no change in whole plasma ex vivo oxidation was observed in this group [44]. In a smaller parallel study of vitamin C supplementation (1 g/d) versus placebo, in which 19 smokers participated for 4 weeks following a 2-week
Ascorbate depletion period (≤ 30 mg/d), there was a significant increase in ex vivo LDL oxidation lagtime in the vitamin-supplemented group [45]. In a subsequent study of 30 young smokers, no effect was observed after 8 weeks supplementation by 1 g/d of vitamin C [46]. In a study with 50 coronary artery disease patients there was no evidence after 6 months that random assignment to vitamin C (1 g/d) together with vitamin E (800 IU/d), or placebo, decreased LDL oxidation or antibodies to oxidised LDL [47]. A borderline effect on LDL oxidation was observed in a similar study with only 18 participants after a shorter period of 12 weeks [48]. No effect on LDL oxidation lag-times of 500 mg/d vitamin C supplementation for 4 weeks as compared with placebo was observed in 30 type II diabetics [49]. In a study without a control group, an increase in ex vivo LDL oxidation lag-time during a 12 week-period was observed in 20 volunteers receiving 260 mg vitamin C in combination with 14 mg iron/d. In another group, receiving only 60 mg vitamin C plus iron per day, no such increase was observed despite changes in plasma ascorbate [50]. Supplementation by 1 g/d ascorbate for 4 weeks in 11 healthy volunteers failed to change the plasma LDL oxidation lag-time as compared with 9 controls [51]. Overall there seems to be no consistent effect of vitamin C supplementation on ex vivo LDL oxidation kinetics.

The oxidizability of LDL is inherently an antioxidant capacity assay for this particular compartment. A variety of antioxidant capacity markers exist for other blood compartments, especially for whole plasma. They are all ex vivo oxidation systems composed of some oxidising system and some relatively simple marker of plasma oxidation, usually one leading to a change in visual or UV absorption of the test matrix [52–55]. There are important differences between the methods which call for caution when they are interpreted [56]. When applied to plasma samples some authors accordingly report poor correlation between methods [57] whereas others observed a high degree of correlation between some of the most commonly applied methods, the ferric reducing ability of plasma (FRAP) assay and the trolox equivalent antioxidant capacity (TEAC) assay which also correlated with ex vivo LDL oxidation [58,59]. These methods generally have interday CVs of less than 10% for repeated measurements of the same sample, the size of the CVs depending on the exact wavelength used for determining the absorbance, the availability of a photometer with exact filters or one equipped with a narrow grid being required.

The infusion of 1000 mg ascorbate for a 1 h period in ten healthy volunteers was found to result in an increased antioxidant capacity as measured repeatedly by two different methods during both the infusion period and the hour following this [60]. Marked increases in antioxidant capacity in the plasma of elderly women during the 4 h period after a dose of 1250 mg ascorbate was given were also observed using three different antioxidant capacity measures [61]. However, only a limited response of this sort was observed with use of the FRAP assay during the 24-hour period following a single dose of 500 mg ascorbate with or without 400 IU vitamin E [62]. At the same time, in a cross-over intervention study involving 48 non-smoking men and women, in which 0 mg, 60 mg or 6 g of vitamin C was administered daily during 14 day-periods separated
by 6 weeks wash-out periods, the plasma antioxidant capacity of these persons was found to be significantly affected [63]. In a 14 d parallel study of 16 women who smoked, half of them treated with 1 g ascorbate daily and half of them given placebo, no effect on the antioxidant capacity of the plasma could be shown [36]. Neither was the plasma TEAC found to be affected by a 150 mg/d vitamin C supplement in a 2-week study involving 18 volunteers [64]. No effect of antioxidants on TEAC could be observed either in 39 lupus erythromatosis patients randomised to being given 500 mg vitamin C together with 800 IU vitamin E a day or a placebo for a 12-week period [65]. In a group of 48 middle-aged male and female participants in a 36-month intervention study in which 500 mg/d of vitamin C, vitamin C plus 182 mg/d dl-α-tocopherol, 182 mg/d dl-α-tocopherol alone, or placebo were given daily in a parallel design, no effect in either of the vitamin C groups on the plasma antioxidant capacity as determined by the TRAP assay was observed at either 12 or 36 months [44]. At the same time, the antioxidant capacity in 6 moderately trained males was found to be significantly affected, following 2.5 h of strenuous exercise stress, by the intake of a drink containing vitamin C (0.15%, approx. 200 mg ascorbate) [66]. In contrast, FRAP was not found to be affected by 1h of hard exercise following the daily administration of 600 mg vitamin C in combination with a range of other micronutrients for a 7-day period [67]. In these various studies, the effect of vitamin C on antioxidant capacity measures could not always be explained simply by an increase in the plasma ascorbate concentration. It appears that, although vitamin C may increase the antioxidant capacity in normal, healthy individuals, the effect is more evident short-term and may be partially due to indirect actions of unknown character.

Although plasma lipid hydroperoxides can be determined individually by HPLC together with electrochemical detection [68,69] or collectively by means of hydroperoxide-sensitive photometric assays [70–72], in most published papers the determination of ‘lipid hydroperoxides’ is a euphemism for TBARS. The effect of ascorbate supplementation on the plasma lipid hydroperoxide concentration in humans under normal conditions has not been frequently reported. The formation of lipid hydroperoxides in LDL was not found to be affected by a 150 mg/d vitamin C supplement in a 2-week randomised study involving 18 volunteers [64]. The effect of an ascorbate supplement in reducing the plasma hydroperoxide level following various conditions of oxidative stress showed a significant effect on this marker. A 50% reduction in exercise-induced total lipid hydroperoxides was observed after acute supplementation of vitamin C [73]. Also vitamin C supplementation in conjunction with biweekly apheresis for 6 months in dyslipidemic and uremic patients markedly increased the efficiency of such treatment in reducing the plasma phosphatidylcholine hydroperoxide level as determined by HPLC [74]. Thus, Vitamin C seems to affect lipid hydroperoxide formation in some oxidative stress conditions.

The least disputed lipid oxidation methods are the isoprostane assays. The determination of plasma isoprostanes can be performed by gas chromatography/mass spectrometry (GC-MS) using electron capture negative ionization (ECNI) or negative ion
chemical ionization detection (NCI). The compounds need to be extracted from the sample and derivatized. The extraction has not always been straightforward and has involved multiple chromatographic steps, including thin layer chromatography, and has led to a poor overall recovery [75]. Improvements have included a combination of solid-phase extraction cartridges and HPLC [76,77], two sequential solid-phase extractions [78] and most recently a single anion exchange cartridge [79]. The derivatization of the compounds is most often done by use of pentafluorobenzyl bromide followed by a silylation step employing bis-(trimethylsilyl) trifluoroacetamide [79]. The overall extraction efficiency is now around 70%, the interday analytical CV% varying from less than 1% to 5–8%, depending on extraction procedures. Since the interindividual variation is much larger, this method has considerable power for detecting the factors causing biological variation. An immunological method for the determination of 8-isoprostane F$_{2alpha}$ having 5–15% analytical variation also exists [80].

The formation of isoprostanes in plasma was found to not be affected by 150 mg/d vitamin C supplementation in a 2-week study involving 18 volunteers [64]. Using the chromatographic method on plasma samples, no change was observed after a daily dose of 272 mg vitamin C in combination with vitamin E (31 mg/d) and folate (400 µg/d) for a 90 day period in elderly male smokers and non-smokers [38]. A dose-response study of hospitalised women in which vitamin C (30–2500 mg/d) was administered to them for 186 days following a short ascorbate-depletion period, gave no evidence of change in the levels of isoprostanes in the urine or plasma [81]. In a study of 30 coronary artery disease patients limited evidence was obtained after 6 months that random assignment to vitamin C (1 g/d) together with vitamin E (800 IU/d) versus placebo led to a decrease in plasma isoprostanes [47]. No studies of the effect of ascorbate supplementation on oxidative stress-induced isoprostanes have been reported. The performance of plasma lipid oxidation markers and the effects on them of vitamin C being administered is summarized in Tables 2.4. and 2.5. Overall, the effect of vitamin C on lipid oxidation markers seems limited to a possible effect in certain oxidative stress conditions.

### Table 2.4. Performance of plasma lipid oxidation markers

<table>
<thead>
<tr>
<th>Test method</th>
<th>Analytical CV%</th>
<th>Normal variation CV%</th>
<th>Speed$^a$ (samples/day)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS (HPLC)</td>
<td>15–20</td>
<td>25</td>
<td>50</td>
<td>[83]</td>
</tr>
<tr>
<td>LDL ex vivo oxidation</td>
<td>5–10</td>
<td>20</td>
<td>10/50</td>
<td>[42,43]</td>
</tr>
<tr>
<td>Antioxidant capacity</td>
<td>&lt;10</td>
<td>20–25</td>
<td>&gt;100</td>
<td>[55]</td>
</tr>
<tr>
<td>Lipid peroxides</td>
<td>&lt;10</td>
<td>60–65</td>
<td>20</td>
<td>[72]</td>
</tr>
<tr>
<td>8-Isoprostane F$_{2alpha}$</td>
<td>5–15</td>
<td>20–50</td>
<td>50–100</td>
<td>[80]</td>
</tr>
<tr>
<td>Isoprostanes GC-MS</td>
<td>&lt;1–6</td>
<td>50</td>
<td>50</td>
<td>[78,79]</td>
</tr>
</tbody>
</table>

$^a$ Estimates in the author’s laboratory with use of standard semiautomated equipment.
Vitamins and selenium: Biomarkers of exposure to and effects of vitamins A, C and E

Vitamin C and protein oxidation

Proteins have no natural carbonyl groups, so such groups are introduced into proteins by oxidative mechanisms, including the oxidative deamination of the e-amino group in lysine and the oxidation of carbons next to the secondary amine functions in proline and arginine. Other assays for oxidatively modified amino acid residues in proteins include the measurement of preformed hydroxytyrosine, dityrosine, and sulphoxides (e.g. methionine sulphoxide), and the loss of protein sulfhydryls. Oxidised proteins can denature, the denatured proteins being quickly chaperoned towards proteolytic degradation in vivo, but the oxidation may also be insufficient to denature the protein so that protein carbonyls are allowed to accumulate to some extent. The steady-state concentration of protein carbonyls and other oxidative modifications in the plasma or in other biological specimens may thus reflect the accumulated oxidative stress in the compartment in question during the lifetime of the protein, thereby providing a potentially valuable biomarker for low-level oxidative stress. Several approaches to developing biomarkers for protein oxidation have been taken, but only few of them have been applied to studying the effects of micronutrient supplementation.

The simplest assay is based on the reaction of carbonyl groups with primary amines to form semi-stable Schiff-bases. The reaction with 2,4-dinitrophenylhydrazine (DNPH) is commonly used for the photometric determination of carbonyls. In its simplest form, it involves the sample reacting with DNPH, precipitation and a washing procedure to remove the unspecifically bound DNPH. After washing, the protein is solubilised to determine the level of specific binding photometrically. This method has the advantage of being fast and of low technical demands. The disadvantages include difficulties in removing the unspecifically bound DNPH, leading to high and variable background readings, a variable loss of protein during washing, and a risk of introducing additional oxidative modification during the procedure, leading to binding of the excess DNPH. The first two disadvantages can be partly overcome by isolating a specific protein fraction prior to precipitation, which leads to a much greater loss of the unspecifically bound DNPH. It also introduces the additional advantage of selecting the specific protein for study, since oxidation can vary between proteins due to differences in the redox micro-environment surrounding the proteins. Another solution is to use an immunoassay procedure involving specific antibodies to the DNPH-modified protein, thus avoiding the unspecifically bound DNPH and extensive washing.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDA</th>
<th>LDL oxidation ex vivo</th>
<th>Antioxidant capacity</th>
<th>Lipid hydroperoxides</th>
<th>Isoprostanes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C</td>
<td></td>
<td></td>
<td>+/-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stress + vitamin C</td>
<td></td>
<td></td>
<td>+/-</td>
<td>+</td>
<td>NR</td>
</tr>
</tbody>
</table>

NR — not reported.
To assess oxidative stress by use of the simplest version of this assay, the influence of taking 500 mg/d versus 1 g/d of vitamin C for 2 weeks prior to 30 min of hard exercise was evaluated in 12 males using a counterbalance design. Vitamin C was found to decrease the exercise-induced increase in protein carbonyls in a dose-dependent manner [83]. Using the same method for protein carbonyls, the effect of taking 272 mg/d of vitamin C supplement in conjunction with vitamin E (800 IU/d) and folate (400 µg/d) for 90 d was assessed in 39 smokers and 38 non-smokers who habitually had a low intake of fruit and vegetables. No effect of the supplement on protein carbonyls was observed [38]. In a very small study, involving seven divers, no effect on the levels of plasma protein carbonyls as a results of diving apnea or of a 1 g/d supplement of vitamin C for a week proceeding a diving experiment was observed [84].

A more advanced approach with an isolated protein fraction was taken in a study of the effect of a 400 mg/d vitamin C supplement given to healthy volunteers for a 15-week period. The level of protein carbonyls in the immunoglobulins was found to decrease after both 10 and 15 weeks of supplementation, whereas no change in the total plasma sulphhydryls was detected [85]. The decrease was confined to individuals with a suboptimal intake of vitamin C.

A third method employed for assessment of protein oxidation takes advantage of the reaction of protein aldehydes with fluorescein and reduction of the product by cyano-borohydride to form a stable adduct. Following protein isolation by a rapid size-exclusion chromatographic step and complete acid hydrolysis, the fluoresceinamine adduct could be detected by HPLC through the use of UV, fluorescence or APCI-MS detection [86]. This procedure has the advantage of being specific for the products of lysine (2-aminoadipic semialdehyde (AAS)) or for proline and arginine (γ-glutamyl semialdehyde) oxidation and of avoiding any further oxidation during workup of the sample. The main disadvantage is the longer time required for analysis and the higher technical demands. The method has been used to study the effect of supplementation by 500 mg/d of vitamin C versus placebo for 30 days in 16 healthy volunteers. A marginal increase in plasma protein AAS was observed (Dragsted, unpublished data). Another line of evidence comes from studies of dietary fruit and vegetable depletion, showing a rapid depletion of plasma ascorbate and a concomitant decrease in AAS, indicating that the depletion of ascorbate might have an antioxidant effect. Use of this approach was found in one study to produce a significant time-dependent decrease in AAS during a 24-day period of fruit and vegetable depletion in the diet, whereas no change was observed after supplementation of the known nutrients from the fruits and vegetables, including 150 mg/d vitamin C. No effect of fruit, vegetables or nutrient supplements was found in that study on the levels of total plasma carbonyls or immunoglobulin carbonyls by the antibody approach in conjunction with Western blotting [85]. None of the volunteers had suboptimal vitamin C levels before intervention. Since vitamin C is a cofactor for the lysine oxidases that cross-bind cartilage in the body, the observed effect of vitamin C on this specific marker may reflect enzyme leakage from the cartilage rather than prooxidative stress.
Overall there appears to be certain but limited evidence for suboptimal vitamin C intake leading to an increase in protein oxidation in the plasma immunoglobulins. Evidence for the prevention of stress-induced carbonyl formation by increasing the intake of vitamin C is controversial and needs further confirmation.

**Conclusions**

Total plasma vitamin C can readily be determined by automated colorimetric assays or HPLC. HPLC has the advantage of having lower detection limits, permitting simultaneous determination of ascorbate and dehydroascorbate, and possibly possessing higher accuracy, whereas the colorimetric assays have much higher throughput. The handling and storage of plasma for vitamin C determination has a strong effect on accuracy. Most of the currently available markers for assessing lipid or protein oxidation in humans have been employed in human intervention studies to assess the effects of vitamin C supplementation. There is only limited evidence that plasma ascorbate is a functional antioxidant in the body as assessed by means of these markers and very limited evidence that high dosages of vitamin C may decrease oxidative stress. No evidence for the antioxidant effect of vitamin C in the dose range of 60–2500 mg/d in connection with mild ascorbate depletion was obtained using the best lipid oxidation marker available, that of the formation of plasma isoprostanes. Clear short-term pro-oxidant effects on lipid oxidation were observed following high-dose vitamin C infusion into the bloodstream. There are no studies currently available on the relationship between lipid or protein oxidation markers, vitamin C and chronic disease.

**Vitamin E**

**Biomarkers for vitamin E in body fluids**

Vitamin E is a collective term for alpha-, beta-, gamma- and delta-tocopherols as well as the tocotrienols, of which RRR-α-tocopherol has the highest vitamin E activity. The reference for the international unit (IU) is 1 mg of all- Rac-α-tocopheryl acetate, 1 IU corresponding to 1.49 mg RRR-α-tocopherol. Since only the four R-forms of α-tocopherol (d-α-tocopherol) are recognised by the human vitamin E transporter in the body, none of the four S-forms of α-tocopherol and none of β-, γ- and δ-tocopherols or of the tocotrienols are thought to have any vitamin E activity in humans as opposed to the rat. This is still controversial, however, since in a study comparing the delivery of RRR-α-tocopherol and all-Rac-α-tocopherol to lipoproteins in humans following 8 weeks of supplementation by 1600 mg/d of either product, no difference in the total vitamin E content of LDL was observed [87]. The relative vitamin E activity of the various tocopherols and tocotrienols in the rat together with their structures are shown in Figure 2.3.

The vitamin E status in rats and possibly in other rodents can be determined as a function of all the active isomers, but in humans vitamin E status is preferably determined as the plasma, serum or erythrocyte concentration of d-α-tocopherol. Various functional tests have also been suggested, including the susceptibility of erythrocytes
to lysis following an *in vitro* challenge with hydrogen peroxide [88–90], or the exhalation of breath pentane [91]. In one study no correlation was found between results of the erythrocyte membrane stability test and plasma or erythrocyte vitamin E concentrations [90]. Also, since these functional tests may be lipid oxidation markers, it seems more appropriate to evaluate the relationship of vitamin E supplementation to currently used lipid oxidation markers. Vitamin E has a physiological transporter in the human that only binds *d*-α-tocopherol, and deletion of this transporter leads to neurological symptoms [92].

![Structures of tocopherols and tocotrienols and their relative potency as vitamin E in the rat (TE; dl-α-tocopherol equivalents). Redrawn from [100].](image-url)
Vitamin E and markers of lipid oxidation

This review concerns only randomised, controlled studies in which the effects of supplementation by vitamin E was investigated on peripheral blood samples using plasma or serum thiobarbituric acid reactive compounds (TBARS), \textit{ex vivo} LDL oxidation, antioxidant capacity markers, lipid hydroperoxides, or isoprostanes (see description in the section on vitamin C above). In a study of 24 diabetic patients assigned to take a capsule each day containing 100 IU vitamin E or a placebo for a period of three months, a significant decrease in plasma MDA was found in the vitamin-supplemented group as determined by HPLC [101]. In a subsequent study of 24 diabetic children by use of the same design, there was a significant decrease in erythrocyte MDA in the vitamin-supplemented group [102]. In 49 diabetic patients given either 504 mg/d \(d\)-\(\alpha\)-tocopherol or a placebo for a 6-month period a decrease in \textit{ex vivo} induced TBARS in the erythrocyte membranes was found for the supplemented group [103]. In a study of 49 HIV patients randomised to supplementation with a placebo or with 800 IU/d vitamin E and 1000 mg/d vitamin C for a 3-month period, a reduction in plasma MDA occurred in the group assigned vitamin supplements [104]. In a double-blind, controlled intervention study, 56 patients with congestive heart failure were given 335 mg/d of the natural \(d\)-\(\alpha\)-tocopherol for a 12-week period and no effect on the plasma MDA level was detected [105]. In a randomised 2-months intervention study of 59 healthy smoking males, MDA was found to increase significantly following daily doses of 200 mg vitamin E combined with 250 mg ascorbate, 50 mg beta-carotene and 100 \(\mu\)g organic selenium, given in a normal formulation, but to be unaffected by a slow-release formulation as compared with placebo treatment [33].

In a study of the effects on 77 smokers of taking vitamins C (272 mg/d) and E (800 IU/d) or a placebo for 90 d, no effect on the plasma MDA was detected [38].

In a trial involving 80 men showing an increase in plasma lipid oxidation from exposure to either olive oil or menhaden oil, giving a 900 IU dose of vitamin E was found to be no better than a placebo in decreasing MDA [106]. In a trial of lipid oxidation induced in 18 untrained men by three sessions of resistance exercises, those assigned to treatment with vitamin E (1200 IU/d starting 7 days before the exercises began) were found to not differ from the placebo group in the levels of plasma MDA [107]. In another study, of a group of 14 runners assigned to either a placebo or a 1200 IU/d dose of vitamin E starting 4 weeks before and continuing during 6 days of increased running training, a decrease in serum MDA was found in the group given vitamin E [108]. In a cross-over intervention study involving 4-week periods of either taking 500 or 1000 IU of vitamin E together with 500 or 1000 mg of vitamin C or taking placebo, no effect on exercise-induced oxidative stress could be shown by serum MDA [109]. In another study, 16 young and 16 older male subjects were first given eccentric exercises for 45 min and were then placed on 1000 IU/d of vitamin E supplementation for 12 weeks before being given a second round of exercises. No change in plasma MDA either within or between the two groups could be shown [110].

In a three parallel groups of 16 middle-aged male and female participants, who were given either 500 mg/d of vitamin C together with 182 mg/d \(dl\)-\(\alpha\)-tocopherol,
182 mg/d \textit{dl-}d\textalpha{}-tocopherol alone, or placebo, no effect on plasma antioxidant capacity was observed at 12 or 36 months [44].

In a double-blind controlled intervention study of 56 patients with congestive heart failure, treatment with 335 mg/d of natural \textit{d-}d\textalpha{}-tocopherol for 12 weeks was found to have no effect on the level of breath pentane exhalation [111]. In a trial involving 80 men who had an increase in plasma lipid oxidation following exposure to olive oil or to menhaden oil, a dosage of 900 IU vitamin E was found to equal placebo in reducing the level of lipid hydroperoxides in the plasma or the exhalation of breath pentane [112]. In a study of 49 HIV patients randomised for supplementation with a placebo or with 800 IU/d of vitamin E and 1000 mg/d of vitamin C over a 3-month period, there was found to be a reduction in plasma lipid peroxides and in breath pentane exhalation in the group to which a vitamin supplement was assigned [106]. In a study without a parallel control group, a 1000 IU/d vitamin E supplement for a 10 d period was found to reduce the exhalation of breath pentane [91].

In a dose-response study of 40 healthy men assigned doses of 60–1200 IU of vitamin E for an 8-week period, vitamin E given at doses higher than 200 IU/d was found to affect the kinetics of \textit{ex vivo} oxidation of LDL [113]. In a group of 45 randomised healthy males and females, the effects of giving mixed supplements of 200 mg/d vitamin E, 900 mg/d vitamin C and 18 mg/d beta-carotene for 6 months were compared with those of a placebo. Non-induced lipoprotein oxidation \textit{ex vivo} was found to be delayed in the group given the vitamin supplement, and the strength of this effect was correlated with the level of plasma \textit{d-}d\textalpha{}-tocopherol [114]. In a group of 48 middle-aged male and female participants in a 36-month intervention study in which either 500 mg/d of vitamin C, this together with 182 mg/d \textit{dl-}d\textalpha{}-tocopherol, 182 mg/d \textit{dl-}d\textalpha{}-tocopherol alone, or a placebo in a parallel design, a significant increase in the susceptibility of isolated LDL or VLDL to oxidation \textit{ex vivo} was observed at 12 and at 36 months in the group given only vitamin E and in group given the combined dosage. A significant change in these groups in whole plasma \textit{ex vivo} oxidation at 36 weeks was likewise observed [44]. In a study comparing the delivery of \textit{RRR-}d\textalpha{}-tocopherol and \textit{all-rac-}d\textalpha{}-tocopherol to lipoproteins in humans following 8 weeks of supplementation with 1600 mg/d of either product, no difference was observed in LDL-MDA, LDL-dienes, or LDL-hydroperoxides induced \textit{ex vivo} [87].

In 49 diabetic patients given 504 mg/d \textit{d-}d\textalpha{}-tocopherol or a placebo for 6 months, no change in the group receiving the supplement was detected in the antioxidant capacity of the erythrocytes as determined on the basis of the glutathione concentration and the glutathione peroxidase activity [103]. In a double-blind controlled intervention study of 56 patients with congestive heart failure, treatment with 335 mg/d of the natural \textit{d-}d\textalpha{}-tocopherol for 12 weeks was found to have no effect on the level of activity of plasma glutathione peroxidase [115].

In a blinded intervention study of 33 triathletes participating in a world championship, who were given 800 IU/d vitamin E or a placebo for 2 months prior to the race, the treatment with vitamin E was found to significantly increase the level of plasma F2-isoprostanes as well as of several markers of inflammation, including IL-6 [115].
In another study, 16 young and 16 older male subjects were given 45 min of eccentric exercise and were then given a 1000 IU/d supplement of vitamin E for a 12-week period before being given a second round of exercises. Following vitamin E supplementation a significant lowering of the plasma isoprostane level both before and 24 h after the exercise was found for the older men. This was not observed for the young men. No other significant differences were observed between the two groups [116]. The study design could not control for period effects, which may have caused the difference. In a third study of the effects of vitamin E on oxidative damage induced by exercise, 21 ultramarathon runners were given either a combination of 300 mg/d \(d\text{-}\alpha\text{-tocopherol}\) and 1000 mg/d vitamin C, or a placebo during the 6 weeks prior to the race. Running as such was found to increase the plasma isoprostane level, particularly in the males, and the vitamin supplement was found to prevent this [117]. The inflammatory markers were also affected by running, but this was not changed by the vitamin supplements. In a study of the combined effects of giving either vitamin C (500 mg/d) together with \(d\text{-}\alpha\text{-tocopherol}\) (400 mg/d), vitamin C (500 mg/d) together with \(d\text{-}\alpha\text{-tocopherol}\) (290 mg/d) and \(d\text{-}\gamma\text{-tocopherol}\) (130 mg/d), or a placebo during a 28 d period on the plasma isoprostane concentrations induced by exercise, no effect of either vitamin treatment was observed, although the treatment by \(g\text{-tocopherol}\) was found to affect the exercise-induced increase in plasma and muscle heat shock protein (HSP72) and also the expression of HSP72 in muscle [118]. In an intervention study of 46 healthy smokers given 0, 300, 600 or 1200 IU/d of vitamin E for 3 weeks, no effect on the excretion of F2-isoprostane could be observed for any of these interventions [119]. In a double-blind controlled intervention study of 56 congestive heart failure patients, treatment with 335 mg/d of natural \(d\text{-}\alpha\text{-tocopherol}\) for a period of 12 weeks was found to have no effect on the plasma F2-isoprostane level [111]. In another double-blind placebo-controlled trial with a crossover design, no effect on the plasma F2-isoprostane level of taking 1000 IU/d of vitamin E was found for 20 patients with endothelial dysfunction [125]. In a group of 33 sclerosis patients, 10 of them were given 500 mg/d and 10 of them 1000 mg/d vitamin E for a period of 3 weeks, the rest being given a placebo. No effect on urinary excretion of F2-isoprostanes was observed [121]. In 43 hypercholesterolemic patients randomised to taking simvastatin, simvastatin together with 600 mg/d vitamin E or a placebo for 2 months, the adding of vitamin E was found to have no further effect on the urinary excretion of F2-isoprostanes [122]. In a small non-blind study of cirrhotic patients, the 9 patients receiving standard medication together with 600 mg/d vitamin E for a 30-day period showed lower urinary excretion of F2-isoprostanes than 5 controls given only standard medication [123].

In a dose-response study, groups of 5 healthy individuals received either 0, 200, 400, 800, 1200 or 2000 IU/d of vitamin E for a period of 8 weeks, followed by 8 weeks of washout. No significant change in the plasma F2-isoprostane level could be detected by GC-MS at any time point, irrespective of the treatment [124]. In a study without a control group, isoprostane excretion was found to be decreased at the end of a 2-month period in a group of 15 healthy individuals receiving 400 IU/d of vitamin E [125].
Effects on protein oxidation
In a study without a control group, the plasma carbonyl content in 15 healthy individuals was found to be unaffected by 400 IU/d of vitamin E for a period of 2 months [125].

Other markers related to vitamin E effect
Intervention trials involving ingestion of synthetic vitamin E either alone or in combination with vitamin C have been performed to assess their effects on various conditions assumed to be caused by oxidative stress. Sperm counts and sperm motility are thought to be partially influenced by oxidative stress. Vitamin E deficiency is also known to cause semen abnormalities and infertility in rats. In two randomised studies, each with 30 men, little effect of taking 600–800 mg/d of vitamin E for 2–3 months was shown. In one study, a significant increase in the binding ratio to the zona pellucida of the unfertilized oocyte in a competitive binding assay was observed, but this parameter was not assessed in the other study [126,127]. No evidence was presented that this effect was related to antioxidation.

Conclusions
In conclusion, present day HPLC and GC-MS methodology has high precision and accuracy in the detection of vitamin E analogues in plasma. Since plasma vitamin E levels are not always correlated with the stability of the erythrocyte membrane or with the exhalation of breath pentane, these proposed functional tests for vitamin E must be regarded as obsolete. There is only limited evidence for a protective effect of vitamin E supplements at levels of 200–2000 IU/d on markers of lipid oxidation, and supposed effects of high levels of vitamin E supplementation either on exercise-induced lipid oxidation as determined by isoprostanes or on inflammatory markers are controversial. Vitamin E intervention has not been shown to decrease the oxidation of plasma proteins.

References


Vitamins and selenium: Biomarkers of exposure to and effects of vitamins A, C and E


2.2. Antioxidant vitamins and cancer risk: is oxidative DNA damage a relevant biomarker?

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The role of oxidative DNA damage in the development of cancer

Cancer is a disease of the genes. An increase in somatic mutations, which are implicated in the development of cancer, has been documented in aging cells and tissues. When mutations accumulate, this can be due to the individual’s overall lifetime exposure to endogenous and exogenous agents that damage the DNA. Food and beverages frequently contain carcinogens, partly those generated in processing, such as in the cooking of meat, and partly carcinogens that occur naturally in the products. It is quite possible, nevertheless, that metabolites of atmospheric oxygen, so-called reactive oxygen species (ROS), though not generally classed as carcinogenic agents, are human carcinogens. ROS, which can be produced both in the biochemical utilization of oxygen and as a by-product of O₂ metabolism in the mitochondria, can damage cellular molecules of different types — in particular proteins, lipids and nucleic acids.

The hydroxyl radicals that ROS creates result in a large number of pyrimidine- and purine-derived lesions being formed in the DNA (as reviewed in [1]). Some of these modified DNA bases have considerable potential for damaging the integrity of the genome (reviewed in [2,3]). One of the most widely studied lesions of this sort is 8-oxo-7,8-dihydroguanine (8-oxoGua). The presence of 8-oxoGua residues in DNA, unless repaired prior to DNA replication, leads to GC→TA transversions [4]. The presence of 8-oxoGua in the cells can thus result in point mutations.

In order to produce mutations, however, oxidative DNA damage needs to occur at a sufficiently high frequency to exceed the cell’s capacity for DNA repair. It is of interest to note in this context that a urinary excretion study showed the average 8-oxoGua and 8-oxodG (7-hydro-8-oxo-2′-deoxyguanosine) excretion in the urine of healthy subjects to be some 2.5 nmol per kg per day, corresponding to about 2000 oxidative modifications of guanine per cell daily.

Not only is DNA mutation a crucial step in carcinogenesis, but the large numbers of oxidative DNA lesions observed in many tumors strongly suggests that such damage is frequently a cause of cancer [5]. Oxidative mechanisms have been shown to have a potential role in the initiation, promotion and malignant conversion (progression) stages of carcinogenesis. Since the cumulative risk of cancer increases at a rate corresponding to the fourth power of age and is associated with accumulated DNA damage, increasing attention has been directed at oxidative DNA damage in relation to cancer. Lesions such as 8-oxodG are used as biomarkers of oxidative stress. This, together with their mutagenicity in mammalian cells, has led to the proposal that they be used as intermediate markers
of a potential disease endpoint such as that of cancer, although their suitability for this has yet to be verified in prospective studies of cancer risk. At the same time, there are serious problems in the chromatographic measurement of 8-oxodG, connected with the spurious oxidation of DNA during sample preparation. On the basis of an extensive investigation by the European Standards Committee on Oxidative DNA Damage (ESCODD) it has been concluded that the true background level of 8-oxodG in mammalian cells is approximately 0.3–4.2 lesions per 10^6 unaltered guanines and that reports of values outside this range should be viewed skeptically [6].

Numerous studies have been concerned with the relationship between the level of oxidative DNA damage and cancer (see [5] for a review). Elevated damage levels have been purported to arise as a result either of (i) the tumour having an environment low in antioxidant enzymes and high in ROS generation, or (ii) there being reduced DNA repair [5].

Elevated ROS levels may activate transcription factors and the corresponding genes being permanently activated. This, together with the increase in DNA damage that occurs, creates a selection pressure for the malignant phenotype seen in the case of cancer. Although studies that indicate this support the hypothesis that oxidative DNA damage can be an important risk factor for carcinogenesis, it has been argued that the mere presence of 8oxodG in DNA is unlikely to be a necessary and sufficient condition for tumour formation. In addition, there are a large number of pathological conditions in which the level of oxidative DNA damage is elevated without any increase in the incidence of carcinogenesis [5]. This raises a number of different issues:

1. Oxidative DNA damage may be an epiphenomenon in relation to the ongoing pathophysiological process, the levels of damage having no causal role in carcinogenesis.
2. Again, regarding the question of ‘cause or consequence’, the mere presence of an elevated level of damage in a tumour does not indicate oxidative damage to have led to the tumourigenic changes that have occurred. An elevated level may be due to some well-established characteristic of the tumour, such as a high level either of metabolism or of cell turnover.
3. For DNA mutations to arise through oxidative damage, the nuclei of the undifferentiated, proliferating stem cells have to be affected. Since tissue samples from both tumours and normal cells represent a heterogeneous mixture of differentiated and undifferentiated cells (the former being likely to predominate), the analytical procedures in current use are unable to quantify the lesion levels found in the most important ‘target’ cells.

In order for a mutation to come about, not only must the DNA of the target cells be affected, but also the damage must be within a coding region of the DNA. Issues of this sort have to be addressed before a causal link between oxidative DNA damage and cancer can be established. There is a need in this context of large prospective studies able to demonstrate to what extent an elevated level of oxidative DNA damage indicates an increased risk of developing cancer. Ultimately, demonstrating that an intervention that reduces oxidative DNA damage also reduces the risk of cancer would provide the evidence needed of the value of such biomarkers in a public health and cancer prevention context.
Summing up, one can say that in light of the data obtained thus far it appears likely that the development of many types of cancer leads to severe oxidative stress, but that it is impossible at present to determine to what extent oxidative stress is directly involved in carcinogenesis, since full development of the disease in response to exposure to a carcinogen may take 20–40 years. It is very difficult, therefore, to establish directly that the DNA lesion responsible for a carcinogenic process is the lesion present in a tumor many cell generations later. One should bear in mind, nevertheless, that DNA damage, altered gene expression and mutations are necessary elements in the process of carcinogenesis. Although these events may come about by way of different mechanisms, oxidants are involved in each case.

**Dietary antioxidants as inhibitors of oxidative DNA damage and as a factor decreasing cancer risk**

There is widely believed to be a link between diet and the incidence of cancer. A plethora of descriptive epidemiological studies have concerned a possible protective effect of a diet rich in fruits and vegetables [7]. Both experimental and epidemiological data indicate vitamin C to protect against both stomach and oesophageal cancer [8]. Nevertheless, large-scale intervention studies have failed to demonstrate use of antioxidant vitamins to reduce the risk of cancer [9]. At the same time, the mode of action of dietary micronutrients is complex and is far from being fully understood. It is reasonable to assume that agents that decrease oxidative DNA damage should also decrease the subsequent development of cancer. One possible mechanism by which the protective effect of fruits and vegetables is exerted could thus be by way of the antioxidative activities of such plant food constituents as vitamins A, C and E or phenolic compounds. These antioxidants are effective free-radical scavengers and should protect the DNA from oxidative damage.

Intuitively, supplementation trials would appear to represent the most relevant way of exploring antioxidant effects, although sampling is usually restricted to use of such surrogate tissues as white blood cells (WBC) and urine. At the beginning of the 1990s, as the possibility of detecting 7-hydro-8-oxo-2'-deoxyguanosine (8-oxodG) appeared, the first of many antioxidant supplementation trials dealing with this lesion in WBC was carried out [10]. Reliable detection of urinary 8-oxodG excretion became possible at about the same time [11], this soon being followed by the performance of antioxidant trials in which urinary 8-oxodG excretion served as the key biomarker. Subsequently, however, the comet assay, used for the detection of DNA strand breaks (SB), and the enzyme-modified version of the comet assay allowing oxidized purines (including 8-oxodG) and pyrimidines to be detected by means of formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (ENDOIII), respectively, have become by far the most popular assays for use in conjunction with antioxidant intervention trials. The literature on biomarkers of oxidative DNA in WBC employed in small-scale intervention studies of antioxidant supplements has been summarized in a series of reviews [9,12].
Many studies suffer, however, from use of a non-optimum design. Table 2.6. provides an overview of intervention studies involving optimal design in which the effects of antioxidants or antioxidant-rich food supplements on oxidative damage to DNA in WBC or in urine have been investigated.

Table 2.6. Multiple administration of dietary antioxidants with assessment of oxidative DNA damage in white blood cells and urine

<table>
<thead>
<tr>
<th>Supplement given per day</th>
<th>Subjects</th>
<th>Age (yr)</th>
<th>Effect</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multivitamin tablet (100 mg vitamin C, 280 mg vitamin E, and 25 mg β-carotene) for 20 wk</td>
<td>100 M (50S)</td>
<td>50–59</td>
<td>A decrease in ENDOIII sites ( Comet) in WBC after 20 weeks</td>
<td>13</td>
</tr>
<tr>
<td>Multivitamin (250 vitamin C, 200 IU α-tocopherol, and 6 mg β-carotene) for 6 mo</td>
<td>63 MF (S)</td>
<td>42±9</td>
<td>No difference in 8-oxodG in WBC (antibody-based detection) between the supplemented and the placebo group, but a decline in the course of the trial in both groups</td>
<td>14</td>
</tr>
<tr>
<td>Carotenoids for 3 wk</td>
<td>32 MF (NS)</td>
<td>32±11</td>
<td>No effect compared with baseline, but a post-supplementation decrease in the urinary excretion of 8-oxodG (ELISA) in the active group</td>
<td>30</td>
</tr>
<tr>
<td>Vitamin C (500 mg) for 3 wk</td>
<td>30 MF (NS)</td>
<td>17–49</td>
<td>No effect of vitamin C supplementation on 8-oxodG (ELISA) in spot urine, but an increase in excretion during the washout period</td>
<td>24</td>
</tr>
<tr>
<td>Six groups receiving combinations of vitamins for 2 mo</td>
<td>116 M (S)</td>
<td>30–65</td>
<td>No effect on the 24-h urinary excretion of 8-oxodG (HPLC)</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin C (1 g) and vitamin E (0.6 g) for 1 mo</td>
<td>13 M (NR)</td>
<td>30±3</td>
<td>Lower 24-h urinary excretion of 8-oxodG (HPLC) in the active group of HIV-infected patients receiving zidovudine therapy</td>
<td>25</td>
</tr>
<tr>
<td>2x2 parallel study of vitamin C (500 mg) and vitamin E (400 IU) for 2 mo</td>
<td>184 MF (NS)</td>
<td>58±14</td>
<td>No effect on the 24-h urinary excretion of 8-oxodG (ELISA)</td>
<td>28</td>
</tr>
<tr>
<td>2x2 parallel study of vitamin C (500 mg) and vitamin E 182 mg) for 12 mo</td>
<td>48 M (22S)</td>
<td>45–69</td>
<td>No effect on the 24-h urinary excretion of 8-oxodG (HPLC)</td>
<td>34</td>
</tr>
<tr>
<td>Multivitamin tablet for 2 wk</td>
<td>30 NR (NR)</td>
<td>22±1</td>
<td>No effect on the 24-h urinary excretion of 8-oxodG (ELISA) in subjects undergoing cold-weather training at a moderate altitude</td>
<td>33</td>
</tr>
<tr>
<td>Multivitamin tablet for 21 d</td>
<td>39 MF (NR)</td>
<td>7±2</td>
<td>No effect on the excretion of 8-oxodG (ELISA) in spot urine samples</td>
<td>37</td>
</tr>
<tr>
<td>Multivitamin tablet for 24 d</td>
<td>40 M (NR)</td>
<td>18–40</td>
<td>No effect on the overnight excretion of 8-oxodG (ELISA) in subjects undergoing cold-weather training at a moderate altitude</td>
<td>36</td>
</tr>
<tr>
<td>Fruit and vegetable capsules for 7 wk</td>
<td>59 MF (11S)</td>
<td>50±6</td>
<td>No effect on the excretion of 8-oxodG (ELISA) in spot urine samples</td>
<td>29</td>
</tr>
</tbody>
</table>
Table 2.6. Multiple administration of dietary antioxidants with assessment of oxidative DNA damage in white blood cells and urine — cont.

<table>
<thead>
<tr>
<th>Supplement given per day</th>
<th>Subjects*</th>
<th>Age (yr)”</th>
<th>Effect</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antioxidant rich diet or tablets for 5 wk</td>
<td>55 MF (NR)</td>
<td>71 ± 6</td>
<td>No effect compared with baseline, but a post-supplementation decrease in the 24-h urinary excretion of 8-oxodG (ELISA) in the active group</td>
<td>32</td>
</tr>
<tr>
<td>Rye crisp bread (76.5 g) or placebo (fiber-free bread) for 2 wk</td>
<td>12 F (NR)</td>
<td>NR</td>
<td>No effect on ENDOIII sites (Comet) in WBC</td>
<td>17</td>
</tr>
<tr>
<td>Flavonol (quercetin) rich diet for 2 wk in crossover design among type 2 diabetes patients</td>
<td>10 MF (3S)</td>
<td>60 ± 7</td>
<td>No effect on ENDOIII sites (Comet) in WBC</td>
<td>16</td>
</tr>
<tr>
<td>Vegetable/fruit (500 g) in crossover design for 3 wk with 2 wk washout</td>
<td>22 M (S)</td>
<td>33 ± 11</td>
<td>No effect on ENDOIII sites (Comet) in WBC</td>
<td>19</td>
</tr>
<tr>
<td>Vegetable/fruit (600 g) or tablets with the same concentration of antioxidants/minerals for 24 days</td>
<td>43 MF (NS)</td>
<td>27 ± 6</td>
<td>No effect on ENDOIII and FPG sites (Comet) in WBC. No effect on the 24-h urinary excretion of 8-oxodG (HPLC), but a decline in all the groups</td>
<td>20</td>
</tr>
<tr>
<td>Cruciferous and legume sprouts (113 g) for 2 wk</td>
<td>18 MF (NR)</td>
<td>21—45</td>
<td>No effect on FPG sites (Comet) in WBC</td>
<td>15</td>
</tr>
<tr>
<td>Kiwi fruit (1–3 pieces) for 3 wk</td>
<td>14 MF (NS)</td>
<td>26—54</td>
<td>A decrease at the ENDOIII and FPG (Comet) sites</td>
<td>22</td>
</tr>
<tr>
<td>Brussels sprouts (300 g) for 1 wk</td>
<td>10 MF (NS)</td>
<td>NR</td>
<td>No effect on the 24-h urinary excretion of 8-oxodG (HPLC)</td>
<td>40</td>
</tr>
<tr>
<td>Brussels sprouts (300 g) for 12 d</td>
<td>10 M (NS)</td>
<td>NR</td>
<td>A decrease in the 24-h urinary excretion of 8-oxodG (HPLC) in the active group</td>
<td>41</td>
</tr>
<tr>
<td>Fruit juice (480 ml) for 4 d</td>
<td>11 M (NR)</td>
<td>21 ± 1</td>
<td>A decrease in the 12-h urinary excretion of 8-oxodG (ELISA) in the active group</td>
<td>26</td>
</tr>
<tr>
<td>Parallel study of blackcurrant juice or anthocyanin drink (475–1000 ml) for 3 wk</td>
<td>57 MF (6S)</td>
<td>19—52</td>
<td>No effect on ENDOIII and FPG sites (Comet) in WBC</td>
<td>18</td>
</tr>
<tr>
<td>Green tea or black tea (4 cups) for 1–4 mo</td>
<td>120 MF (S)</td>
<td>18–79</td>
<td>A decrease in the excretion of 8-oxodG (ELISA) in spot urine samples in the green tea group after 4 mo, but not earlier</td>
<td>27, 45</td>
</tr>
<tr>
<td>Two interventions of green tea with 300 ml for 7 d or 32 oz for 7 days</td>
<td>68 MF (13S)</td>
<td>18–45</td>
<td>A decrease in the 12-h urinary excretion of 8-oxodG (HPLC)</td>
<td>317</td>
</tr>
<tr>
<td>Green tea extract* for 3 wk</td>
<td>16 M (8S)</td>
<td>20–31</td>
<td>No effect of supplementation on the 24-h urinary excretion of 8-oxodG (HPLC) but a decrease in excretion during the study in all groups</td>
<td>44</td>
</tr>
<tr>
<td>Soya-hypocotyl tea (&gt; 1 l) for 1 mo</td>
<td>38 F (NR)</td>
<td>NR</td>
<td>A decrease in the excretion of 8-oxodG (ELISA) in the active group (statistical test not reported)</td>
<td>42</td>
</tr>
</tbody>
</table>
Table 2.6. Multiple administration of dietary antioxidants with assessment of oxidative DNA damage in white blood cells and urine — cont.

<table>
<thead>
<tr>
<th>Supplement given per day</th>
<th>Subjects(^a)</th>
<th>Age (yr)(^b)</th>
<th>Effect</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy milk, rice milk, or cow milk (1 l) for 4 wk</td>
<td>10 M (NS)</td>
<td>20–50</td>
<td>A decrease in ENDOIII sites (Comet) for soy milk</td>
<td>21</td>
</tr>
<tr>
<td>Polyphenol-rich olive oils (25 ml) for 40 d</td>
<td>12 M (NS)</td>
<td>20–22</td>
<td>A decrease in the excretion of 8-oxodG (HPLC) in spot urinary samples following supplementation in a dose-dependent manner</td>
<td>43</td>
</tr>
</tbody>
</table>

\(^a\) Number of subjects indicated as males (M) and females (F). Smokers (S) and nonsmokers (NS) are indicated in brackets.

\(^b\) Age is shown as range or as mean ± standard deviation.

\(^c\) Supplement consisting of β-carotene (6 mg), α-carotene (1.4 mg), lycopene (4.5 mg), bixin (11.7 mg), lutein (4.4 mg), and paprika carotenoids (2.2 mg).

\(^d\) The six groups received daily supplements of 1) 200 mg vitamin E; 2) 500 mg plain-release vitamin C; 3) 500 mg slow-release vitamin C; 4) 90 mg coenzyme Q10 in oil; 5) 30 mg coenzyme Q10 granulate; 6) placebo.

\(^e\) Tablets containing β-carotene (12 mg), vitamin E (400 IU), vitamin C (500 mg), selenium (100 mg), and zinc (30 mg).

\(^f\) Tablets containing micronutrients similar to those of 3 fruits and 3 vegetables (containing 107 mg vitamin C and 83 mg vitamin E).

\(^g\) Consisted of β-carotene (20050 IU), vitamin C (330 mg), tocopherols (650 IU), selenium (167 mg), catechin (13.2 mg), lutein (500 mg), lycopene (100 mg), N-acetyl-1-cysteine (181 mg), and pomegranate extract (5 mg).

\(^h\) The fruit capsules were made from juiced apple, orange, pineapple, papaya, cranberry and peach. The vegetable capsules were made from carrots, parsley, beet, broccoli, kale, cabbage, spinach, and tomato. The daily dose consisted of 200 mg vitamin E, 60 mg vitamin C, and 15 mg β-carotene.

\(^i\) Consisted of tablets containing vitamin antioxidants (400 mg vitamin C, 150 mg vitamin E, 4 mg β-carotene), capsules (containing 90 mg vitamin C, 18 IU vitamin E, 2.4 mg β-carotene, and powder or extract of fruits and berries), or a carotenoid-rich diet.

\(^j\) Containing vitamin A (240 mg), β-carotene (399 mg), vitamin C (219 mg), vitamin E (1.44 mg) per day.

\(^k\) Consisted of 1000 mg extract/kg body weight in meat patties (total phenolics 2.55 mg/10 MJ).

**Effects of antioxidant supplementation on oxidative DNA damage in WBC**

Single doses of simple antioxidants have been found to generally reduce the level of oxidative DNA damage temporarily [9,12]. The effect of a single dose of vitamin C seems to disappear after several hours, whereas tocopherols and carotenoids exert their effects somewhat longer, possibly because of differences in the respective rates of bioavailability and elimination. Among studies in which multiple doses of single antioxidants are given, however, there are fewer that reveal protective effects than those that report only negative results [9,12]. This suggests that the protective effect of a single antioxidant is relatively short. In two well-designed studies of the effects of administering a combination of antioxidant vitamins, a protective effect could be shown after only 20 weeks of supplementation but no effect after 10 weeks (or 6 months of supplementation) [13,14]. Accordingly, the question of whether multiple vitamin supplementation provide better protection against oxidative DNA damage than a single dose does cannot be answered unambiguously.

**Antioxidant-rich foods**

Ingestion of a diet rich in flavonols (including quercetin) and of one rich in cruciferous and legume sprouts (113 g/d for 2 wk) was not found to alter the frequency of ENDOIII and of FPG sites, respectively [15,16]. Also, being given rye crisp
Vitamins and selenium: Antioxidant vitamins and cancer risk

bread (76.5 mg/d for 2 wk) as a source of lignans was not found to be associated with any increase in the plasma enterolactone concentration, or to have any effect on the ENDOIII sites [17]. The lack of any effect of lignans in the WBC would seem reasonable in view of the low bioavailability of the active substances in rye crisp bread, and their effects in the gastrointestinal tract are easier to comprehend. Drinking black currant juice or an anthocyanine drink (475–1000 ml/d for 3 wk) was also not found to have any beneficial effect on ENDOIII- or FPG-sensitive sites, there in fact being a tendency in the group of subjects drinking black currant juice for the number of FPG sites to increase [18]. Anthocyanines have low bioavailability and the dose provided here was rather high. It can be speculated that the subjects suffered a slight, unintentional intoxication of the gastrointestinal tract (some of those in the active groups complained of nausea, for example). Two studies concerned the effects of an intervention of providing vegetables and fruits. The one investigation, a cross-over study of male smokers, showed no effect on the ENDOIII sites of ingesting 500 g/d of such food for 3 wk [19]. The other, a placebo-controlled parallel study in which non-smoking subjects of both sexes were given 600 g/d of fruits and vegetables for 24 days, was negative with respect to effects on the ENDOIII and FPG sites [20]. Five studies carried out in which a reduction in the level of oxidative DNA damage was observed involved providing very different antioxidant-rich foods that were not easy to compare. Drinking soy milk (1000 ml/d for 4 wk) as a source of phytoestrogens in the one study increased the plasma levels of genistein and daidzein but not of enterolactone; assessment of the DNA damage occurring showed the levels of ENDOIII to be reduced [21]. The only study showing consistent effects involving more than one endpoint was a study of the effects of kiwi fruit supplementation (1–3 kiwi fruits/d for 3 wk), which showed the numbers of ENDOIII and FPG sensitive sites to be reduced [22].

An overall summary of the studies showed that six investigations reported beneficial effect of antioxidant supplementation, whereas 13 studies reported null effect. There is little support for the notion that ingestion of antioxidant-rich foods is associated with lower spontaneous level of oxidative DNA damage in WBC than intake of single antioxidants.

**Effect of antioxidant supplementation on 8-oxodG in urine**

Measurement of the urinary excretion of 8-oxodG in antioxidant intervention studies is connected with the idea that it decreases following a steady state ingestion of antioxidants due to the rate of generation of oxidative DNA damage in the body being decreased. For 24 studies of the effects of antioxidant supplementation on the urinary excretion of 8-oxodG in which a controlled design was employed [20,23–44], no appreciable difference was present in terms of duration of the intervention period, number of subjects, or power to detect a 50% change between these studies reporting beneficial effects and those reporting no effects.
Four studies of the supplementation of a single carotenoid showed there in each case to be no effect on the urinary excretion of 8-oxodG [23,35,38,39]. A comparable study involving supplementation of a mixture of carotenoids (daily intake: α-carotene (1.4 mg), β-carotene (6.0 mg), lycopene (4.5 mg), bixin (11.7 mg), lutein (4.4 mg), and paprika carotenoids (2.2 mg)) revealed a statistically significant difference between the active and the placebo group in the delta values obtained (i.e. the difference between results at the end of supplementation and at baseline) but no difference between the two groups at baseline [30]. The statistically significant difference in the delta values reflected a marked increase in 8-oxodG excretion in the placebo group and a slight decrease in the excretion of it in the group given mixed carotenoids. In four additional studies, neither supplementation of vitamin C or vitamin E or a combination of them was found to have any effect in healthy subjects [24,28,34,35], whereas in still another study a beneficial effect of the supplementation of high doses of vitamin C (1000 mg/d) and vitamin E (600 mg/d) was found for HIV-infected patients treated with zidovudine [25]. Further studies showed multi-vitamin tablet supplementation to provide no beneficial effect either in normal subjects [20,32,37] or in subjects undergoing cold-weather field training at a moderate altitude [33,36].

Investigations of the effects of natural food products are distributed about equally between studies reporting beneficial and those reporting null effects. Ingestion of olive oils with a high content of phenolic compounds was found to be associated with a lowering of the urinary excretion of 8-oxodG [43]. A number of studies have involved supplying antioxidants in the form of berries, fruits, tea, and vegetables. Taking capsules containing extracts of fruits and berries and eating diets rich in carotenoids were both found to lower the excretion of 8-oxodG [32]. A positive effect of vegetable juice consumption on 8-oxodG excretion was also observed in subjects enrolled in a soccer summer training camp [26]. On the other hand, neither eating 600 g of fruits and vegetables nor consuming corresponding amounts of minerals and vitamins in tablet form was found to be associated with a lower level of the urinary excretion of 8-oxodG than in a placebo group, whereas this intervention was found to have a pronounced period effect [20]. No effect on the urinary excretion of 8-oxodG was found for the ingestion of capsules containing juices and powders of fruit (apple, orange, pineapple, papaya, cranberry, and peach) and of vegetables (carrot, parsley, beet, broccoli, kale, cabbage, spinach, and tomato) [29].

Mixed results concerning the effects of a dietary supplementation of Brussels sprouts (300 g/d) were obtained in two studies [40,41]. In the first study, that involved only male subjects, the Brussels sprouts supplementation was found to lower the urinary excretion of 8-oxodG [41]. In the subsequent study, in which both sexes were included, the effect was less clear, there being a tendency for only the male subjects to benefit from ingestion of Brussels sprouts, but the results were uncertain because of the only small number of subjects tested and of one of the male subjects showing an unexpectedly high urinary 8-oxodG excretion level [40]. Three additional studies in this area concerned the effect of drinking green tea [see 27,31,44,45]. In the one study a beneficial effect was found for drinking 300 ml/d for a week [31], whereas in one of the other two studies
no effect of ingesting green tea extract in meat patties for 3 wk was obtained [44]. Although the unadjusted data of the third study indicated no beneficial effect of drinking green tea, adjustment of the data for a number of variables, including baseline 8-oxodG levels, revealed a statistically significant positive effect of drinking green for 4 months [27,45]. In still a further study, drinking soya hypocotyl tea was found to be associated with a lower urinary excretion of 8-oxodG, although it should be emphasized that the fate of the antioxidants in this investigations was inconclusive since 1) the plasma concentration of carotenoids decreased, 2) the putative active constituents (isoflavones) were not measured in the plasma, and 3) the alterations in the urinary concentration could not be assessed due to insufficient information [42].

There are a number of possible reasons for the failure of a positive effect of vitamin supplementation to be shown in connection with the cancer risk that oxidative DNA damage creates:

1. It is possible that a preventive effect of the vitamins can be only be seen when their basal levels are very low, such as in the case of severe oxidative stress. Indeed, vitamin supplementation of HIV-infected patients showing very low levels of antioxidant vitamins and significantly increased lymphocyte amounts of 8-oxoGua (as well as other base modifications) was found in one study to result in vitamin restoration to levels characteristic for the control subjects. The authors also noted a significant decrease in the levels of the modified bases in patients who were thus treated as compared with those who received only a placebo. It is possible, therefore, that the presence of oxidative stress, which might fail to be recognized, could increase the likelihood of detecting a protective effect.

2. Under some circumstances, the prooxidative properties of certain of the antioxidant vitamins (vitamins C and A) may take effect. Experimental data suggest that supplementation of vitamin C to iron-overload subjects may increase the oxidative DNA damage that occurs [46]. It is worthy of note in this context that presumably healthy men may have the hereditary disease idiopathic haemochromatosis, which leads to iron overload, such that iron catalytic to free-radical reactions (a so-called labile iron pool — LIP) is present in the blood plasma [47]). Interestingly, a positive correlation has been shown between LIP and the oxidatively modified nucleoside in human lymphocytes [48]. The absorption of non-haem iron has also been found to be affected by ascorbic acid [49].

3. The question can be raised as to whether antioxidants in the blood and 8-oxodG in the DNA of lymphocytes and leukocytes are representative of the situation in the target tissue.

4. It is possible that the antioxidants themselves may allow clonal expansion to occur and promote tumour growth by protecting initiated cells from excessive oxidant toxicity and apoptosis that would otherwise kill them [50].

5. Paradoxically, antioxidant vitamins may have biological activities, such as those of regulating changes in gene expression, that are separate from their direct antioxidant effects [51].
Comments

There are numerous experimental studies published each year on the potential of antioxidants or antioxidant-rich foods for preventing the oxidation of DNA. On an experimental basis, it is easy to understand the ingestion of antioxidants being associated with lower levels of oxidative DNA damage. At the same time, many of the studies performed are of only limited value due to methodological problems related to the study design and the assays, and many of them lack the power of detecting 50% differences between two separate groups. Although single studies may possess considerable power due to specific aspects of their design such as use of repeated measurements, a major problem is that many of these studies have too few subjects. The most likely effect ratio in healthy subjects involves a difference of less than 10%, which means that, to obtain significant results, hundreds of subjects would be needed, most studies encompassing far fewer subjects than this.

At present, no firm conclusions can be reached on the basis of antioxidant intervention studies. There is a tendency for supplementation with use of antioxidant-rich food to decrease the urinary excretion of 8-oxodG, which is not the case with use of single antioxidants. WBC studies provide little support for the idea that long-term antioxidant supplementation lowers the basic level of oxidative DNA damage, although there may be a beneficial effect in the first few hours after ingestion. This can be interpreted as antioxidants having an overall beneficial effect on the body as a whole, yet the use of WBC as a surrogate tissue may not be particularly well suited for detection of this effect. It should also be borne in mind that the majority of studies involve healthy individuals, whereas the protective effect of antioxidants may be more easily detected in subjects who are suffering from oxidative stress. These could be either healthy subjects exposed to oxidative stress (such as exhaustive exercise or hyperbaric oxygen treatment) or patients subject to oxidative stress on the basis of some given disease. The few well-controlled studies that have reported realistic levels of oxidative DNA damage in the WBC of oxidatively stressed subjects lend little support to the notion that such a population benefits more from antioxidant supplementation than a normal study population. It should be noted, however, that most of the studies reported have used supplements of vitamin E, which is considered to be the least effective form of antioxidant supplementation. There is an obvious need for controlled antioxidant intervention studies encompassing subjects who are oxidatively stressed and in whom oxidative DNA damage is measured by enzymic or chromatographic methods.

In the future, more attention should probably be devoted to alternative chemopreventive mechanisms such as the upregulation of DNA repair systems, and to other types of DNA damage, such as those involving bulky DNA adducts. Also the chemopreventive effect of antioxidants on non-lymphatic tissue, which has been only sparsely investigated, should be addressed more thoroughly before the idea of antioxidants having clearly beneficial effects is abandoned. Hopefully, such studies will benefit from the lessons learned from antioxidant intervention studies, particularly as regards the need of proper investigative designs and of the validity biomarkers, which are of pivotal importance for such studies.
References


2.3. Measurement of serum 25-hydroxycholecalciferol as marker of vitamin D status

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Vitamin D₃, or cholecalciferol, is synthesized in the skin. Its precursor, 7-dehydrocholesterol, is converted by UV light from the sun (UVB 290–315 nm) into previtamin D₃ which slowly isomerizes to vitamin D₃. In addition, the human diet contains vitamin D in the form of vitamin D₃ (in food of animal origin) and vitamin D₂ (ergocalciferol contained in plant food, arising from the irradiation of ergosterol). In the blood vitamin D and its metabolites are bound to the vitamin D binding protein. Vitamin D is metabolised by hepatic 25-hydroxylase into 25-hydroxycholecalciferol (25-(OH)D), which is the major circulating metabolite. Further hydroxylation to 1,25-dihydroxycholecalciferol (1,25-(OH)₂D or calcitriol) occurs primarily in the kidney (1α-hydroxylase). The biologically most active vitamin D metabolite is 1,25-(OH)₂D whereas 25-(OH)D has only limited biological activity [1,2].

There is overall agreement that 25-(OH)D is the appropriate biomarker to measure vitamin D-status in humans. Because of its serum half-life time of about three weeks [3], this metabolite is a good indicator of the vitamin D-stores obtained both from exposure to sunlight and ingestion of vitamin D [2]. As compared with 25-(OH)D, the half-life time of its precursor, vitamin D (cholecalciferol), is rather short (24 h in the blood circulation), its thus being more strongly affected by very recent sun exposure or dietary intake of vitamin D [4]. Plasma 1,25-(OH)₂D, with an even shorter serum half-life time of 4–6 h, is also not regarded as a suitable biomarker of vitamin D status [5]; since its conversion from 25-(OH)D is also tightly regulated, the circulating 1,25-(OH)₂D level does not provide valid information on the individual’s vitamin D status [6].

Determination of 25-(OH)D

As reflected in the recent scientific literature, the determination of circulating 25-(OH)D is not an easy task [7]. One of the major problems in measuring 25-(OH)D lies in the molecule itself. First of all it is a very hydrophobic compound and secondly it exists in two forms, 25-(OH)D₂ and 25-(OH)D₃. Because of its hydrophobic nature, 25-(OH)D measurements are quite vulnerable to matrix effects, e.g. to lipids. Such matrix effects can markedly diminish the validity of assays carried out [8]. Extraction from the matrix (serum or plasma) is thus required for most analytical procedures (see Table 2.7.).

Considerable interest has developed in assays for measuring 25-(OH)D. Since the time of first assays in 1971, strong efforts have been made to simplify them for routine use [6]. Measurement by means of HPLC is considered by most to be the gold standard here [6,8]. Although such measurement is very accurate, it has to be performed by experienced personnel, requires expensive equipment (HPLC) and is more time-consuming than other
methods. Radioimmunoassays (RIA), enzyme immunoassays (EIA) and chemiluminescence assays (CLPBA) are easier to handle, as well as being fast, and are thus more frequently used. There are important differences in the performance of the various detection methods, however. Table 2.7. summarizes some of the performance characteristics of most of the assays that are available commercially.

Table 2.7. Commercially available 25-hydroxyvitamin D assays [6]

<table>
<thead>
<tr>
<th>Test principle, Manufacturer</th>
<th>Sample type and volume*</th>
<th>Extraction</th>
<th>Range of detection (nmol/l)</th>
<th>Sensitivity (nmol/l)</th>
<th>Intra-assay CV (%)</th>
<th>Inter-assay CV (%)</th>
<th>Assay time**</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIA, DiaSorin</td>
<td>Serum or plasma, 50 µL</td>
<td>Acetonitrile</td>
<td>≤6</td>
<td>&lt;8</td>
<td>&lt;12</td>
<td>2 h, 10 min</td>
<td>Calibrators and controls in the serum matrix; no yield determination required</td>
<td></td>
</tr>
<tr>
<td>RIA, IDS</td>
<td>Serum or plasma 50 µL</td>
<td>Two-step reagent extraction</td>
<td>4—400</td>
<td>≤5</td>
<td>5.5</td>
<td>7.9</td>
<td>3 h, 0 min</td>
<td>Calibrators and controls in the serum matrix; no yield determination required</td>
</tr>
<tr>
<td>ELISA, IDS</td>
<td>Serum or plasma 50 µL</td>
<td>None</td>
<td>6—360</td>
<td>≤5</td>
<td>&lt;6</td>
<td>&lt;9</td>
<td>3 h, 0 min</td>
<td>No radioactive waste; 100% cross-reactivity with 24,25 (OH2)D</td>
</tr>
<tr>
<td>ELISA, Biomedica</td>
<td>Serum or plasma 50 µL</td>
<td>Proprietary extraction reagent</td>
<td>6.3—250</td>
<td>2.4</td>
<td>9</td>
<td>11</td>
<td>5 h, 0 min</td>
<td>The primary antibody is the vitamin D binding protein from serum</td>
</tr>
<tr>
<td>Chemiluminescence, Nichols Institute Diagnostics</td>
<td>Serum or plasma 20 µL</td>
<td>Unknown</td>
<td>17.5—300</td>
<td>18</td>
<td>6.6</td>
<td>11.2</td>
<td>75 min</td>
<td>Fully automated; requires luminometer</td>
</tr>
<tr>
<td>HPLC, IDK</td>
<td>Serum 500 µL</td>
<td>Acetonitrile and C18 cartridge extraction</td>
<td>15—150</td>
<td>4.0</td>
<td>5.2</td>
<td>8.4</td>
<td>20 min</td>
<td>The laboratory must have an HPLC unit with a silica column</td>
</tr>
<tr>
<td>CPB, Immundiagnostics</td>
<td>Serum, plasma or urine 500 µL</td>
<td>Acetonitrile</td>
<td>8—312</td>
<td>2.5</td>
<td>9.9</td>
<td>14</td>
<td>1 h, 10 min</td>
<td>Uses vitamin D binding protein</td>
</tr>
</tbody>
</table>

* Represents the initial starting volume of the sample.

** Does not include extraction, counting or microplate reading times. CV — coefficient of variation; RIA — radioimmunoassay; ELISA — enzyme-linked immunosorbent assay; HPLC — high-performance liquid chromatography; CPB — competitive protein binding; 24, 25 (OH2) D, 24,25-dihydroxyvitamin D.
Validity of different assays

Only few studies have compared the different commercially available assays [9–13]. Binkley et al. [11] reported recently not only immense variation between different assays but also variability between different laboratories in using a given assay. In their study, the serum of 10 subjects was sent to six different laboratories. Table 2.8 lists the methods and the normal range for 25-(OH)D measurement in the laboratories that participated. The mean serum 25-(OH)D concentrations differed 2-fold between laboratories (Figure 2.4), and the proportion of subjects below an arbitrary threshold of insufficiency (32 ng/ml) varied between 17 and 90%. After spiking of the serum samples with a defined quantity of 25-(OH)D, a broad range (17–95%) of the expected concentration was found by the different laboratories [11] (Figure 2.5). On the basis of their results, Binkley et al. [11] recommended the use of RIA techniques for 25-(OH)D measurement until other methodologies have been developed further.

Table 2.8. 25-(OH)D assay methodology and the normal range employed in different laboratories [11]

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Methodology</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Acetonitrile extraction followed by in-house RIA</td>
<td>10–55 ng/ml</td>
</tr>
<tr>
<td>B</td>
<td>DiaSorin (RIA)</td>
<td>10–40 ng/ml</td>
</tr>
<tr>
<td>C</td>
<td>Acetonitrile extraction followed by DiaSorin (RIA)</td>
<td>8–38 ng/ml</td>
</tr>
<tr>
<td>D</td>
<td>Chemiluminescent assay</td>
<td>20–57 ng/ml</td>
</tr>
<tr>
<td>E</td>
<td>Acetonitrile extraction followed by DiaSorin (RIA)</td>
<td>NA</td>
</tr>
<tr>
<td>F</td>
<td>Chemiluminescent assay</td>
<td>6–54 ng/ml</td>
</tr>
<tr>
<td>G</td>
<td>Chemiluminescent assay</td>
<td>10–68 ng/ml</td>
</tr>
<tr>
<td>H</td>
<td>Ethyl acetate extraction followed by normal phase HPLC</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA — not applicable; RIA — radioimmunoassay; HPLC — high-performance liquid chromatography.

Fig. 2.4. Mean (SEM) results for the serum 25-(OH)D concentrations for 10 subjects as estimated by six different laboratories (for abbreviations see Table 2.8). The means varied widely between laboratories from 17.1 to 35.6 ng/ml (p < 0.005) [11].
Fig. 2.5. Mean (SEM) results for the 25-(OH)D concentrations in the serum samples of 10 subjects spiked with the 25-(OH)D standard, as estimated by five different laboratories (for abbreviations see Table 2.8). The means varied widely between laboratories from 27.4 to 44.6 ng/ml (p < 0.05). The increment was less than anticipated and varied between laboratories (p < 0.005) from 7.7 to 18 ng/ml, the greatest increment being detected by HPLC [11].

Another study compared the DiaSorin RIA assay with the Nichols chemiluminescence assay, finding discordant serum 25-(OH)D-values. Both assays also underestimated the 25-(OH)D-concentration as compared with the HPLC-method [10].

In accordance with these findings, several authors have called for international standardization of the vitamin D assays that are available and that are affordable for practicing clinicians. Hollis also emphasizes the need for validation of the user’s assay system in the laboratory in question regardless of the claims made by the manufacturers [8].

According to the latest DEQAS (Vitamin D External Quality Assessment Scheme) report, 59% of the participating laboratories were found to have met the target [12,13]. Each of the laboratories was given five serum samples quarterly, and the requirement for meeting the target being to get 80% or more of the results obtained be within ± 30% of the All-Laboratory Trimmed Mean (ALTM). Figure 2.6 shows the latest results of this external quality control project. These give the impression that only method 6 exceeded the limit. However, the authors of the report state that the validity of the 25-(OH)D results that are obtained will justifiably continue to be questioned.

There has been discussion regarding the importance of the assays detecting 25-(OH)D₂ as well as 25-(OH)D₃. The HPLC method enables 25-(OH)D₂ and 25-(OH)D₃ to be quantified whereas there are concerns regarding the detection of 25-(OH)D₂ with other assays (Nichols procedure and IDS RIA) [9]. However, a subsequent study refuted these findings and also concluded that vitamin D₂ is less potent and has a shorter duration of action than vitamin D₃ [14]. This suggests that the contribution of 25-(OH)D₂ to the overall 25-(OH)D supply is less important than had been previously thought. Patients, however, especially those in the US, are still treated with 25-(OH)D₂ and the monitoring of vitamin D therapy is complicated by the presence of assays that underestimate 25-(OH)D₂.
Having an adequate definition of the normal range is essential in routine clinical practice in order to be able to define groups that are at risk for the negative consequences of vitamin D deficiency. In a recent publication, Hollis defined 25-(OH)D levels below 80 nmol/L as being deficient, due to the serious health risks encountered at levels less than this [7]. This is in contrast with most of the published studies, that set the cut-off points at much lower levels, around 25–40 nmol/L [1,15,16]. It is important to note that “normal” is not defined as the median population level, but the level of circulating 25-(OH)D sufficient to maintain good health and avoid clinical symptoms or consequences due to an inadequate vitamin D supply. In addition to the high degree of uncertainty that the divergence of the results for different techniques and different laboratories creates, epidemiological variability needs to also be taken into account, as shown by the seasonal variation in vitamin D status that occurs [17,18].

Apart from the analytical problems, the serum 25-(OH)D concentrations in different populations vary with latitude, season, race, age, dietary intake, and the composition of the population being studied. There is growing evidence that there is not only a specific seasonal decline in serum 25-(OH)D during the winter months, but there may also be a significant proportion of the population that exhibits asymptomatic subclinical vitamin D insufficiency. Populations at risk include nursing home residents and the elderly, especially the home-bound elderly. In epidemiologic studies, suggested cut-offs for insufficiency range from 10 to 30 ng/ml (25–80 nmol/l). Recent reports suggest European and North American populations to have a higher degree of insufficiency than had previously been thought [15,18–23]. A summary of studies on vitamin D status in the elderly is given elsewhere [1].
Conclusions

In conclusion, the determination of vitamin D status is still a challenging task. Several problems are important to bear in mind including the different forms of vitamin D (vitamin D$_2$, vitamin D$_3$), the measurement variation introduced by different assays and different laboratories, epidemiologic variation in vitamin D status and its determinants, and problems in defining the appropriate threshold for vitamin D sufficiency. Nonetheless, vitamin D metabolites have various important biological effects. This makes strong scientific research efforts needed. Epidemiologic studies in particular can contribute knowledge concerning the association between vitamin D insufficiency and the risk of disease, including the risk of cancers at various sites.

References


2.4. Selenium and cancer — selenoproteins as biomarkers
in relation to selenium supplementation

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and Department of Clinical Nutrition, Lund University Hospital, Lund, Sweden

Introduction

The relationship between selenium and cancer involves many different factors. These include the forms of selenium present in the body and in the diet, their functions and mechanisms of action, and methods employed in assessing an individual’s selenium nutritional status — both generally, and in epidemiological studies of the risk of cancer, as well as in connection with long-term trials for investigating the disease-preventive potential of selenium supplementation. A variety of issues connected with this are reviewed in two of the chapters. The present chapter concerns the various forms of selenium and their metabolism, the occurrence of different selenoproteins used as biomarkers of selenium status, and the use of intervention trials to study the cancer-preventive effects of selenium supplementation. In the chapter thereafter (Gromadzińska et al.), the mechanisms of action involved, together with epidemiological findings on relations between the selenium status in the body and risk of cancer are reviewed.

Different forms of selenium

With few exceptions, nearly all of the selenium in animals, plants and microorganisms is bound within proteins, several protein-bound forms having been identified. A major part of the selenium in mammals is specifically incorporated into proteins of defined biological function, so-called selenoproteins, containing the amino acid selenocysteine (Sec; analogous to cysteine in which sulphur is replaced by selenium) (Table 2.9.). In the diet, selenoproteins are largely found in animal foods. Another selenium-containing amino acid is selenomethionine, which is synthesized by plants and by yeast. When ingested by humans or other organisms, it is incorporated non-specifically into proteins as an analogue of methionine [1]. The replacement of methionine by selenomethionine appears to be random and to be dependent on the relative concentrations of these amino acids [2]. Other proteins can also bind selenium as a ligand [3], but little is known of the role it has here.

Selenite and selenate are inorganic forms of selenium used as dietary supplements. It is uncertain whether they occur naturally in foods to any major extent. In addition to the forms of selenium mentioned, several low-molecular-weight selenium compounds have been shown to be present in different foods, some of these compounds being uncharacterized [4–6].
Metabolism of selenium

Different chemical forms of selenium are involved in metabolic pathways (Figure 2.7.). Selenate and selenite are reduced by glutathione to hydrogen selenide, which is either transformed into selenophosphate for incorporation into selenoproteins (see below) as Sec or is methylated to selenosugar (1-β-methylseleno-N-acetyl-D-galactosamine), dimethylselenide or trimethylselenonium ions for excretion. Selenomethionine and selenocysteine can also be converted to hydrogen selenide. The major selenium metabolite excreted in urine is selenosugar, a much lesser amount being excreted as trimethylselenonium ions [7]. At toxic doses, selenium is removed as dimethylselenide via exha-

<table>
<thead>
<tr>
<th>Organic</th>
<th>Inorganic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenocysteine (Sec)</td>
<td>HSeCH₂CH(NH₂)COOH</td>
</tr>
<tr>
<td>Selenomethionine</td>
<td>CH₃Se(CH₂)₂CH(NH₂)COOH</td>
</tr>
</tbody>
</table>

Table 2.9. Some major inorganic and organic forms of selenium

Fig. 2.7. Metabolic pathways of selenium. Selenomethionine, selenocysteine and selenite can be converted into the key metabolite hydrogen selenide (H₂Se), which is turn is the precursor of selenocysteine in selenoproteins and various excreted forms of selenium. Several compounds can be converted into methylselenol (from [8]).
lation. Several studies have suggested that methylated Se derivatives, such as Se-methylselenocysteine and selenomethionine, are the selenium compounds most effective in cancer prevention [8]. Several of these compounds can be converted to methylseleninic acid or methylselenol which have anticarcinogenic effects \textit{in vitro} [8].

**Selenoproteins**

A total of 25 selenoprotein genes were discovered in the human genome several years ago by sequence analysis, yet the functions of many of the proteins involved are still unknown (Table 2.10.) [1,9]. The distribution and concentrations of selenoproteins

<table>
<thead>
<tr>
<th>Selenoprotein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 kDa</td>
<td>Involved in protein folding in the ER</td>
</tr>
<tr>
<td>DI1</td>
<td>Thyroid hormone control</td>
</tr>
<tr>
<td>DI2</td>
<td>Thyroid hormone control</td>
</tr>
<tr>
<td>DI3</td>
<td>Thyroid hormone control</td>
</tr>
<tr>
<td>cGSHPx</td>
<td>Peroxide reduction in the cytoplasm</td>
</tr>
<tr>
<td>eGSHPx</td>
<td>Peroxide reduction in plasma and other extracellular fluids</td>
</tr>
<tr>
<td>giGSHPx</td>
<td>Peroxide reduction, mainly in the gastrointestinal tract</td>
</tr>
<tr>
<td>phGSHPx</td>
<td>Reduction of phospholipid hydroperoxides</td>
</tr>
<tr>
<td>GSHPx 6</td>
<td>Peroxide reduction, found in embryos and in the olfactory epithelium</td>
</tr>
<tr>
<td>SelH</td>
<td>Unknown</td>
</tr>
<tr>
<td>SelI</td>
<td>Unknown</td>
</tr>
<tr>
<td>SelK</td>
<td>Unknown, a membrane protein</td>
</tr>
<tr>
<td>SelM</td>
<td>Involved in protein folding in the ER</td>
</tr>
<tr>
<td>SelN</td>
<td>Unknown, mutations in gene associated with muscular diseases</td>
</tr>
<tr>
<td>SelO</td>
<td>Unknown</td>
</tr>
<tr>
<td>SeP</td>
<td>An antioxidant and selenium transport protein</td>
</tr>
<tr>
<td>SelR</td>
<td>Reduces methionine-R-sulfoxides</td>
</tr>
<tr>
<td>SelS</td>
<td>A membrane protein, involved in the elimination of misfolded proteins from the ER</td>
</tr>
<tr>
<td>SelT</td>
<td>Unknown</td>
</tr>
<tr>
<td>SelV</td>
<td>Unknown, only found in the testes</td>
</tr>
<tr>
<td>SelW</td>
<td>Can reduce peroxides using glutathione as an electron donor</td>
</tr>
<tr>
<td>SPS2</td>
<td>Catalyses the formation of selenophosphate</td>
</tr>
<tr>
<td>TrxR 1</td>
<td>Cytoplasmic thioredoxin reductase, involved in many biological pathways</td>
</tr>
<tr>
<td>TrxR 2</td>
<td>Mitochondrial thioredoxin reductase, involved in many biological pathways</td>
</tr>
<tr>
<td>TrxR 3 (TGR)</td>
<td>Thioredoxin/glutathione reductase found mainly in testes</td>
</tr>
</tbody>
</table>
in different tissues are also not well known. The cytosolic or cellular form of glutathione peroxidase (cGSHPx) was the first selenoprotein identified [10,11]. It is found in almost all tissues and is believed to play a part in the body’s antioxidant defence. Several other glutathione peroxidases containing selenocysteine have been found since then. The other two major groups of known selenoprotein enzymes are the iodothyronine deiodinases, that regulate thyroid hormones, and the thioredoxin reductases, catalysing the reduction of oxidised thioredoxin and other substrates [1,12].

Many of the known selenoproteins catalyse redox reactions in which selenium is at the active site. Several of the selenoproteins have an homologue protein containing Cys instead of Sec, but the catalytic ability of these latter proteins is much lower. There are no selenoproteins known to be present in yeast or higher plants, these organisms tending to have homologue proteins containing Cys instead of Sec [13].

**Biosynthesis of selenoproteins**

Translation of the codon UGA to selenocysteine (Sec) and its insertion into proteins is a complicated process (Figure 2.8.). In the first step, serine is bound to tRNA\textsubscript{Sec} and then transformed to selenocysteine [13]. The next major step is the interpretation of UGA as a signal to insert Sec instead of stopping the translation. This takes place when a special stem-loop structure called the selenocysteine insertion sequence (SECIS) is present in the mRNA. This structure is located in the 3’-untranslated region of the mRNA in eukaryotes and immediately after the UGA codon in prokaryotes [14,15]. All known eukaryotic selenoproteins have one SECIS-element, except for SeP, which has two [15].

Except for the SECIS element there are no features in the DNA-sequence that selenoprotein genes are known to have in common, selenoproteins also differing markedly in the nucleotide sequence of their SECIS elements. The SECIS consensus sequence consists of two helices, one internal loop, one apical loop, and the SECIS core, which is a short sequence of non Watson-Crick paired nucleotides that appear to exist in all selenoprotein mRNA:s. Preservation of the SECIS core and of the length of the helix between the first internal loop and the second internal loop or the apical loop are important for the functioning of SECIS [16].

In addition to the SECIS element, additional factors are required for the insertion of Sec in eukaryotes such as the SECIS binding protein 2 (SBP2), the Sec-specific elongation factor (EFsec), and the recently discovered L30 protein, but the mechanisms involved have not yet been elucidated (Figure 2.8.). In eukaryotes the Sec codon and the SECIS element are located at some distance from each other in the mRNA. The current conception is that the mRNA and the SBP2-EFsec loops back towards the translation complex [13,17].
The stability of selenoprotein mRNAs is affected by the amount of selenium present in the cell. A special feature is that transcripts of some selenoproteins are much more stable than those of others, there being a selenoprotein hierarchy. This hierarchy is particularly noticeable in the glutathione peroxidase family, where the order of stability is as follows: giGSHPx ≥ phGSHPx > cGSHPx = eGSHPx, but it is not yet known how this regulation operates [18,19]. The role of the SECIS for mRNA stability was studied by combining the coding regions of giGSHPx, phGSHPx and cGSHPx with the 3’UTRs (3’-untranslated regions) of each mRNA and then to study the system during selenium deficiency. It was shown that giGSHPx and phGSHPx containing each other’s 3’UTRs would remain stable, but not with that from cGSHPx. cGSHPx could not be stabilised by replacing its 3’UTR with those from more stable glutathione peroxidase mRNAs. This indicates that in the cGSHPx mRNA, at least there are factors in both the coding and the non-coding mRNA regions that influence its stability [19].

There is also a tissue selenium hierarchy that controls the retention of selenium in the tissues and organs under selenium-deficient conditions. In rats and mice fed a selenium-deficient diet, the brain and the testes retain selenium whereas the selenium concentrations in the liver and the kidneys decrease markedly [20]. The links between selenium and cancer probably involve different selenoproteins. Accordingly an overview of the individual selenoproteins is provided.

**Individual selenoproteins**

**The glutathione peroxidase family**

The glutathione peroxidases generally catalyse the reduction of peroxides using mainly glutathione as the electron donor, thus contributing to the body’s defence against free radicals. Five selenium-dependent glutathione peroxidases are known to date [9].
Cellular (or cytosolic) glutathione peroxidase (cGSHPx), which is found in most tissues, catalyses the reduction of hydrogen peroxide and organic peroxides. It consists of four identical subunits, each containing one selenium atom. Under conditions of severe selenium deficiency, the level of cGSHPx in most tissues decreases considerably, without any obvious damages to the host organism, which has led to speculations that this enzyme is a form of selenium storage to some extent [21]. cGSHPx knock-out mice have been used to explore the effects of complete loss of cGSHPx activity. These mice appear phenotypically normal but when challenged by viruses or oxidising poisons such as paraquat they are more severely affected than mice of the wild type are. In model systems with an over-expression of cGSHPx, protective adaptations against paraquat and other challenges were shown to take place, there also being a number of negative effects such as increased obesity and insulin resistance [22].

Gastrointestinal glutathione peroxidase (giGSHPx), which consists of four subunits, is mainly found in the gastrointestinal tract and also in the human liver and in mammary cells and tissue according to certain studies [23,24]. Putative functions of this enzyme are those of protecting against ingested lipid hydroperoxides and reducing susceptibility to colon cancer [21]. giGSHPx knock-out models show no particular changes in phenotype to occur compared with the wild type, but knock-out of both cGSHPx and giGSHPx leads to colitis in mice [25].

Extracellular glutathione peroxidase, eGSHPx, is a tetrameric protein produced mainly in the kidney and then excreted into the extracellular environment [26]. Other tissues such as liver, skeletal muscle, pancreatic, thyroid, placental and mammary gland tissue produce this enzyme to a lesser extent. This glutathione peroxidase isoenzyme is also found in extracellular fluids such as blood plasma, milk, amniotic fluid, lung lavage and the aqueous humour [21,27–29]. This is the only selenoprotein that has been identified in milk thus far. Although the glutathione concentration in the plasma is very low eGSHPx can also use other electron donors such as the thioredoxin system [30]. The postulated roles of eGSHPx include control of peroxide transport and of the extracellular ‘peroxide tone’ [18,21].

Phospholipid hydroperoxide glutathione peroxidase, phGSHPx, catalyses the reduction of phospholipid hydroperoxides and is expressed in a wide range of tissues. It differs from the three glutathione peroxidases just mentioned by being a monomer and having a different substrate specificity. This enzyme has been implicated in inflammation and molecular signalling. Disruption of the phGSHPx gene is lethal embryonically, unlike disruption of cGSHPx or giGSHPx. phGSHPx occurs in mitochondrial, non-mitochondrial and sperm-nuclei-specific forms produced from the same gene [31] and has an important role in sperm functioning [32].

The most recently discovered member of the glutathione peroxidase family is glutathione peroxidase 6, which has thus far only been found in olfactory epithelium and in embryos, and its functional significance is unclear. In mouse and rat, the selenocysteine in this enzyme is replaced by cysteine [9].
Vitamins and selenium: Selenium and cancer — selenoproteins as biomarkers

The thioredoxin reductase family
The thioredoxin reductases (TrxR) catalyse the reduction mainly of thioredoxin, but in mammals they can also reduce other substrates, such as vitamin C. Thioredoxin catalyses the reduction of protein disulfides and is involved in a number of vital processes, such as DNA synthesis and the regulation of apoptosis. There are three main isoforms of thioredoxin reductases, but a number of splice variants of TrxR 1 and 2 have also been reported. TrxR 1 and 2 are ubiquitous being located in the cytosol and the mitochondria, respectively [12]. The third isoform, thioredoxin/glutathione reductase (TGR), is expressed in small amounts in many tissues but is primarily found in the testis [33]. Targeted disruption of the TrxR 1 or 2 genes in mouse models is embryonically lethal [22].

The iodothyronine deiodinase family
The iodothyronine deiodinase family consists of three enzymes, iodothyronine deiodinase 1, 2 and 3 (DI1, DI2, DI3), which catalyse the removal of different iodine groups from the thyroid hormones, thus activating or deactivating them. The iodothyronine deiodinases have high priority in the selenium hierarchy, particularly DI2 and DI3, their levels remaining virtually unaltered in the case of selenium deficiency. In some tissues, DI1 decreases during selenium deficiency, but not in the thyroid. DI1 is found in the liver, kidneys and thyroid, for example, and expression of DI2 and 3 having been found in many tissues, including bovine mammary tissue [34–36].

Selenoprotein P
Selenoprotein P (SeP), the second selenoprotein to be discovered, was designated “P” because of its being found in the blood plasma. It can contain from 1 to 17 selenocysteines, depending on the animal species. Truncated isoforms of this protein have also been found. SeP is expressed in most tissues, but is produced primarily in the liver and is secreted then into the plasma. Selenoprotein P is the major form of selenium in the plasma and is involved in selenium transport [37,38]. There are indications that it also acts as an antioxidant in the extracellular space. It is localised in the endothelium, binding to heparin and related carbohydrates [38]. It can reduce peroxynitrite and phospholipid hydroperoxides [39,40], can also form complexes with mercury and cadmium [41], and can stimulate the survival of nerve cells in culture [42]. SeP knock-out mice exhibit low levels of selenium in the brain and testes, organs that normally are highly prioritised during selenium deficiency. These mice die after weaning of the young, unless they are rescued by a high-selenium diet [43].

Additional selenoproteins
Selenophosphate synthetase 2 (SPS2) catalyses the formation of selenophosphate, which is the selenium donor for the formation of selenocysteine from serine bound in tRNA Sec. SPS2 is a selenoprotein, this property indicating the existence of a feedback step in the production of selenoproteins. Selenophosphate synthetase 1, which is not a selenoprotein, also catalyses the formation of selenophosphate [44]. The 15 kDa selenoprotein,
located in the endoplasmatic reticulum (ER), is believed to be involved in protein folding [45]. Selenoprotein M (SelM) is structurally similar to the 15 kDa protein and is supposedly also involved in protein folding in ER [46]. Selenoprotein R (SelR) reduces methionine-R-sulfoxides. This is an important step in the regulation of biological processes and the management of oxidative stress in the cell. It has been identified in prokaryotes, eukaryotes and archaea [15]. Selenoprotein N (SelN) is a 70 kDa protein located in the ER. Although its catalytic function is still unknown, mutations in the gene have been associated with various muscular diseases, such as rigid spine muscular dystrophy. To date, SelN is the only selenoprotein in which a mutation of it has been shown to cause a disease [47]. Selenoprotein S (SelS, also known as VIMP) is a membrane protein in the ER, one that has been associated with the process of eliminating misfolded proteins by transferring them to the cytosol [48] and also with inflammation [49]. The function of selenoprotein W (SelW) has not been elucidated fully but it has been implicated in white muscle disease [50]. SelW occurs mainly in muscle and brain and has been shown to act as an antioxidant, utilising glutathione to reduce peroxides [51]. A number of other selenoproteins have been discovered in man but information regarding their function is very limited.

Indices of selenium status

The most commonly used methods for assessing the selenium status in humans involve analysis of selenium concentrations in the blood or blood fractions. In addition, the determination of selenium in the hair, nails and urine has been employed. Selenium in the plasma or serum is the best known and most accessible index, usually responding rapidly to changes in selenium status or in the dietary intake of selenium [52]. The responses to selenium intake obtained if other blood fractions are analysed can differ. For instance, the selenium levels in whole blood or the erythrocytes appear to primarily reflect the long-term intake of selenium [53], since the turnover of erythrocyte selenium is slower.

Selenoproteins as biomarkers

The use of selenoproteins as markers of selenium status has only been exploited thus far in a few large epidemiological studies. In contrast, the activity of GSHPx in plasma has been used by a variety of laboratories in selenium supplementation studies, and it responds rapidly to changes in selenium status, and may thus be suitable as an indicator of short-term changes.

In general, the measurement of selenoproteins can be expected to provide information on specific selenium functions, as compared with plasma selenium, which also includes non-specifically bound selenium. Another important conception is that plasma selenoproteins may not be suitable biomarkers under conditions of high selenium status, since above a certain selenium level they tend to reach saturation. When data from
different cross-sectional studies was combined eGSHPx was found to approach a plateau at a plasma selenium concentration of approximately 1 mmol/l [54]. Supplementation by selenate resulted in glutathione peroxidase activity plateauing at a plasma selenium concentration of 1.2 mmol/l [55].

Selenoprotein P, glutathione peroxidase and protein-bound selenomethionine are the major selenium fractions contained in plasma [56]. Selenoprotein P accounts for at least 40% of the plasma selenium [37]. Immunoassays for measurement of this protein have been developed [57–59]. In the following various results from their use in the authors’ laboratory are summarized (Table 2.11).

<table>
<thead>
<tr>
<th>Study</th>
<th>n</th>
<th>SeP (a.u.)</th>
<th>Plasma selenium (µmol/l)</th>
<th>eGSHPx (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>European countries</td>
<td>414</td>
<td>1.41 (1.39, 1.44)</td>
<td>1.10 (1.08, 1.12)</td>
<td>—</td>
</tr>
<tr>
<td>Prior to LDL-apheresis</td>
<td>13</td>
<td>1.07 (0.92, 1.22)</td>
<td>0.73 (0.60, 0.86)</td>
<td>352 (306, 397)</td>
</tr>
<tr>
<td>After LDL-apheresis</td>
<td>13</td>
<td>0.55 (0.44, 0.66)</td>
<td>0.41 (0.33, 0.51)</td>
<td>302 (259, 346)</td>
</tr>
<tr>
<td>Finland, Trial I baseline</td>
<td>50</td>
<td>1.03 (0.98, 1.07)</td>
<td>0.86 (0.83, 0.88)</td>
<td>6.51 (6.28, 6.74)</td>
</tr>
<tr>
<td>Finland, Trial II baseline</td>
<td>45</td>
<td>1.77 (1.69, 1.85)</td>
<td>1.38 (1.34, 1.43)</td>
<td>—</td>
</tr>
<tr>
<td>Cancer cases</td>
<td>302</td>
<td>1.20 (1.16, 1.24)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Controls</td>
<td>406</td>
<td>1.23 (1.21, 1.25)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Elderly subjects (Malmö Food Study)</td>
<td>126–205</td>
<td>1.47 (1.43, 1.52)</td>
<td>1.14 (1.11, 1.16)</td>
<td>4.13 (4.0, 4.27)</td>
</tr>
<tr>
<td>Patients on HPN</td>
<td>38</td>
<td>0.69 (0.56, 0.83)</td>
<td>0.52 (0.41, 0.64)</td>
<td>1.91 (1.51, 2.31)</td>
</tr>
<tr>
<td>Latvians with differing fish intake</td>
<td>21</td>
<td>0.83 (0.54–1.15)</td>
<td>0.69 (0.30–1.14)</td>
<td>2.78 (1.20–4.32)</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>1.00 (0.63–1.83)</td>
<td>0.91 (0.46–1.47)</td>
<td>3.38 (2.31–4.65)</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>1.38 (0.75–2.21)</td>
<td>1.18 (0.66–1.56)</td>
<td>3.95 (2.69–5.73)</td>
</tr>
</tbody>
</table>

Values are means (95% CI).

* GSHPx activity, U/l.

* GSHPx activity, mU/mg protein.

* Data from subgroups having (top to bottom) low, medium and high fish intake, respectively. Median values (and range) are given.
Selenoprotein P as a biomarker

Selenoprotein P levels were measured in healthy adults from 17 European Regions [60]. Considerable variation between different regions was found. There was a close correlation between selenoprotein P and plasma selenium values, with some indication of a plateau. In the Malmö Food Study [61] the relationship between the intake of different foods and selenium status was investigated. For women, significant relationships between milk intake versus selenoprotein P and urinary selenium, and also between fish intake versus serum and urinary selenium were found. In a study of Latvian fisherman [62], a highly significant positive relationship between fish intake and selenium status was obtained as well as an inverse relationship between the plasma selenium and thyroid stimulating hormone levels. Home parenteral nutrition patients with a variety of gastrointestinal diseases showed much lower levels of extracellular GSHPx, total selenium and selenoprotein P than control subjects did [64]. Concerning the association of selenium status and exposure to toxic metals, studies of lead-exposed children in Katowice, Poland revealed an inverse relationship between the blood lead level and the level of selenoprotein P and of extracellular GSHPx [65]. Since the direction of causality was uncertain, however, and it is not possible to conclude that lead exposure produces a decrease in the selenoprotein concentration or that a poor selenium status increases susceptibility to high blood lead concentrations. Regarding the relationships between SeP, eGSHPx and plasma selenium as markers of selenium status, it was found generally speaking that the selenoprotein P level correlated more highly with the plasma selenium and eGSHPx level at low selenium status than at high selenium status, but that at normal selenium status selenoprotein P usually correlated more highly with plasma selenium than eGSHPx did. The levels of selenoprotein P and eGSHPx were found to vary markedly for subjects from different regions and with different diseases.

Biomarkers of selenium status in relation to selenium supplementation

Many studies on efforts to improve selenium status through selenium supplementation have been performed and a review of different modes of selenium supplementation has been assembled [66]. Considerable attention has been directed at two studies conducted in Finland regarding the use of different forms of selenium [67,68]. The scientists there compared the responses to oral supplementation of 200 mg selenium per day in healthy subjects on two occasions. In the first study, in which the subjects were low in selenium status, the selenoprotein P values increased after selenium supplementation, plateauing within two weeks [69] (Figure 2.9.). In the second study, performed after the introduction of selenium-enriched fertilizers in Finland, the selenium status of the subjects thus being higher, no significant increase in selenoprotein P levels was observed after selenium supplementation and no differences were found between groups given different forms of selenium [69]. A summary of the responses shown by the different selenium indices in the first of these two studies is provided in Figure 2.9. In a recent study of Chinese subjects of low selenium status given different doses of selenite and selenomethionine,
full expression of glutathione peroxidase was achieved with use of 37 µg Se/d in the form of selenomethionine and with 66 µg Se/d in the form of selenite. Full expression of selenoprotein P was not achieved at the highest dose of either form (66 µg/d). This suggested that selenoprotein P is a better indicator of selenium nutritional status than glutathione peroxidase is [70].

Generally speaking, in view of the many regulatory mechanisms that exist for the incorporation of selenium into selenoproteins and the varying effects of different forms of dietary selenium on indices of the selenium status, it appears that a more adequate assessment of the selenium status would be obtained through the use of several biomarkers being employed concurrently than through analysis of only total selenium or of a single selenoprotein.

Fig. 2.9. Percentual changes in selenium status variables after supplementation of Finnish subjects of low selenium status with 200 µg/day of selenium in different forms (from ref [67,69]).

**Biomarkers of selenium status and cancer**

Experimental studies have shown that the addition of high (0.5–2 ppm) levels of selenium to the diet has a carcinostatic effect in animals treated with carcinogenic chemicals [71,72]. Interest in the preventive role of selenium supplementation in humans was stimulated markedly by results from two intervention studies. Blot and coworkers [73] found that a mixture of selenium, β-carotene and α-tocopherol reduced the total cancer mortality and stomach cancer rate in a Chinese population. Clark and associates [74], in turn found selenium supplementation to reduce total incidence and mortality of cancer in an American study group (see below).

In a number of case-control studies, lower prediagnostic plasma selenium was found for cases of cancer than for controls, particularly among men [75–81], whereas in other studies no significant differences in this respect were found between cases and controls [82–86]. The calculated degree of protection was found to differ between cancer sites [81,87]. The strongest associations between premorbid plasma selenium levels and risk of cancer were observed for cancer of the respiratory and digestive tracts. These matters are reviewed in greater detail in the chapter that follows (Gromadzińska et al.).
Relations between the selenoprotein P level and risk of cancer

In most studies of the association between selenium status and risk of cancer, plasma selenium has been used as a marker of selenium status. More recently the premorbid level of SeP in the plasma of subjects who had developed cancer at different sites was studied in a nested case-control study [88]. When cases were divided into subgroups according to cancer site, the SeP levels for cancer of the respiratory tract were found to be significantly lower than in matched healthy control subjects. The association between the relative risk of getting cancer and the SeP concentration found was also estimated from quintiles of the SeP level. For increasing quintiles, the ORs (adjusted for smoking) were 5.2, 2.3, 2.9, 2.0, and 1.0, respectively (p for trend = 0.01). In addition, the ORs (adjusted for smoking) in tertiles of the SeP level were calculated for the respiratory, digestive and urinary tract and for cancer of other sites. These were 6.0, 3.4, 0.2, and 0.6 respectively, in the lowest tertile as compared with the cases in the highest tertile. In Figure 2.10, the case-control differences of plasma selenium and SeP are compared for major cancer sites in several different study populations. The previously reported association of plasma selenium levels with cancer risk can very likely be explained by the corresponding association of SeP levels. This is probably due to SeP constituting at least 40% of the total selenium in human plasma [37].

Fig. 2.10. Percentage differences in the prediagnostic selenium and SeP plasma levels between cancer cases and controls (set at 0) for cancer at different sites. The circles represent selenium and the squares SeP. Bold contour: p < 0.05, normal contour: p ≥ 0.05. The individual references are cited in [63].

Smoking and biomarkers of selenium status

Several factors other than selenium intake may affect biomarkers of selenium status. Smokers were found to have significantly lower levels of selenoprotein P than non-smokers [88]. In other studies, lower values of plasma selenium [77,85,89], whole blood selenium [89,90], erythrocyte selenium [89], and toenail selenium [90–92] were observed in smokers than in non-smokers. The factors contributing to the lower selenium status in smokers are unclear. One possible explanation to the lower selenoprotein P level in smokers would be that smoking contributes to chronic low-grade inflammation due to its irritating effect on the respiratory tract and on the vascular endothelial cells.
The finding that selenoprotein P is positively correlated with albumin level and negatively correlated with \( \alpha_1 \)-antitrypsin, both being acute-phase reactants, and that these correlations are higher (more significant) in smokers, suggests that the selenoprotein P levels are reduced by inflammatory activity. This agrees with findings of Dreher and co-workers [93] indicating that the human selenoprotein P promoter is rendered less active by cytokine treatment, which suggests a repression of selenoprotein P expression during an acute phase reaction. Smoking may also increase oxidative stress since cigarette smoke is a rich source of reactive nitrogen species, which together with superoxide can produce peroxynitrite [39]. As reported by Sies and coworkers [94], selenomethionine and glutathione peroxidase can scavenge peroxynitrite. It has also been shown that selenoprotein P plays a role in the defence against peroxynitrite [39]. This may also explain the slightly lower selenoprotein P concentration in smokers.

The natural presence of cadmium in tobacco smoke may also contribute to the lower selenium status found in smokers. It has been shown that the concentration of selenium in the blood is significantly lower in subjects smoking more than 50 g tobacco per week than in never-smokers, whereas the concentration of cadmium in the blood is significantly higher in smokers [95]. Multiple linear regression analysis of the data also suggested a depressive effect of cadmium on the concentration of selenium in the blood, whereas smoking alone did not serve as a true predictor of this effect. In another study, the blood levels of selenium and cadmium and the plasma levels of selenoprotein P were measured in children from the Katowice industrial area in Poland [65]. The cadmium blood level was found to be negatively associated both with selenium in the blood and with selenium and selenoprotein P in the plasma. Multiple regression analysis also indicated the blood cadmium to increase significantly with a decrease in the selenoprotein P level, although this association disappeared when lead was included in the model, a result that could possibly be explained by the covariance of lead and selenium in the blood [65].

It has been reported that smokers eat less selenium than non-smokers do, which could probably in part explain their lower selenium level [90]. In addition, in a meta-analysis of 51 published nutritional surveys of the relationship between smoking status and nutrient intakes, smoking was found to be significantly associated with an unhealthy pattern of nutrient intake, which could exacerbate the risk of cancer associated with smoking [96].

**Intervention trials on the effect of selenium supplementation on cancer**

No definite proof of a protective effect of selenium in connection with cancer has been presented as yet in human investigations but there has been increasing interest in the cancer preventive action of selenium supplementation, several intervention studies having indicated beneficial effects [73, 74]. In the Linxian trial involving supplementation of \( \beta \)-carotene, \( \alpha \)-tocopherol and selenium given for a 5.25-year period, a small but significant reduction in total cancer mortality was obtained (RR = 0.91), in particular mortality due to stomach cancer [73]. Patients with a history of skin carcinomas who
were treated with 200 mg selenium per day showed significant reductions in total cancer mortality (RR = 0.50) and in incidence of lung (RR = 0.54), colorectal (RR = 0.42) and prostate (RR = 0.57) cancer [74], whereas later results showed selenium supplementation to be ineffective in preventing basal cell carcinoma and that it increased the risk of squamous cell carcinoma and of total nonmelanoma skin cancer [97]. Experimental findings also indicate that in some experimental systems selenium can both promote and inhibit cancer [98]. Recent results of the SUVIMAX study showed supplementation with vitamin C, vitamin E, β-carotene, selenium and zinc to reduce the rate of prostate cancer in men having normal levels of prostate-specific antigen in their plasma [99]. In another Chinese investigation, selenomethionine supplementation of subjects with mild to moderate esophageal squamous dysplasia showed there to be a nonsignificant trend toward an increased regression and decreased progression of dysplasia as well as a significant beneficial effect in the subgroup showing mild esophageal squamous dysplasia [100]. A summary of results of the first generation of nutritional intervention studies to prevent cancer have been presented [101] and future directions and criteria for evaluating the efficacy of such interventions have been proposed [101,102]. In addition, evaluation of health claims by the FDA in the U.S. concerning the purportedly positive effects of selenium in connection with the prevention of cancer provided certain evidence for permitting a qualified health claim regarding selenium and cancer [103].

It is apparent from this review that selenium can play an important role in cancer prevention, but additional studies are needed to determine whether there is also an increased risk of some forms of cancer after selenium supplementation. The type of selenium supplements best employed is also in need of further investigations. A better understanding of the mechanisms by which selenium interferes with the carcinogenesis process is a necessary focus for future research also for the evaluation of selenium related biomarkers.

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2.5. Anticancerogenic activity of selenium — molecular mechanisms and epidemiological data

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Introduction

There are many different aspects of the relationship between selenium and cancer, including the forms of selenium present in the diet and in the body, their functions and mechanisms of action, the methods used in assessing the nutritional status of selenium — in general, in response to selenium supplementation, and in epidemiological studies of risk of cancer — and the outcome of long-term trials concerned with the disease-preventive potential of selenium supplementation. Two of the chapters deal with these and related matters. In the present chapter the mechanisms behind the role that selenium can play in cancer prevention, as well as epidemiological findings regarding the relationship between selenium level and risk of cancer are reviewed. In the previous chapter the forms and metabolism of selenium, in addition to the occurrence of different selenoproteins, their use as biomarkers of selenium status, and intervention trials to investigate the effect of selenium supplementation on the incidence of cancer are summarized.

History

Historically, interest in selenium (Se) as a toxic trace element dates back to 1937 [1], and concern for it as a carcinogenic agent dates to 1943 [2]. Nelson et al. [2] observed that liver tumours occurred in rats fed protein-deficient fodder and grains originating from Se-rich (5–10 ppm) regions. Of 53 animals that survived for 18 months when thus fed, 11 were found to develop liver tumours, whereas such tumours were found in less than 1% of the animals in the control group. Shapiro [3], however, suggested that the pathological changes that occurred reflected regeneration of the liver rather than a carcinogenic process. Researchers of the former Soviet Union [4] feeding Se (as selenite) to rats at a 4.3 ppm level, observed the development of different forms of liver tumours. Seifter et al. [5] published the alarming finding that feeding rats 0.05–0.1% bis-(4-acetamine)-phenylselenyl hydroxide, equivalent to inclusion of 103–207 ppm Se in the diet for a 10-day period, induced thyroid adenoma. It should be noted, nevertheless, that since no studies have been reported of animals administered an analogous compound that did not contain selenium, it is quite possible that the organic selenium compound employed there had carcinogenic properties not linked with the element per se. In 1963, however, Tscherkers et al. [6] observed the development of tumours in male rats fed a diet containing 4.3 ppm Se, which suggests that the neoplastic changes that occurred could be attributed to Se.
The anticancerogenic effect of selenium in animals was first reported in 1911, when Wassermann et al. (cf. [7]) managed to inhibit the development of placental tumours in mice by use of Se compounds. Another early observation of such an effect was that of Clayton and Bauman [8], who reported that in rats a diet enriched with 5 ppm Se decreased the incidence of liver tumours brought on by 3’-methyl-1,4-dimethylamino-benzene (3’-MeDAB). These findings were confirmed in a study showing that the number of liver tumours induced in male Spraque-Dawley rats by 3’MeDAB decreased from 92% in rats fed simply a control diet to 46% and 67%, respectively, in rats to which either of two different forms of Se were administered [9]. Supplementing a standard diet with administration of sodium selenite was also found to reduce the number of tumours that developed in rats that were given 2-acetyloaminofluorene (2-AAF) as a carcinogenic agent [10–12].

Ip and Sinha [13] investigated the effect of various concentrations of Se on the development of breast cancer induced in rats by 7,12-dimethylbenzantracene (DMBA). Two groups of animals received a diet containing maize oil. The maize oil content in the one case was high (25%) and in the other case low (5%). In both groups the incidence of cancer was found to decrease with increasing level of Se in the diet. At the same time, the Se concentration influenced neither the level of malondialdehyde (a final product of lipid peroxidation) in the breast carcinoma cells nor the level of glutathione peroxidase (GSH-Px) activity there [13]. The authors concluded that the protective role of selenium was not due to its being able to inhibit lipid peroxidation or to its having any antioxidative function in fat metabolism [13]. Ip [14] also studied the effect of selenium on different stages of cancer development in rats given 5 ppm Se at different time intervals after the administration of 10 mg DMBA. Supplying Se was found to decrease the number of tumours induced (from 97 to 46%), particularly when it was provided at both the initiation and the promotion stage of chemical carcinogenesis. Se supplied during only one of these two phases had a much weaker effect [14].

Harr et al. [10] showed that adding 0.1 to 0.5 ppm Se to the diet of rats given a carcinogenic substance led to a decrease in the occurrence of tumours from 80% (in the non-supplemented group) to 10% (in the group receiving Se). Providing selenium at a still higher level (2.5 ppm) reduced the incidence of cancer even more, to 3%. An interesting study of the anticarcinogenic role of selenium was carried out by Schrauzer et al. [15], using female mice of the C3H strain, a strain characterized by the spontaneous development of breast adenoma in 80% or more of the cases. The animals were divided into several groups, a control group receiving a basal diet that contained 0.15 ppm Se and the other groups receiving, in addition to this, a supply of 0.1 to 1.0 ppm Se in their drinking water. In the control group, the first tumours developed when the animals were 4 months of age, whereas in the group receiving the largest amount of Se in its drinking water (1.0 ppm) they first developed at 17 months of age. In the groups given extra Se in their drinking water, tumours not only developed later, but they also decreased in number with an increase in the amounts of selenium provided. These results indicated that supplying Se in larger amounts protects animals from developing cancer or delays
the carcinogenic processes. The anticarcinogenic effect of Se has been found to depend on the chemical form in which the element is administered, its dose and the agent that induces the development of cancer [16]. The anticarcinogenic effect of selenium has also been found to reach an optimum if it is given prior to the onset of the disease or in an early phase of its development. Cells adequately supplied with Se have been shown to be less sensitive to effects of both endogenous and exogenous carcinogens [17].

About 200 experiments have been performed aimed at assessing the effects of Se given to laboratory animals in doses higher than those usually employed in standard diets used to counteract the development of cancer induced by chemicals, viruses or transplanted tumours [18]. Two thirds of these experiments provided evidence for high doses of Se reducing cancer development to a moderate extent (15–35% in relation to controls), in the majority of cases the reduction being quite significant [19]. Experiments in which no effect of Se was observed were rather rare. The experiments as a whole strongly suggest that consumption of Se in doses higher than those customarily given in such cases appreciably reduces the development of neoplastic tumours. At the same time, one should bear in mind that the results of animal studies cannot be directly extrapolated to humans.

**Mechanisms responsible for the link between selenium and cancer prevention**

Experimental studies have thus shown that adding high levels of selenium to the diet of animals treated with carcinogenic chemicals has a carcinostatic effect [20]. Regarding the mechanisms involved, it has been postulated that the chemopreventive effect is related to the toxicity of selenium and the oxidative stress it induces, since reactive oxygen species can promote apoptosis *in vitro* [21]. It has also been shown, however, that selenium compounds can induce cell death through a mechanism distinct from oxidant toxicity [22,23]. In addition, high levels of selenium compounds in the diet can reduce both the extent to which DNA adducts are formed and the extent to which DNA damage by carcinogens occurs. In several studies, the activity of xenobiotic-metabolizing enzymes *in vivo* has been reported to increase when selenium compounds are given, resulting in more efficient carcinogen detoxification [24,25]. It is also possible that selenium in the form of glutathione peroxidases, and perhaps selenoprotein P and other selenoproteins as well, can prevent mutations by serving as free radical scavengers [26–28].

Thus a number of mechanisms for the anticarcinogenic effects of selenium and of many selenocompounds have been proposed, including their providing protection against oxidative damage, altering the metabolism of carcinogens, enhancing immune responses, affecting the cell cycle, and inhibiting angiogenesis [29,30] (Tables 2.12. and 2.13.). Some reports suggest that the action of selenium toward cells that have been transformed earlier and toward normal cells differ [32]. The anticarcinogenic effects of Se depend upon the chemical form of the Se-compound involved and the nature and dosage of the carcinogen. The effects can occur at a systemic, cellular or nuclear level. The activity of many cellular targets, in which the metabolism, proliferation and differentiation of the cells can be affected, depends on the cellular GSH/GSSG ratio. Glutathione
peroxidases and thioredoxin reductases are involved in ROS scavenging. This suggests that selenoproteins participate in the regulation of intracellular signal transduction [33]. Yet non-protein selenium metabolites may also be important in the regulation of intracellular communication and metabolism. Se and the selenoproteins play a regulatory role in the following processes, for example:

— ROS-activation of protein kinases in the cytoplasm and nucleus.
— ROS-activated modification of the thiol and hydroxyl groups in the Cys and Tyr.
— Controlling changes in the cell redox potential through inducing activation of the transcription factors and initiating *de novo* gene expression.
— Regulating the expression of membrane and nuclear receptors responsible for cell maintenance, intercellular communication, and changes in cell growth.
— Affecting apoptosis, necrosis and cell survival processes [34].

**Table 2.12.** The effects of selenium compounds on cell growth and on the molecular targets of cancerogenesis (ref. [16])

<table>
<thead>
<tr>
<th>Form of selenium</th>
<th>Parameter</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenite</td>
<td>Growth of Ehrlich ascites tumour cells in mice</td>
<td>I</td>
</tr>
<tr>
<td>Selenite</td>
<td>Growth of L1210 leukemic cells</td>
<td>I</td>
</tr>
<tr>
<td>Selenodiglutathione</td>
<td>Growth of L1210 leukemic cells</td>
<td>I</td>
</tr>
<tr>
<td>Selenite, Selenodiglutathione</td>
<td>Cell growth (<em>in vitro</em>)</td>
<td>I</td>
</tr>
<tr>
<td>p-XSC, BSC, selenite</td>
<td>DNA, RNA, protein synthesis</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Apoptosis</td>
<td>E</td>
</tr>
<tr>
<td>Selenite</td>
<td>DNA synthesis (<em>in vitro</em>)</td>
<td>I</td>
</tr>
<tr>
<td>Selenite</td>
<td>RNA and protein synthesis</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Cell death (necrosis SSB)</td>
<td>E</td>
</tr>
<tr>
<td>Selenite</td>
<td>Cell growth</td>
<td>I</td>
</tr>
<tr>
<td>Selenite</td>
<td>Cell cycle</td>
<td>Block S/G2-M</td>
</tr>
<tr>
<td>Selenite</td>
<td>p53</td>
<td>E</td>
</tr>
<tr>
<td></td>
<td>AP-1</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>NFκB</td>
<td>I</td>
</tr>
<tr>
<td>CH₃SeCN, p-XSC, Se-methylselenocysteine</td>
<td>DNA synthesis (<em>in vitro</em>)</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Cell cycle</td>
<td>Block G1</td>
</tr>
<tr>
<td></td>
<td>Apoptosis</td>
<td>E</td>
</tr>
<tr>
<td>BSC and ist glutathione conjugates</td>
<td>ACF</td>
<td>I</td>
</tr>
<tr>
<td>p-XSC, BSC PKC, PKA</td>
<td></td>
<td>I</td>
</tr>
</tbody>
</table>
Table 2.12. The effects of selenium compounds on cell growth and on the molecular targets of cancerogenesis (ref. [16]) — cont.

<table>
<thead>
<tr>
<th>Form of selenium</th>
<th>Parameter</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenite</td>
<td>PKC</td>
<td>I</td>
</tr>
<tr>
<td>p-XSC</td>
<td>TK</td>
<td>I</td>
</tr>
<tr>
<td>Selenite, selenium dioxide, selenic acid</td>
<td>Phospholipid/Ca²⁺ dependent PKC</td>
<td>I</td>
</tr>
<tr>
<td>Ebselen</td>
<td>PKC</td>
<td>I</td>
</tr>
<tr>
<td>P-XSC, BSC</td>
<td>JNK</td>
<td>Dose dependent E/I</td>
</tr>
<tr>
<td>p-XSC, BSC, selenite</td>
<td>DNA cytosine methyltransferase</td>
<td>I</td>
</tr>
<tr>
<td>Se-methylselenocysteine</td>
<td>PKC (in vitro)</td>
<td>I</td>
</tr>
<tr>
<td>Se-methylselenocysteine, triphenylselenium chloride</td>
<td>Cell proliferation and cell cycle biomarkers (in vitro)</td>
<td>I/E/NE</td>
</tr>
<tr>
<td>p-XSC</td>
<td>PKC and isoprostane (in vivo)</td>
<td>I</td>
</tr>
</tbody>
</table>

I — inhibition; E — enhancement, W — weak, NE — no effect; AP-1 — activator protein 1; NF-κB — nuclear factor κB; ACF — aberrant crypt foci; COX-2 — cyclooxygenase; PKA, PKC — protein kinase A and C; TK — thymidine kinase; JNK — Jun-N-kinase; p-XSC — 1,4-phenylenebis(methylene)selenocyanate; BSC — benzyl selenocyanate.

Table 2.13. *In vitro* effects of selenite and methylated selenocompounds [31]

<table>
<thead>
<tr>
<th>Endpoints</th>
<th>Selenite</th>
<th>Methyleneselenocyanate or Se-methylselenocysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>Extensive cytoplasmic vacuolisation, cell detachment</td>
<td>Normal</td>
</tr>
<tr>
<td>Membrane damage</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Cell growth inhibition</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>DNA synthesis inhibition</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Cell cycle block</td>
<td>S/G2:M</td>
<td>G1</td>
</tr>
<tr>
<td>DNA single strand breaks</td>
<td>+++</td>
<td>None</td>
</tr>
<tr>
<td>Cell death</td>
<td>Necrosis</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Gadd gene induction</td>
<td>Late</td>
<td>Early</td>
</tr>
</tbody>
</table>

Fibronectin is an extracellular component that plays an important role in intercellular communication. Inorganic Se compounds such as selenite reduce the number of fibronectin receptors at the cell surface. Since this is an immediate action, it cannot come about through activity of the selenoproteins. Selenite activates the protein kinases involved in the pathways of cellular response to stimulation by inflammatory agents, whereas these processes can be inhibited by selenate [34]. Oxidation of the thiol groups
in the transductional proteins results in their being modified structurally, which can lead to their becoming activated. This process in turn results in the activation of AP-1 and of such transductional proteins as ras/rac, fos, myc and c-Jun N kinase [35,36]. In human hepatoma cells, selenite inactivates the c-myc oncogene and activates the c-fos genes that lead to the growth of normal cells [37]. In human colon cancer cells, Se inhibits the expression of one of the activating zinc finger proteins that regulates the activation of the c-myc oncogene [38].

The redox potential is an important factor in regulating the nuclear factor κB (NF-κB) and the activation of AP-1 [39]. NF-κB is a crucial target in the pathogenesis of various chronic inflammatory, degenerative and neoplastic diseases. It regulates the effector genes in the promoter regions and can thus activate or repress gene expression. The up-or downregulation of the effector genes can modulate many different cell pathways, such as those of proliferation, growth suppression, differentiation and senescence. NF-κB is also an important factor in regulating activation of the genes involved in the control of cell necrosis and apoptosis, the cell cycle, the immune response, and repair processes [39]. NF-κB activation involves changes in protein conformation and in the binding of numerous genes, such as cytokines, for example, to the promotor regions. Reactive oxygen species (ROS) activates the phosphorylation of one of the NF-κB subunits, I-κB, directly. GSH-Px overexpression inhibits the translocation of another NF-κB subunit and reduces the phosphorylation of I-κB [40–42]. This process has been found to be inhibited in cells cultured in an Se-supplemented medium and to be intensified in the case of Se deficiency [43]. The process the translocation of NF-κB and its binding to DNA is multiphasic and highly complex. The phosphorylation of mitogen-activated protein kinases (MAPK) represents one of these phases. The activation of individual kinases of the MAPK family triggers the transcription factors involved in changes in chromatine conformation and in the expression of numerous genes of proinflammatory and antioxidative proteins, as well as the genes involved in apoptosis activation, and in cellular proliferation or differentiation [35]. MAPK inactivation inhibits the cellular proliferation occurring in the course of the carcinogenic process. GSH-Px and SeP are considered to have a particular role in MAPK inactivation [44]. GSH-Px in particular can act as a suppressor of protein kinases. The breakdown of hydroperoxides by GSH-Px and the resulting decrease in the cellular hydroperoxide level inhibit the Se-activated kinase p38 [34].

The strength of the binding of NF-κB to DNA is influenced by Se, which modifies the structure of Cys in the regulatory subunit of NF-κB so as to prevent oxidation of the thiol group located within this factor [34]. The reduced thiol group in Cys is essential for maintaining the activity of numerous transcription factors [45]. Thioredoxin (Trx) plays a key role in this process. Oxidative stress induced by chemical or physical factors translocates Trx to the nucleus, intensifying the binding of NF-κB and AP-1 to the DNA. This process is made possible by intracellular reduction in the disulfide bridges (-S-S-), mainly in regions in which DNA binding to nuclear factors occurs [46]. Through acting as a protein disulfide reductase, TrxR thus affects the redox regulation of a variety of enzymes and receptors, as well as such transcriptional and nuclear factors as NF-κB
and AP1 [47]. Trx inhibits certain kinases of the MAPK family (e.g., ASK1) directly. Se is also known to arrest several kinase pathways important in signal transduction, such as protein kinase A, Ca^{2+}-dependent and -independent kinase C (PKC), diacylglycerol kinase and thymidyl kinase [48].

It has been shown that selenium creates a dose-dependent increase in TrxR activity and in the expression and stability of TrxR mRNA in different cancer-cell lines [39]. Trx limits DNA synthesis, whereas Trx/TrxR activates ribonucleotide reductase, a key enzyme in the deoxyribose formation needed for DNA synthesis. TrxR also regulates gene expression by affecting the cellular redox status and activating numerous DNA-binding transcriptional factors: NF-κB, AP-1, ref-1, p53, and the glucocorticoid receptors [34,49]. At the same time, not only the selenocysteine incorporated into the protein structure, but also its metabolites, affect cellular metabolism. The various chemical forms of Se have been shown to differ widely in their anticancer properties. Some low molecular weight Se compounds have been shown to influence the regulation of gene expression, reduction in the oxidative DNA damage that occurs, the bioactivation of carcinogens, modulation of tumour angiogenesis, cellular growth, etc. [50,51]. It is important to note that different selenocompounds are essential for the growth and differentiation of both normal and neoplastic cells [52].

**Effects of selenium on apoptosis and necrosis**

In 1992 it was shown that Se(IV) compounds can induce the necrotic death of a cell by damaging a single DNA strand [53]. It was also observed that methylated Se derivatives can induce apoptosis [48]. The anticancerogenic activities of various Se compounds are summarized in Table 2.12. [16]. It has been found that both sodium selenite and selenomethionine (SeMet) suppress tumour growth in many animal models involving a dose-dependent response [20,54,55]. Selenite has been shown to be more effective than selenomethionine in the inhibition of cancer cell growth during chemically induced carcinogenesis [31,54], the concentration of Se in the tissues also being found to be higher after the administration of selenomethionine than after selenite supplementation. From this it can be concluded that a high concentration of Se is not crucial for the inhibition of carcinogenesis, but it is likely that one of Se metabolites is essential to this process.

**Effect of different selenium compounds in cancer prevention**

The chemoprevention of cancer by selenium can involve the action of different selenometabolites. Selenite, selenodiglutathione, selenomethionine and Se-methylselenocysteine can be transformed into selenides. In cells with a high Se level, high concentrations of selenides tend to also be generated. When selenides are generated in sizeable amounts, they react with oxygen to produce superoxide and hydrogen peroxide [56]. It is believed that the role of selenium in the inhibition of carcinogenic processes is associated with oxidative damages caused by the redox cycle of selenides [57].
When Se is supplied in high concentrations, however, any surplus of it is excreted from the body. Over 90% of the circulating Se is incorporated into the structure of the selenoproteins, only about 5% of it being found in other metabolites [58]. It appears then that the anticarcinogenic action of Se is due to the combined effect of selenium and selenoproteins [33].

Regarding the selenocompounds, it is the actions of sodium selenite (Se IV), responsible for single and double DNA strand breaks, and of selenomethionine, which is not a DNA-damaging agent, that have been investigated most frequently [49]. Inorganic Se compounds added to cell cultures at a concentration of 5–10 µM can induce 8-OHdG lesions or DNA single-strand breaks and cell death by necrosis. Experimental studies have shown that the organic Se compounds induce apoptosis without DNA damage, even at rather high concentrations (10–50 µM) [29]. Ip and Ganther [59,60], in connection with studies of Se metabolism they conducted, suggested that methylated Se derivatives are the most effective selenium compounds for cancer prevention [61]. Such methylated selenium compounds as methylselenocysteine and selenomethionine show powerful anticarcinogenic activity and also lack some of the toxic effects produced by other Se forms, DNA strand breaks after selenite treatment being an example. The metabolic pathways of Se-methylselenocysteine and selenobetaine involve the release of methyl-selenol or methylseleninic acid derivatives, which have been shown in in vitro experiments to affect apoptosis or to arrest the cell cycle (see Figure 2.7. in the previous chapter). The results of in vitro experiments on cells treated with selenite and methylated selenocompounds are summarized in Table 2.13. [31]. Methylselenocysteine is one of the most important selenocompounds for chemopreventive activity [32]. It is twice as active as selenomethionine in suppressing mammary carcinogenesis in rodents [62].

Selenium compounds can also affect activation of the p53 gene since the main dietary selenium compound, selenomethionine, modulates p53 activity. In normal cells, p53 regulates the activity of genes involved in DNA repair processes. In human cancer cells, p53 is often mutated and its functional activity suppressed. Selenomethionine has been shown to act together with the p53 tumour suppressor protein in affecting the redox status. SeMet oxidizes the thiols of the p53 molecules at 275 and 277 cysteine residues. Seo et al. [49] demonstrated that the Se concentration is a determinant of basal p53 activity, p53-dependent DNA repair being activated within the concentration range of 10–20 µM. Fiala et al. [63] showed that the selenium compounds as selenite, 1,4-phenylenebis-(methylene)selenocyanate and benzyl selenocyanate inhibit the activity of DNA cytosine methyltransferase, an enzyme involved in DNA repair processes. They suggested that this pathway may be the major mechanism involved in the chemoprevention that selenium compounds provide after the initiation of carcinogenesis. Two other Se compounds, sodium selenite and methylseleninic acid, were found to cause phosphorylation of the serines and threonines contained in the p53 molecule. This modulation of the p53 molecule may be a specific mechanism of p53 activation. Methylseleninic acid in a range of concentration of 0.5–1 µM was found to promote DNA repair processes by increasing the expression of two proteins associated with the p53
Vitamins and selenium: Anticancerogenic activity of selenium

gene, and to be involved in the pathway connected with the p53-dependent activation of DNA repair processes [64]. There is a need of explaining how triggering of the p53-dependent DNA repair process by two different Se compounds can be achieved when a 10–20-fold higher Se concentration is employed. One possibility is that a small fraction of the SeMet is metabolized to a low-molecular-weight compound [32]. Seo et al. [49] showed that in normal human fibroblasts, selenomethionine protects the cells from DNA damage through the induction of DNA repair. Limitations in the DNA repair capacity appear to be a key determinant of predisposition to cancer [49, 65]. Enhanced formation of the DNA repair complex in cells treated with selenomethionine has been considered to be a possible mechanism for the inducible capacity for repair of DNA [49, 66]. Blessing et al. [67] described the incorporation of Se into the factors involved in DNA repair regulation. Reducible selenium compounds (phenylseleninic acid, ebselen, selenocystine, and 2-nitrophenylselenocyanate) were found to cause a dose-dependent decrease in the activity of formamidopyrimidine-DNA glycosylase (Fpg), one of the enzymes involved in DNA repair processes. These selenocompounds affect the DNA repair processes through oxidation of the zinc finger structures and the release of zinc from DNA, as well as damaging the integrity of the genes of the DNA repair enzymes [67, 68]. They also affect the integrity of the XPA protein (xeroderma pigmentosum group A protein), which has an essential role in recognizing DNA lesions in the nucleotides and in the excision of damaged nucleotides in mammalian cells [69].

Studies conducted in cell cultures suggest that selenocompounds may exert their chemopreventive effects via induction of apoptosis and cell growth inhibition in the transformed cells. It has been found that Se derivatives can activate the p53 gene, but that the induction of apoptosis is not a simple result of the regulation of p53 by selenium [16]. In investigating the ability of inorganic Se compounds to induce apoptosis, it was found that the human oral squamous cell carcinoma line (HSC-3) lost >80% of its GSH after treatment by 10 µM selenite or 100 µM selenodioxide for 72 h. This decreased GSH concentration in the cells induced apoptosis, which is a dominant mechanism of the cell death these compounds bring about [70].

Selenium can also affect DNA methylation, an important epigenetic mechanism that exerts control over gene expression [30]. The postsynthetic methylation of DNA is catalyzed by the family of S-adenosylmethionine-dependent DNA methyltransferases [71]. The methylation of Se compounds to Se(CH₃)₂ and Se(CH₃) results in a decrease in methyl donation, this preventing DNA methylation from occurring. DNA methylation is one of the first steps in the carcinogenesis induced by certain chemicals, such as benzo(a)pyrene and arsenic [30].

Selenium can also act as an inductor of the enzymes participating in phase II detoxification [48] and it modulates expression of the enzymes involved in phase I detoxification. In vitro studies of a mammary cell line exposed to DMBA showed 1,4-phenylenebis(methylene)selenocyanate and its putative glutathione conjugate to inhibit the expression of various CYP450 isoenzymes and to induce the expression of various enzymes involved in phase II or DMBA detoxication [72].
**Effects of selenium on immune functions**

Certain anticancer properties of the selenocompounds may be associated with their effects on cellular immunity. Selenium compounds can activate cytotoxic cells, stimulate the expression of cytokine receptors and the proliferation of lymphocytes [73,74]. Low Se concentrations in the tissues and in human body fluids also appear to be associated with numerous changes in the immune system, such as suppression of the host immune response to bacterial and viral infections, the inhibition of prostaglandin and immunoglobulin synthesis, reduction in the activity of T lymphocytes, NK cells and macrophages, and impairment of the body’s ability to reject implants and to destroy neoplastic tumours [75,76]. Cellular membranes of the T lymphocytes are particularly sensitive to Se deficiency, due to the large amounts of unsaturated fatty acids present in their structure. The decrease in the number and the activity of cytotoxic T lymphocytes (CTL) is accompanied by the reduced excretion of lymphotoxins and the inhibition of both leukocyte and macrophage migration. CTL activity has been found to be significantly increased in Se (SeIV) supplemented patients with cancer located in the head and neck region who were given standard anticarcinogenic therapy. Insufficient dietary intake of selenium results in many types of defects of the immune processes, such as in connection with antibody production and specific cell immunity [77]. Selenium regulates the immune response by stimulating natural killer cells and activating antigens of various types to destroy the tumour cells [78]. Se can stimulate the expression of IL-2 receptors found on activated T lymphocytes and on NK cells [79]. The anticarcinogenic effect of selenium is also partly based on its ability to produce antitumour metabolites (e.g. methylselenol). These metabolites that are synthetised in the cell can be involved in the cell’s metabolic pathways and destroy the integrity of tumour cells or induce apoptosis in these cells [51,80].

**Selenium and cancer risk — epidemiological results**

Several studies have shown the development of cancer in humans to be inversely related to the intake of specific dietary components, including nutrients, micronutrients and phytochemicals [81,82]. Research over the last decade points to a significant protective role of Se in preventing the development of malignant neoplasms. Low plasma selenium concentrations are thought to be associated with increased morbidity and mortality from cancer. In a Chinese study, a statistically significant increase in morbidity from oesophagus and stomach cancer was noted in a population with low levels of selenium, but no such relationship was found for lung cancer [83]. An inverse association between selenium and risk of cancer has been reported both in case-control studies and in follow-up cohort studies. Prospective cohort studies appear to show in a more distinct manner the possible association found between the prediagnostic selenium concentration level and risk of cancer. Case-control studies tend to reflect the short-term Se concentrations in the organism, such as the Se level associated with the dietary pattern at the time of sampling in cases and controls. To date, however, the results of epidemiologic studies
have been rather inconsistent. Some authors have reported there to be an association between cancer risk and Se status, whereas others have obtained null results [84,85]. The findings for three major forms of cancer are summarized below.

**Lung cancer**

Studies to test the hypothesis that low Se concentrations contribute to the development of lung cancer have been conducted in many countries (Table 2.14.). The significant, dose-dependent protective activity of Se was documented in Finnish and Dutch studies [86,87] in which Se in both serum and toenail samples was analyzed, whereas no association between selenium level and risk of lung cancer was found in two studies of non-European populations [88,89]. It is notable that of the women investigated within the Nurses Health Study, those with high toenail Se levels were found to have a particularly high relative risk of lung cancer (RR: 4.33, 95% CI: 0.54–34.60) after adjustments for smoking status were made [90].

A review of epidemiologic studies of lung cancer and of the selenium concentration found in biological material, undertaken by Zhou et al [91], indicated selenium to have a certain protective effect, but only in populations with a low mean Se level. In a metaanalysis of 14 epidemiologic studies, 11 of which had a prospective design, the risk of lung cancer at high Se concentrations was found to be RR= 0.74 (95% CI: 0.57–0.97) (Table 2.14.). Interestingly, when the study population was divided up according to Se level, the mean value obtained for groups showing a high basic concentration of Se was RR = 0.86 (95% CI: 0.61–1.22), whereas the value for groups with a low basic level of Se was RR = 0.72 (95% CI: 0.45–1.16). In the control group of non-cancer patients the mean serum Se concentration was 100 ng/ml blood serum, representing a daily Se intake of 55 mg. Of the studies considered in the meta-analysis, only the findings for the two Finnish populations (RR = 0.41; 95% CI: 0.17–0.94 and RR = 0.20; 95% CI: 0.09–0.44) and for the Dutch population (RR = 0.50; 95% CI: 0.30–0.81) revealed a statistically significant protective effect of high Se concentrations. In most of the reports, the protective activity of Se could be clearly discerned when the reference group consisted of subjects showing the lowest level of this trace element.

The protective effect of Se in connection with lung cancer could also be noted in studies examining Se concentration in the toenails. The total RR values for lung cancer in patients showing high Se levels ranged from 0.46 (95% CI: 0.24–0.87) when toenail Se was used as a marker of the concentration of Se in the body, to 0.80 (95% CI: 0.58–1.10) for the serum Se level, to 1.00 (95% CI: 0.77–1.30) in studies of Se intake based on use of a questionnaire. These finding confirm the assumption that the Se level in the toenails can be used as a marker of long-term Se concentration [91].
Table 2.14. Epidemiological studies of the selenium level in the body and risk of lung cancer (according to Zhuo et al. [91])

<table>
<thead>
<tr>
<th>Author</th>
<th>Study</th>
<th>Se index</th>
<th>Gender</th>
<th>Population</th>
<th>Years of follow up</th>
<th>No. cases (mean Se level)</th>
<th>No. controls (mean Se level)</th>
<th>RR (95% CI) (high vs. low Se)</th>
<th>P for dose-response trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comstock et al. [92]</td>
<td>NCC</td>
<td>Serum</td>
<td>All</td>
<td>USA</td>
<td>15–18</td>
<td>258 (0.108 ppm)</td>
<td>515 (0.110 ppm)</td>
<td>0.65 (0.41–1.02)</td>
<td>0.08</td>
</tr>
<tr>
<td>Goodman et al. [89]</td>
<td>NCC</td>
<td>Serum</td>
<td>All</td>
<td>USA</td>
<td>5–14</td>
<td>356 (119.1 (19.6) µg/l)</td>
<td>356 (117.7 (18.5) µg/l)</td>
<td>1.20 (0.77–1.88)</td>
<td>0.49</td>
</tr>
<tr>
<td>Kabuto et al. [93]</td>
<td>NCC</td>
<td>Serum</td>
<td>All</td>
<td>Japan</td>
<td>11–13</td>
<td>77 (113.0 µg/l)</td>
<td>120 (119.1 (2.0) µg/l)</td>
<td>0.56 (0.20–1.43)</td>
<td>NA</td>
</tr>
<tr>
<td>Knekt et al. [94]</td>
<td>NCC</td>
<td>Serum</td>
<td>Male</td>
<td>Finland</td>
<td>8–12</td>
<td>153 (57.0 (16.7) µg/l)</td>
<td>153 (61.0 (13.5) µg/l)</td>
<td>0.66 (0.37–1.19)</td>
<td>0.001</td>
</tr>
<tr>
<td>Knekt et al. [86]</td>
<td>NCC</td>
<td>Serum</td>
<td>All</td>
<td>Finland</td>
<td>16–19</td>
<td>77 (53.2 (24.3) µg/l)</td>
<td>145 (57.8 (16.9) µg/l)</td>
<td>0.41 (0.17–0.94)</td>
<td>0.46</td>
</tr>
<tr>
<td>Ratnasinghe et al. [88]</td>
<td>NCC</td>
<td>Serum</td>
<td>Male</td>
<td>China</td>
<td>4–5</td>
<td>108 (46.5 µg/l)</td>
<td>216 (45 µg/l)</td>
<td>1.20 (0.60–2.40)</td>
<td>0.52</td>
</tr>
<tr>
<td>Garland et al. [90]</td>
<td>NCC</td>
<td>Toenail</td>
<td>Female</td>
<td>USA</td>
<td>3.5</td>
<td>47 (0.811 (0.166) µg/g)</td>
<td>47 (0.897 (0.308) µg/g)</td>
<td>4.33 (0.54–34.60)</td>
<td>0.17</td>
</tr>
<tr>
<td>Kromhout et al. [97]</td>
<td>cohort</td>
<td>Diet</td>
<td>Male</td>
<td>The Netherlands</td>
<td>25</td>
<td>63 (NA)</td>
<td>870 (64.6 (15.2) µg/day)</td>
<td>0.98 (0.41–2.36)</td>
<td>&gt;0.01</td>
</tr>
<tr>
<td>Zhou et al. [98]</td>
<td>CC</td>
<td>Diet</td>
<td>Female</td>
<td>China</td>
<td>–</td>
<td>290 (36.10 (16.0) µg/day)</td>
<td>290 (39.80 (33.0) µg/day)</td>
<td>0.76 (0.47–1.15)</td>
<td>0.11</td>
</tr>
</tbody>
</table>

* Nested case-control; b data from 189 sets; c data from 91 individuals; d data from 177 individuals; e male subjects randomized in the earliest in the trial; f male subjects randomized in the 5th year; g data from 370 individuals.
Prostate cancer

A majority of prospective and case-control studies show high levels of selenium intake to have a protective role in preventing the development of prostate cancer (Table 2.15.). Several studies have shown that selenium can be of specific help in combatting prostate cancer [99]. In the Health Professionals Follow-Up Study, in which the Se concentration in the toenails served as a measure of long-term Se intake, the odds ratio (OR) for the development of cancer that was associated with a high level of Se intake was 0.49 (95% CI: 0.25–0.96) [100]. In the Netherlands Cohort Study, which involved a 6.3 years’ follow-up period, high Se concentration in the toenails was associated with an appreciably lower risk of prostate cancer [101]. In two large case-control studies of male populations in Great Britain and Canada, however, the Se level in the toenails was not found to be associated to any marked degree with the level of risk of prostate cancer [102,103]. Similar results were obtained in a 6-year follow-up nested case-control study in which the mean toenail Se concentration in prostate cancer cases and in matching controls were not found to differ significantly, although a protective effect of a high Se level (fifth quintile) was found (OR = 0.38, 95%CI: 0.17–0.85). In addition, the protective effect of high Se levels and high α-tocopherol concentrations in the plasma was only particularly strong when the γ-tocopherol level was high [104]. A nested case-cohort study conducted on Japanese-American men (at a > 20-year follow-up) also confirmed the association between a high Se level in the plasma and a decreased risk of prostate cancer (OR = 0.5; 95% CI: 0.3–0.9) [105]. A high prediagnostic Se level was found, in the Physicians’ Health Study, to be associated (at a 13-year follow up) with a significantly reduced risk of prostate cancer, especially men who had a PSA concentration equal to or higher than 4 ng/ml [106]. The lack of a protective effect of Se in connection with risk of prostate cancer risk was observed in the men participating in the Carotene and Retinol Efficacy Trial (CARET) [89]. A systematic review of sixteen studies (11 cohort studies and 5 case-control studies) was conducted recently to investigate the association between selenium level and risk of prostate cancer. The findings of this review showed that the pooled RR of prostate cancer for a particular Se intake, defined as the average of the 1st and 4th quintiles or the 1st and 3rd quartiles, was 0.72 (95% CI: 0.61-0.84) in cohort studies and 0.74 (95% CI: 0.61–1.39) in case-control studies, indicating that the intake of selenium was able to reduce the risk of prostate cancer [107].

Colorectal cancer

Results of prospective and case-control studies of the risk of colorectal cancer in relation to the selenium level in the body are summarised in Table 2.16. In a US case-control study of the relation between the risk of colonic malignant or benign tumour and the serum Se level, no protective effect of higher Se levels was found in either of the groups investigated [108]. A lower Se concentration in the serum was found to be associated with a stronger tendency to be afflicted with a colorectal tumour [109]. Also, colorectal cancer patients with low Se concentrations were found to have a significantly lower survival time
Table 2.15. Epidemiological studies of the selenium level in the body and risk of prostate cancer

<table>
<thead>
<tr>
<th>Author</th>
<th>Study</th>
<th>Se indicator</th>
<th>Population</th>
<th>Years of follow up</th>
<th>No. cases (mean Se level)</th>
<th>No. controls (mean Se level)</th>
<th>RR (95% CI) (high vs. low Se)</th>
<th>P for dose-response trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knekt et al. [94]</td>
<td>NCCb</td>
<td>Serum</td>
<td>Finland</td>
<td>8–12</td>
<td>46 (59.6 (19.4) µg/l)</td>
<td>46 (58.3 (14.8) µg/l)</td>
<td>1.00 (0.42–2.40)</td>
<td>0.707</td>
</tr>
<tr>
<td>Goodman et al. [89]</td>
<td>NCC</td>
<td>Serum</td>
<td>USA</td>
<td>5–14</td>
<td>235 (114.8 (19.6) µg/l)</td>
<td>456 (114.3 (20.4) µg/l)</td>
<td>1.02 (0.65–1.60)</td>
<td>0.69</td>
</tr>
<tr>
<td>Yoshizawa et al. [100]</td>
<td>NCC</td>
<td>Toenail</td>
<td>USA</td>
<td>5</td>
<td>181 (0.82 µg/g)</td>
<td>181 (0.96 µg/g)</td>
<td>0.39 (0.18–0.84)</td>
<td>0.05</td>
</tr>
<tr>
<td>Helzlsouer et al. [104]</td>
<td>NCC</td>
<td>Toenail</td>
<td>USA</td>
<td>1–6</td>
<td>117 (0.77 µg/g)</td>
<td>233 (0.79 µg/g)</td>
<td>0.38 (0.17–0.85)</td>
<td>0.12</td>
</tr>
<tr>
<td>Van den Brandt et al.</td>
<td>Cohort</td>
<td>Toenail</td>
<td>The Netherlands</td>
<td>6.3</td>
<td>540 (0.530 (0.090) µg/g)</td>
<td>1211 (0.547 (0.126) µg/g)</td>
<td>0.69 (0.48–0.99)</td>
<td>0.008</td>
</tr>
<tr>
<td>Nomura et al. [105]</td>
<td>Case-control</td>
<td>Serum</td>
<td>USA (Japanese-Americans)</td>
<td>–</td>
<td>249 (128.0 µg/l)</td>
<td>249 (131.6 µg/l)</td>
<td>0.5 (0.3–0.9)</td>
<td>0.02</td>
</tr>
<tr>
<td>Li et al. [106]</td>
<td>NCC</td>
<td>Plasma</td>
<td>USA</td>
<td>13</td>
<td>586 (0.106 (0.018) µg/g)</td>
<td>577 (0.108 (0.018) µg/g)</td>
<td>0.78 (0.54–1.13)</td>
<td>0.16</td>
</tr>
<tr>
<td>Allen et al. [103]</td>
<td>Case-control</td>
<td>Nails</td>
<td>UK</td>
<td>–</td>
<td>300 (0.622 µg/g)</td>
<td>300 (0.611 µg/g)</td>
<td>1.24 (0.73–2.10)</td>
<td>0.581</td>
</tr>
</tbody>
</table>

a Nested case-control; b data from 51 sets.
Table 2.16. Epidemiological studies of the selenium level in the body and risk of colorectal cancer

<table>
<thead>
<tr>
<th>Author</th>
<th>Study</th>
<th>Se indicator</th>
<th>Gender</th>
<th>Population</th>
<th>Years of follow up</th>
<th>No. cases (mean Se level)</th>
<th>No. controls (mean Se level)</th>
<th>RR (95% CI) (high vs. low Se)</th>
<th>P for dose-response trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knekt et al. [94]</td>
<td>NCC</td>
<td>Serum</td>
<td>Male</td>
<td>Finland</td>
<td>8–12</td>
<td>29 (63.3 (18.9) µg/l)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29 (64.0 (18.2) µg/l)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.01 (0.18–5.65)</td>
<td>0.643</td>
</tr>
<tr>
<td>Knekt et al. [94]</td>
<td>NCC</td>
<td>Serum</td>
<td>Female</td>
<td>Finland</td>
<td>8–12</td>
<td>48 (64.0 (18.7) µg/l)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48 (65.3 (15.3) µg/l)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.10 (0.42–2.92)</td>
<td>0.724</td>
</tr>
<tr>
<td>Wallace et al. [112]</td>
<td>NCC</td>
<td>Serum</td>
<td>All</td>
<td>USA</td>
<td>1–4</td>
<td>276 (131.5 (19.7) µg/l)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>276 (130.3 (17.8) µg/l)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.76 (0.44–1.30)</td>
<td>0.50</td>
</tr>
<tr>
<td>Nelson et al. [108]</td>
<td>Case-control</td>
<td>Serum</td>
<td>All</td>
<td>USA</td>
<td>–</td>
<td>25 (138 µg/l)</td>
<td>138 (134 µg/l)</td>
<td>1.7 (0.5–5.9)</td>
<td>NA</td>
</tr>
<tr>
<td>Garland et al. [90]</td>
<td>NCC</td>
<td>Toenail</td>
<td>Female</td>
<td>USA</td>
<td>3.5</td>
<td>89 (0.863 (0.146) µg/g)</td>
<td>47 (0.843 (0.186) µg/g)</td>
<td>2.04 (0.88–4.75)</td>
<td>0.12</td>
</tr>
<tr>
<td>Van den Brandt et al. [111]</td>
<td>Cohort</td>
<td>Toenail</td>
<td>Male</td>
<td>The Netherlands</td>
<td>3.3</td>
<td>Colon 121 (0.535 (0.092) µg/g)</td>
<td>1209 (0.547 (0.123) µg/g)</td>
<td>0.82 (0.43–1.58)</td>
<td>0.326</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rectal 76 (0.593 (0.411) µg/g)</td>
<td></td>
<td>0.91 (0.41–2.00)</td>
<td>0.131</td>
</tr>
<tr>
<td>Van den Brandt et al. [111]</td>
<td>Cohort</td>
<td>Toenail</td>
<td>Female</td>
<td>The Netherlands</td>
<td>3.3</td>
<td>Colon 112 (0.560 (0.106) µg/g)</td>
<td>1246 (0.575 (0.108))</td>
<td>0.77 (0.41–1.45)</td>
<td>0.733</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rectal 36 (0.578 (0.091))</td>
<td></td>
<td>1.58 (0.59–4.22)</td>
<td>0.273</td>
</tr>
</tbody>
</table>

<sup>a</sup> Nested case-control; <sup>b</sup> data from 32 sets; <sup>c</sup> data from 59 sets.
and a lower cumulative cancer-related survival rate than such patients with higher Se concentrations did [109]. However, in a recent study no differences between healthy individuals and individuals with adenomatous colon polyps or colorectal cancer were found in terms of Se level, selenoprotein P (SeP) concentration or glutathione peroxidase activity in the plasma [110]. Colorectal cancer risk was also analyzed in a 41-month follow-up of the Nurses’ Health Study [90], no significant association being found between risk of cancer and toenail Se status. A similar result was obtained in a 3.3-year follow-up study of a Dutch cohort in which toenail Se level was used as an indicator [111]. In a Canadian case-control study, however, a significant inverse association was obtained between toenail Se level and risk of colon cancer (OR = 0.42; 95% CI: 0.19–0.93) [102]. Two studies in which the serum Se status was measured did not indicate there to be a clear association between serum Se levels and risk of colorectal cancer [94,112].

To gain further insight into the anti-carcinogenic potential of selenium, a pooled analysis of data from three clinical trials of colorectal adenoma was conducted: the Wheat Bran Fiber Trial, the Polyp Prevention Trial, and the Polyp Prevention Study. The selenium level was measured in blood specimens of 1763 trial participants. After adjustment for age, gender, smoking status and study site, there was found for each of the three studies to be a lower risk of recurrence of an adenoma in patients with blood selenium levels in the highest quartile than in those in the lowest quartile, although this result was only statistically significant in the case of the Polyp Prevention Study (OR: 0.57, 95% CI: 0.34–0.95) [113].

To sum up, a number of epidemiologic studies show a low Se level, especially in males, to be associated with an elevated risk of lung and prostate cancer. No inverse association of Se level and cancer risk was found for lung cancer in females, possibly due in part to the rather low proportion of women in the study population [94]. In contrast to prostate cancer, breast cancer was not found to be influenced by the selenium level [114–116]. The majority of studies on relations between selenium level and occurrence of colorectal cancer have yielded null results for both males and females. However, there are some reports of a significant inverse relationship between blood Se levels and the prevalence of adenomatous polyps [117,118]. The potential role of dietary Se in the early prevention of colorectal neoplasms would need to be confirmed, and the preventive role of Se regarding cancer of this type, as found in the Polyp Prevention Study [113], would need further verification as well.

Summary

Results of epidemiologic and laboratory studies have indicated selenium to have a protective role in counteracting or preventing the development of cancer. A low level of selenium concentration in the body was shown to be associated with a higher risk of lung, prostate or colorectal cancer. However, a variety of confounding factors such as geographical location, gender, age, environmental exposure, genetic susceptibility, and the like need to be taken into account. There is a need of clarifying the role of Se
in the etiology of certain types of cancer through further epidemiologic investigation. We now have evidence from laboratory studies of selenium compounds affecting cell growth, the cell cycle, DNA repair, gene expression, and signal transduction. In the experiments involved, the effects of both organic forms of Se (SeMet and methylated selenocompounds) and inorganic forms of it (selenites and selenates) were evaluated. The metabolic pathways of the action of the two forms differ and depend on the basic levels of the compounds in question. Various hypotheses endeavoring to explain the connection between the metabolic pathways in which Se is involved and the effects these can have on chemically induced cancerogenesis, such as in connection with the regulation of cell signaling and of the redox status, the modulation of transcriptional factors, and the activation of DNA repair. Results of animal and in vitro studies have shown that the form of the selenium compound (methylated selenocompound) in question may be of critical importance to the chemoprotective actions it can perform.

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Vitamins and selenium: Anticancerogenic activity of selenium


3. Bioactive components in foods

3.1. Anticarcinogenic effects of flavonoids

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Cancer Biomarkers and Prevention Group, University of Leicester, Leicester, UK

Introduction

Many thousands of different flavonoids are found in plant species, with major dietary sources including fruit, vegetables, tea, chocolate and soy. Total daily intake can range from 50–800 mg. Those flavonoids which have been studied in most detail exhibit many properties which could be protective against heart disease, ageing and cancer. These polyphenolic compounds are classified, according to structure, as flavonols (quercetin, kaempferol), flavones (luteolin, apigenin), flavanones (myricetin, naringin, hesperetin), isoflavones (genistein, daidzein), anthocyanins (cyanidin, pelargonidin, petunidin), catechins (epicatechin, epicatechin-3-gallate) and chalones (xanthohumol). Amongst their health-promoting properties are antioxidant, antiviral, anti-allergic, anti-inflammatory, and anticancer activities. Such chemopreventive agents can be effective at different stages of the carcinogenic process, both by blocking initiation and by suppressing the later stages involving promotion, progression, angiogenesis, invasion and metastasis. Several recent reviews have summarised the potential chemopreventive mechanisms for a number of flavonoids [1–4]. Some recent data for the well-studied flavonoids apigenin, epigallocatechin gallate (EGCG), genistein, resveratrol, quercetin, the chalone, xanthohumol and the novel flavonol, tricin, are summarised here.

Blocking mechanisms

Possible ways in which initiation of carcinogenesis can be blocked include prevention of reactive oxygen species attack on DNA, altered metabolism of procarcinogens in favour of conjugation and excretion of reactive metabolites, inhibition of carcinogen uptake into cells and enhanced DNA repair.

Many flavonoids possess antioxidant or free radical scavenging potential, which varies depending on the hydroxylation status of the benzene rings. Examples include quercetin (a flavonol in vegetables, apples and onions), xanthohumol (a chalone in hops and beer) and genistein (an isoflavone in soy). An early study by Duthie et al [5] reported that quercetin protected human lymphocytes from hydrogen peroxide-induced DNA damage. Similar findings were reported by Wilms et al [6], who also found that quercetin protected human lymphocyte DNA from bulky adduct formation following treatment with benzo[a]pyrene. Also in this study, volunteers consumed quercetin-rich blueberry/apple juice for 4 weeks, which led to a significant increase in antioxidant capacity of plasma.
Flavonoids can interact with the aryl hydrocarbon receptor (AhR) as agonists or antagonists, depending on structure and cell context. Such interactions influence the expression of drug metabolising enzymes such as cytochromes P450 [7]. They have also been shown to influence the multi-drug resistance phenotype acquired by many tumour cells. Quercetin and silymarin were found to inhibit MRP1/4/5-mediated drug transport from intact erythrocytes with high affinity, in a manner which suggested that they interact at the substrate-binding sites. Such interactions might influence bioavailability of anti-cancer drugs in vivo and could be considered for combination therapies [8]. In another recent study [9], the flavonols, quercetin and kaempferol, reduced P-glycoprotein expression and function in multi-drug resistant human cervical carcinoma KB-IV cells, while the isoflavones, genistein and daidzein, modulated intracellular drug levels by inhibiting function, without affecting expression.

Xanthohumol possesses several useful properties to block carcinogenesis including modulation of enzymes involved in carcinogen metabolism and detoxification (inhibition of Cyp1A, induction of quinone reductase activity), scavenging of ROS, including hydroxyl and peroxyl radicals, along with inhibition of superoxide anion radical formation and nitric oxide production [10].

**Suppressing mechanisms**

Mechanisms which result in suppression, or even better, elimination of tumour cells, include growth inhibition by induction of cell cycle arrest or apoptosis. A significant number of flavonoids, alone and in combination, have been shown to induce G2/M arrest in SW480 and CaCo2 human colon carcinoma cells [11]. Tricin, a novel flavonol in rice bran, was shown to inhibit the growth of breast tumour cells, causing G2/M arrest, but not apoptosis [12]. In a subsequent study by the same group [13], tricin decreased the number of intestinal adenomas in Apcmin mice by 33%, with inhibition of COX-1 and COX-2 activity. The latter led to a 34% reduction of PGE2 levels in small intestinal mucosa and blood. Xanthohumol was also found to inhibit COX-1 and COX-2 activities, and to be antiestrogenic [10]. The inhibitory effect of other flavonoids on COX-2 expression and activity has been reviewed by O’Leary et al. [14]. During later stages of carcinogenesis additional useful mechanisms include inhibition of angiogenesis, invasion and metastasis.

A range of tumour suppressing activities is shown for apigenin (Table 3.1.) and quercetin (Table 3.2.). Resveratrol, genistein and EGCG (reviewed in [2]) have a number of effects in common with those detailed here for apigenin and quercetin, namely inhibition of signalling through the EGFR family, NF-κB, and pAkt, induction of cell cycle arrest involving a decrease in cyclin D1 and phosphorylation of Rb, accompanied by upregulation of p21 and p27, and induction of apoptosis involving release of cytochrome c from mitochondria, activation of caspases 3 and 9 and downregulation of Bcl family members. However, depending on cell type and experimental conditions, flavonoids can both up- and down-regulate key molecules, including JNK, AP-1, p21, p27, cdc2, cyclin D1, p53, and PI3K.
Table 3.1. Chemopreventive suppressing effects of apigenin

<table>
<thead>
<tr>
<th>Tissue/cell type</th>
<th>Mechanism</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jurkat T cells</td>
<td>↓ chymotrypsin-like activity of 20S and 26S proteosomes; ↑ Bax; ↑ IκBα</td>
<td>Apoptosis</td>
<td>[15]</td>
</tr>
<tr>
<td>PWR-1E, LNCaP, PC-3, DU145 prostate tumour cells</td>
<td>↑ ROS; ↓ Bcl2; ↑ cleavage of caspases 3, 7, 8, 9 and cIAP-2</td>
<td>Apoptosis</td>
<td>[16]</td>
</tr>
<tr>
<td>Breast and prostate cancer cells</td>
<td>↓ fatty acid synthase activity</td>
<td>Growth inhibition and apoptosis</td>
<td>[17]</td>
</tr>
<tr>
<td>NUB-7, LAN-5, SK-N-BE neuroblastoma cells</td>
<td>↑ p53; ↑ p21; ↑ Bax; ↑ cleavage of caspase 3</td>
<td>Apoptosis (p53-dependent)</td>
<td>[18]</td>
</tr>
<tr>
<td>Breast cancer cells</td>
<td>↓ Her2; ↓ Akt; ↑ cleavage of caspase 3; ↑ DFF-45 cleavage; ↓ cyclin D1/D3 &amp; cdk4; ↑ p27</td>
<td>Apoptosis; ↓ colony formation</td>
<td>[19]</td>
</tr>
<tr>
<td>PC-3 prostate cancer cells</td>
<td>↓ p50; ↓ p65; ↓ NF-κB-DNA-binding; ↓ IκBα degradation and phosphorylation; ↓ JκKα activity; ↓ TNFα activation of NF-κB</td>
<td>Apoptosis; Bcl2, cyclin D1, COX-2, MMP9, NOS-2, VEGF</td>
<td>[20]</td>
</tr>
<tr>
<td>DU145 prostate cancer cells</td>
<td>↓ cyclin D1/2 &amp; E; ↓ CDK2/4/6; ↑ p21, p27, p16, p18; altered Bax:Bcl2 ratio; ↑ cyt c release; ↑ APAF-1; ↑ IκBα; ↓ NF-κB p50 &amp; p65</td>
<td>Growth inhibition, G1 arrest, apoptosis</td>
<td>[21]</td>
</tr>
<tr>
<td>HER2-overexpressing breast tumor cells</td>
<td>↓ PI3K &amp; Akt activity; ↓ PI3K-HER2 docking; ↓ HER2/neu phosphorylation; ↑ HER2 degradation</td>
<td>Apoptosis</td>
<td>[22]</td>
</tr>
<tr>
<td>A549 lung cancer cells in vitro and in nude mice</td>
<td>↓ Akt; ↓ p70S6K1; ↓ HIF-1α; ↓ VEGF</td>
<td>Growth and angiogenesis inhibition</td>
<td>[23]</td>
</tr>
<tr>
<td>OVCAR-3 and A2780/CP70 ovarian cancer cells</td>
<td>↓ Akt; ↓ p70S6K1; ↓ HIF-1α; ↓ VEGF; ↑ p53; ↓ HDM2</td>
<td>Angiogenesis inhibition</td>
<td>[24]</td>
</tr>
<tr>
<td>HCT 116 colon carcinoma cells</td>
<td>↑ ERK &amp; p38 phosphorylation and activity</td>
<td>↑ phosphorylation of Elk &amp; ATF2</td>
<td>[25]</td>
</tr>
<tr>
<td>HCT 116, HT-29 colon cancer cells</td>
<td>↓ CK2; ↓ TNF α-induced NF-κB activation</td>
<td>Apoptosis</td>
<td>[26]</td>
</tr>
</tbody>
</table>
Table 3.2. Chemopreventive suppressing effects of quercetin

<table>
<thead>
<tr>
<th>Tissue/cell type</th>
<th>Mechanism</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL60 human myeloid leukemia cells</td>
<td>↑Bax; ↑phosphoBcl2; ↓Pgp</td>
<td>Apoptosis</td>
<td>[27]</td>
</tr>
<tr>
<td>Jurkat T cells</td>
<td>Inhibiting chymotrypsin-like activity of 20S and 26S proteosomes; ↑Bax; ↑IkBα</td>
<td>Apoptosis</td>
<td>[15]</td>
</tr>
<tr>
<td>Breast and prostate cancer cells</td>
<td>↓fatty acid synthase activity</td>
<td>Growth inhibition and apoptosis</td>
<td>[17]</td>
</tr>
<tr>
<td>Colonic aberrant crypt foci</td>
<td>↑Bax; ↓Bcl2; ↑ cleavage of caspase 9</td>
<td>Suppression by 4-fold; apoptosis ↑3-fold</td>
<td>[28]</td>
</tr>
<tr>
<td>MiaPaCa pancreatic tumour cells</td>
<td>↓phosphoFAK</td>
<td>Decreased invasion</td>
<td>[29]</td>
</tr>
<tr>
<td>MCF7 breast tumour cells</td>
<td>↑PTEN; ↑p27; ↓Akt</td>
<td>Growth inhibition and apoptosis</td>
<td>[30]</td>
</tr>
<tr>
<td>LNCaP, PC3 prostate tumour cells</td>
<td>↓Sp1 interaction with AR; ↑c-jun</td>
<td>Inhibition of androgen receptor activity</td>
<td>[31]</td>
</tr>
<tr>
<td>HT29, SW480 colon cancer cells</td>
<td>↓ErbB2/3; ↓Bcl2; ↓phosphoAkt</td>
<td>Growth inhibition and apoptosis</td>
<td>[32]</td>
</tr>
<tr>
<td>SW480 colon cancer cells</td>
<td>↓β-catenin/Tcf transcriptional activity</td>
<td>↓c-myc</td>
<td>[33]</td>
</tr>
<tr>
<td>A549, H1299 human lung carcinoma cells</td>
<td>↑cyclin B1; ↑phospho cdc2; ↑survivin; ↑p53; ↑p21</td>
<td>Growth inhibition; G2/M arrest</td>
<td>[34]</td>
</tr>
<tr>
<td>PC3 prostate cancer cells</td>
<td>↓HSP70</td>
<td>Apoptosis</td>
<td>[35]</td>
</tr>
</tbody>
</table>

One recent report by Fenton and Hord [36] has suggested a novel chemopreventive mechanism for flavonoids. In normal colon, epithelial cells migrate to the apex of the crypt, a process involving the APC gene, which is often mutated in colon cancer. These authors reported that apigenin, epicatechin, naringin and hesperidin induced a greater migratory response in APC<sup>min/+</sup> cells compared to those expressing wild type APC. Such flavonoid-induced migration was dependent on matrix metalloproteinase activity.

During the carcinogenic process, both hypermethylation of the promoter regions of tumour suppressor genes and hypomethylation of oncogenes can occur, resulting in under- or over-expression. Both EGCG [37] and genistein [38] have been shown to reactivate a number of key genes, such as the cell cycle inhibitor p16 and the retinoic
acid receptor (RARβ), in several different cancer cell types. The mechanism proposed was through inhibition of DNA methyltransferase, which, in the case of EGCG, involved direct interaction with the enzyme.

**Combined effects**

There is now accumulating evidence for the additive, synergistic or antagonistic effects of combinations of more than one chemopreventive agent. For example Mertens-Talcott et al. [39], using MOLT-4 human leukaemia cells, showed that quercetin and ellagic acid acted synergistically in inducing apoptosis. Ellagic acid potentiated the inducing effect of quercetin on levels of p21 and phosphorylation of p53 at serine 15. Phosphorylation of JNK1/2 and p38 was increased by the combination, while quercetin alone only induced p38 phosphorylation. Neither the generation of ROS, nor quercetin stability were affected by ellagic acid. Combinations of flavonoids were found to have an inhibitory effect on the breast cancer resistance protein (ABCG2), suggesting the potential use of ‘flavonoid cocktails’ to reverse multi-drug resistance in treatment of this cancer [40].

**Conclusions**

Flavonoids, like other types of dietary chemopreventive agents, exhibit a wide range of potentially useful activities for cancer prevention. Their blocking activities include antioxidant effects, modulation of drug metabolising enzymes and multidrug resistant genes. Suppressing activities include inhibition of signalling pathways responsible for cell proliferation and survival, and induction of apoptosis, mainly through intrinsic pathways involving Bcl family members, mitochondrial membrane depolarisation, cytochrome c release and activation of caspases. They can also induce cell cycle arrest by modulating key components of cell cycle regulation, including cyclins, cyclin dependent kinases and inhibitors.

**References**


7. Zhang S, Qin CH, Safe SH. Flavonoids as aryl hydrocarbon receptor agonists/antagonists: effects of structure and cell-context. Env Health Persp 2003;111:1877–82.


3.2. Biomarkers of dietary polyphenol intake for studying diet-cancer relationships

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Division of Clinical Epidemiology, German Cancer Research Centre, Heidelberg, Germany

Introduction

The primary aim of analytical cancer epidemiology is to detect associations between exposure variables and disease endpoints, associations that are accompanied and supported by basic research findings enabling one to identify and understand insofar as possible the steps contained in the causal chain of disease development. Nutritional epidemiology deals with dietary factors that are difficult to assess. Long-term dietary habits are usually explored in epidemiological studies by use of food frequency questionnaires (FFQ). Dietary measurements tend to suffer, however, from imprecision, particularly concerning dietary components provided by only certain kinds of foods, and components for which bioavailability is low, such as various secondary plant products. The use of biomarkers for such compounds should overcome some of the methodological problems in nutritional epidemiology just referred to. The analytical data obtained are objective and more precise, measurement error being independent of that contained in the corresponding questionnaire data. The validity and reproducibility of nutritional biomarker measurements, however, needs to be demonstrated in advance of their use in epidemiological and etiological studies [1].

Polyphenols

Polyphenols are provided by plant-derived foods, including beverages. The basic flavonoid structure and various examples of them are given in Figure 3.1. Some of the polyphenols are found in a rather wide variety of foods (kaempferol contained in many vegetables, for example), whereas others are limited to only a few kinds of food (such as apigenin found in parsley and celery). Flavonoids are classified in several different sub-groups, these including the flavonols, flavones, flavanols, flavanones, isoflavones, lignans, anthocyanins and proanthocyanins; phenolic acids consist of two main subgroups, the hydroxybenzoic and the hydroxycinnamic acids.

Estimates of dietary intake estimations are hampered by incomplete or missing data in food composition tables. Even more problematic, is the fact that the polyphenols markedly differ from one another in their bioavailability and intestinal metabolism. Current evidence from bioavailability studies suggests that the bioavailability varies from about 0.5 to 43% (based on urinary excretion) of the dose administered, reaching plasma concentrations of 0–4 µmol/l at an intake of 50 mg aglycone equivalents [2]. Bioavailability is determined by different factors, the sugar moiety of the compound in question and its further metabolism by the gut microflora, for example. Isoflavones and gallic acid are polyphenols that are absorbed to the highest extent, followed
by the catechins, flavanones and quercetin glucosides, although the kinetics involved
differ considerably. The proanthocyanidins, the galloylated tea catechins and the antho-
cyanins are much less available. Thus far, data on the phenolic acids are very limited.
Flavonoids undergo extensive first-pass phase II metabolism in the intestinal epithelial
cells and the liver, their being substrates for methylation, sulfation, and glucuronidation.
For many of the polyphenols, their half-life time in the plasma is short, concentrations
of them reaching baseline levels within 24 hours [3]. The steady-state levels of the plasma
polyphenols should only be achievable through regular intake of the foods in question.
The plasma concentrations present in free-living (fasting-status) subjects are even lower
than the abovementioned range obtained after intervention. Measurement of the
compounds found in urine samples, ideally 24-hour samples, is a promising approach,
due mainly due to the higher concentrations of polyphenols — at least after extraction
and enrichment — as compared with plasma samples. The polyphenol concentrations
contained in biological specimens are estimated in most studies after hydrolysis
of the glycosides involved, the aglycones being quantified.

Fig. 3.1. The basic structure of flavonoids and the chemical structure of selected polyphenols.
Laboratory techniques

Two recent reviews provide an overview of the analytical methods used to determine the structure and content of the flavonoids and phenolic acids contained in foods and in food-based matrices [4,5]. Although in principle these methods can be applied to human specimens, the clean-up procedures for this type of samples may be more complicated than required for many food systems.

Hydrolysis

In foods, the flavonoids are usually glycosylated and the phenolic acids are ester-bound. Hydrolysis (acidic or enzymatic) is frequently used to simplify the analytical procedure, the respective aglycones and acids being subsequently detected and quantified. In biological specimens, flavonoids mainly undergo modification by means of phase II enzymes leading to methylated, sulfated and glucuronidated compounds. Accordingly, frequent use is made of enzymic hydrolysis by means of sulfatase and glucuronidase unless a study aims at determining the exact metabolites. Phenolic acids undergo further metabolism and degradation, although a part of them is excreted unmodified.

Clean-up procedures

For human specimens, such as plasma, serum, or urine samples, solid phase extraction (SPE)-columns provide the most convenient solution for removing from a sample any matrix compounds that would disturb the analysis. Some techniques such as liquid chromatography-mass spectrometry (LC-MS), do require only minor sample-preparation steps.

Separation and detection systems

The two major separation techniques for the quantification of polyphenolics are HPLC and GC, combined with different detection systems (Table 3.3.), although the use of LC-MS is becoming increasingly common. For the flavonoids, HPLC has become the method of choice, and the phenolic acids are generally quantified by means of GC after derivatisation. Identification of compounds by means of mass fragmentation is used as a gold standard. However, a single mass-selective detector often fails to fulfil the requirements for sensitivity. Thus, new developments include the creation of HPLC-ESI-MS-MS systems and similarly coupled devices. For the structural characterisation of compounds, mass spectrometric techniques need to be used.

The availability of antibodies for isoflavones and lignans has enabled the antibody-based assays with a high degree of sensitivity to be developed [6]. For the purpose of quantification, the fluorescence emitted is recorded by means of a plate reader, with the option of time-resolved measurement. Thus far, the scientific literature contains no report on use of the metabolomics techniques (NMR, LC-MS) to characterise clusters of polyphenolic compounds that can potentially be used as biomarkers.
Bioavailability studies and other short-term interventional studies

A report was published very recently summarizing all scientific studies (n = 97) that has been conducted thus far on the bioavailability of polyphenols [7]. Most of the studies concerned only one or some few compounds within a given subclass of polyphenols. Kinetic data from the experiments in question are summarized in Table 3.4. (according to [7]). Relatively low plasma concentrations were obtained in each case, even after the administration of polyphenol preparations or polyphenol-rich food corresponding to 50 mg aglycone equivalents. Differences between the various compounds and of classes of polyphenol are striking nevertheless.

Table 3.3. Principal techniques and detection systems used for the identification and quantification of flavonoids and phenolic acids in human specimens (metabonomics/NMR excluded)

<table>
<thead>
<tr>
<th>Separation</th>
<th>Detection system</th>
<th>Substances class technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>UV/VIS spectroscopy (Diode array detection)</td>
<td>Flavonoids, Phenolic acids</td>
</tr>
<tr>
<td></td>
<td>Mass spectrometry</td>
<td>Flavonoids, Phenolic acids</td>
</tr>
<tr>
<td></td>
<td>Electrochemical</td>
<td>Flavonoids, Phenolic acids</td>
</tr>
<tr>
<td></td>
<td>Fluorometric</td>
<td>Flavonoids, Phenolic acids</td>
</tr>
<tr>
<td>GC</td>
<td>Mass spectrometry</td>
<td>Phenolic acids</td>
</tr>
<tr>
<td>LC</td>
<td>Mass spectrometry</td>
<td>Flavonoids, Phenolic acids</td>
</tr>
<tr>
<td>Immunoassays</td>
<td>Time-resolved fluorescence</td>
<td>Isoflavones, lignans</td>
</tr>
</tbody>
</table>


Table 3.4. Pharmacokinetic data from 97 studies concerning the bioavailability of polyphenols¹ (according to Manach et al. [7])

<table>
<thead>
<tr>
<th></th>
<th>$T_{\text{max}}$ (h)</th>
<th>$C_{\text{max}}$ (µmol/l)</th>
<th>Urinary excretion² (% of intake)</th>
<th>Elimination half-life (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SEM)</td>
<td>Range</td>
<td>Mean(SEM)</td>
<td>Range</td>
</tr>
<tr>
<td>Daidzin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.3 (0.6)</td>
<td>4.0–9.0</td>
<td>1.92 (0.25)</td>
<td>0.36–3.14</td>
</tr>
<tr>
<td>Daidzein</td>
<td>4.9 (1.0)</td>
<td>3.0–6.6</td>
<td>1.57 (0.52)</td>
<td>0.76–3.00</td>
</tr>
<tr>
<td>Genistin</td>
<td>6.5 (0.6)</td>
<td>4.4–9.3</td>
<td>1.84 (0.27)</td>
<td>0.46–4.04</td>
</tr>
<tr>
<td>Genistein</td>
<td>4.1 (0.6)</td>
<td>3.0–5.2</td>
<td>2.56 (1.00)</td>
<td>1.26–4.50</td>
</tr>
<tr>
<td>Glycitin</td>
<td>5.0</td>
<td>1.88 (0.38)</td>
<td>1.50–2.26</td>
<td>42.9 (12.0)</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>5.5 (0.1)</td>
<td>5.4–5.8</td>
<td>0.46 (0.21)</td>
<td>0.21–0.87</td>
</tr>
</tbody>
</table>
Table 3.4. Pharmacokinetic data from 97 studies concerning the bioavailability of polyphenols (according to Manach et al. [7]) — cont.

<table>
<thead>
<tr>
<th>Compound</th>
<th>(T_{\text{max}}) (h)</th>
<th>(C_{\text{max}}) (µmol/l)</th>
<th>Urinary excretion (^1) (% of intake)</th>
<th>Elimination half-life (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SEM)</td>
<td>Range</td>
<td>Mean(SEM)</td>
<td>Range</td>
</tr>
<tr>
<td>Naringin</td>
<td>5.0 (0.1)</td>
<td>4.6–5.5</td>
<td>0.50 (0.33)</td>
<td>0.13–1.50</td>
</tr>
<tr>
<td>Quercetin gluc.</td>
<td>1.1 (0.3)</td>
<td>0.5–2.9</td>
<td>1.46 (0.45)</td>
<td>0.51–3.80</td>
</tr>
<tr>
<td>Rutin</td>
<td>6.5 (0.7)</td>
<td>4.3–9.3</td>
<td>0.20 (0.06)</td>
<td>0.09–0.52</td>
</tr>
<tr>
<td>(Epi)catechin</td>
<td>1.8 (0.1)</td>
<td>0.5–2.5</td>
<td>0.40 (0.09)</td>
<td>0.09–1.10</td>
</tr>
<tr>
<td>EGC</td>
<td>1.4 (0.1)</td>
<td>0.5–2.0</td>
<td>1.10 (0.40)</td>
<td>0.30–2.70</td>
</tr>
<tr>
<td>EGCG</td>
<td>2.3 (0.2)</td>
<td>1.6–3.2</td>
<td>0.12 (0.03)</td>
<td>0.03–0.38</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>1.6 (0.2)</td>
<td>1.3–1.5</td>
<td>4.00 (0.57)</td>
<td>2.57–4.70</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>1.0</td>
<td>0.26</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>1.4 (0.6)</td>
<td>0.7–2.0</td>
<td>0.96 (0.26)</td>
<td>0.45–1.35</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>2.0</td>
<td>0.03</td>
<td>27.6 (17.6)</td>
<td>3.1–61.7</td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>1.5 (0.4)</td>
<td>0.7–4.0</td>
<td>0.03 (0.02)</td>
<td>0.001–0.20</td>
</tr>
<tr>
<td>Proanthocyanidins</td>
<td>2.0</td>
<td>0.02 (0.01)</td>
<td>0.008–0.03</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) All the data were converted so as to correspond to a supply of 50 mg aglycone equivalent.
\(^2\) Usually represent 24-h urine samples.

A review of short-term intervention studies (n = 93) in which polyphenols were administered (either as isolated compounds or in the form of foods or food extracts) to human subjects was published recently [8]. The authors examined evidence relating to the effects of these polyphenols on biomarkers used to test various pathophysio logically relevant hypotheses in vivo.

**Observational studies: cross-sectional studies and studies related to cancer**

Various studies have dealt with the suitability of using fasting plasma or urinary concentrations of polyphenols (mainly flavonols, flavanones or isoflavones) as biomarkers of polyphenol intake [9–16]. The results of these studies as a whole suggest biomarker measurements of this sort to reflect in an adequate way the degree of short-term intake of the polyphenols that were investigated, although one study in particular failed to support this conclusion [12]. The magnitude of the variation in the plasma polyphenol concentrations found between free-living subjects following their usual diet habits was
found to be rather high (Table 3.5.), the intra-individual variation also being high [14].
The correlation coefficients between estimates of the dietary intake of polyphenols of the day before blood sampling took place and fasting plasma concentrations polyphenols were as high as 0.75, but it should be emphasized that the validity of such correlations may be limited by a lack of precision in the estimates of dietary polyphenol intake, and that the correlations are much lower when intake calculations are based on data obtained either three or seven days prior to blood sampling. Due to the short half-life time of most polyphenols, close to of steady-state plasma concentrations of them can only be achieved if the substance in question is consumed regularly, a precondition most likely to be fulfilled by compounds such as kaempferol that are widely distributed in plant foods, and if the bioavailability of the compound is not particularly low.

Table 3.5. Concentrations (nmol/l) of selected flavonoids and phenolic acids in plasma samples of 41 men on their usual diet, participating in a cross-sectional study (according to Bolarinwa and Linseisen [16].

<table>
<thead>
<tr>
<th>Compound</th>
<th>Median</th>
<th>Mean (SD)</th>
<th>Min. (nmol/l)</th>
<th>Max.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallocatechin</td>
<td>68.6</td>
<td>91.2 (17.1)</td>
<td>0.0ª</td>
<td>561.9</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>413.0</td>
<td>520.8 (77.2)</td>
<td>48.0</td>
<td>2641.9</td>
</tr>
<tr>
<td>Epigallocatechin</td>
<td>742.8</td>
<td>1031.3 (153.4)</td>
<td>0.0ª</td>
<td>4528.3</td>
</tr>
<tr>
<td>Catechin</td>
<td>82.1</td>
<td>107.8 (15.6)</td>
<td>0.0ª</td>
<td>388.1</td>
</tr>
<tr>
<td>Gentisinic acid</td>
<td>1357.8</td>
<td>2160.6 (275.9)</td>
<td>0.0ª</td>
<td>6849.7</td>
</tr>
<tr>
<td>Epigallocatechingallate</td>
<td>158.9</td>
<td>267.7 (48.6)</td>
<td>0.0ª</td>
<td>1348.7</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>372.4</td>
<td>477.3 (67.2)</td>
<td>0.0ª</td>
<td>2290.8</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>159.5</td>
<td>260.3 (42.7)</td>
<td>0.0ª</td>
<td>1121.6</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>114.0</td>
<td>277.7 (78.5)</td>
<td>0.0ª</td>
<td>2542.9</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>478.3</td>
<td>908.1 (203.5)</td>
<td>0.0ª</td>
<td>7103.0</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>644.6</td>
<td>1304.6 (254.3)</td>
<td>0.0ª</td>
<td>6103.4</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>460.6</td>
<td>652.1 (94.6)</td>
<td>58.8</td>
<td>2904.4</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>339.1</td>
<td>6990.0 (3077.9)</td>
<td>26.2</td>
<td>104468.3</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>53.8</td>
<td>140.1 (41.4)</td>
<td>0.0ª</td>
<td>1253.4</td>
</tr>
<tr>
<td>Daidzein</td>
<td>0.0ª</td>
<td>10.1 (2.8)</td>
<td>0.0ª</td>
<td>59.9</td>
</tr>
<tr>
<td>Quercetin</td>
<td>78.5</td>
<td>108.7 (15.9)</td>
<td>19.7</td>
<td>563.8</td>
</tr>
</tbody>
</table>
The urinary excretion of polyphenols in subjects on habitual diets was also shown to be correlated significantly with estimates of the short-term of fruits and vegetables, the correlation coefficients for selected flavonols and flavanones being between 0.28 and 0.38 [13]. The urinary polyphenol excretion rates show a high degree of variability, just as was indicated already for the plasma concentrations. To give an example of the polyphenol concentrations found in 24-h urine samples of subjects on a habitual diet, Nielsen and coworkers [13] reported average (SD) concentrations of quercetin, kaempferol, naringenin, phloretin, and total flavonoids as being 25(23), 50(32), 701(659), 76(110), 1638(1316) µg/24 h, respectively.

Plasma and urinary polyphenol concentrations are not expected to reflect long-term or habitual dietary intake, although this has not been investigated extensively. One study reported correlation coefficients of between 0.24 and 0.74 for plasma isoflavone concentrations, the dietary intake estimates being based on FFQ data [11].

In large-scale epidemiologic (etiological) studies of disease-related effects of dietary polyphenol levels, little use has been made of biomarker measurements. Hertog and colleagues were the first to analyze commonly consumed foods in terms of their flavonol and flavone content by means of HPLC. Their work provided the basis for the estimation of dietary flavonol and flavone intake. In the following, several studies on associations with the risk of cardiovascular diseases and with cancer at different sites, some of which appear very promising, are taken up. Only few studies concerning the use of biomarkers of polyphenol intake were found to be available (Table 3.6), except as regards the intake of phytoestrogens (isoflavones and lignans).

### Table 3.5. Concentrations (nmol/l) of selected flavonoids and phenolic acids in plasma samples of 41 men on their usual diet, participating in a cross-sectional study (according to Bolarinwa and Linseisen [16] — cont.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Median</th>
<th>Mean (SD)</th>
<th>Min.</th>
<th>Max.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(nmol/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naringenin</td>
<td>79.7</td>
<td>122.5 (21.7)</td>
<td>0.0⁺</td>
<td>533.1</td>
</tr>
<tr>
<td>Luteolin</td>
<td>388.0</td>
<td>545.8 (80.8)</td>
<td>0.0⁺</td>
<td>2555.2</td>
</tr>
<tr>
<td>Genistein</td>
<td>108.1</td>
<td>157.1 (24.1)</td>
<td>0.0⁺</td>
<td>639.7</td>
</tr>
<tr>
<td>Hesperetin</td>
<td>27.6</td>
<td>37.2 (4.9)</td>
<td>0.0⁺</td>
<td>151.7</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>36.8</td>
<td>126.1 (40.4)</td>
<td>0.0⁺</td>
<td>1356.8</td>
</tr>
<tr>
<td>Apigenin</td>
<td>5.3</td>
<td>9.3 (1.9)</td>
<td>0.0⁺</td>
<td>52.5</td>
</tr>
<tr>
<td>Isorhamnetin</td>
<td>14.2</td>
<td>31.0 (7.3)</td>
<td>0.0⁺</td>
<td>204.8</td>
</tr>
</tbody>
</table>

* Below the detection limit.
Table 3.6. Epidemiologic (observational) studies of the risk of cancer in which biomarkers of dietary polyphenol intake (other than that of isoflavones and lignans) were employed

<table>
<thead>
<tr>
<th>Author</th>
<th>Type</th>
<th>N, cases/controls</th>
<th>Specimen; Flavonoid</th>
<th>Cancer site</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dai et al. 2002 [17]</td>
<td>CCS</td>
<td>250/250</td>
<td>Urine; citrus flavonoids</td>
<td>Breast</td>
<td>NS</td>
</tr>
<tr>
<td>Zheng et al. 1999 [18]</td>
<td>CCS</td>
<td>60/60</td>
<td>Urine; total phenols</td>
<td>Breast</td>
<td>NS</td>
</tr>
<tr>
<td>Sun et al. 2002 [19]</td>
<td>Cohort</td>
<td>232/772</td>
<td>Urine; tea polyphenols</td>
<td>Gastric and esophageal</td>
<td>Significant inverse association</td>
</tr>
</tbody>
</table>

CCS — case-control study; NS — not significant.

One of the major reasons for the frequent use of biomarkers of phytoestrogen intake in epidemiological studies (see Table 3.7.) may be the ready availability of appropriate immunoassays suitable for measurements on large numbers of samples, enabling the attainment of the requirement of sufficient statistical power.

Table 3.7. Epidemiological studies of the risk of breast cancer risk in which biomarkers of dietary isoflavone and mammalian lignans intake were employed.

<table>
<thead>
<tr>
<th>Author</th>
<th>Type</th>
<th>N, cases/controls</th>
<th>Specimen; Phytoestrogen</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingram et al. [21]</td>
<td>CCS</td>
<td>144/144</td>
<td>Urine; equol, enterolactone</td>
<td>Significant inverse association</td>
</tr>
<tr>
<td>Zheng et al. [18]</td>
<td>CCS</td>
<td>60/60</td>
<td>Urine; isoflavones</td>
<td>NS</td>
</tr>
<tr>
<td>Murkies et al. [22]</td>
<td>CCS</td>
<td>18/20</td>
<td>Urine; isoflavones</td>
<td>Significant inverse association</td>
</tr>
<tr>
<td>Dai et al. [17]</td>
<td>CCS</td>
<td>250/250</td>
<td>Urine; isoflavones, lignans</td>
<td>Significant inverse association</td>
</tr>
<tr>
<td>Piller et al. [23]</td>
<td>CCS</td>
<td>220/237</td>
<td>Plasma; enterolactone</td>
<td>Significant inverse association</td>
</tr>
<tr>
<td>Pietinen et al. [24]</td>
<td>CCS</td>
<td>194/208</td>
<td>Serum; enterolactone</td>
<td>Significant inverse association</td>
</tr>
<tr>
<td>den Tonkelaar et al. [25]</td>
<td>Cohort: nested CCS</td>
<td>88/268</td>
<td>Urine; genistein, enterolactone</td>
<td>NS</td>
</tr>
<tr>
<td>Hulten et al. [26]</td>
<td>Cohort: nested CCS</td>
<td>248/492</td>
<td>Plasma; enterolactone for highest and lowest categories</td>
<td>Significant positive association</td>
</tr>
<tr>
<td>Grace et al. [27]</td>
<td>Cohort: nested CCS</td>
<td>97/187</td>
<td>Serum, Urine; isoflavones, lignans</td>
<td>Higher risk when isoflavone estimates are higher</td>
</tr>
</tbody>
</table>

CCS — case-control study; NS — not significant.
In view of the usually rather low sample volumes available in epidemiologic studies, the analysis of polyphenol content is restricted in most cases to only a few compounds or classes of compounds. Accounts of analytical procedures that permit a wide variety of polyphenols to be determined in a single run were published recently [16,20].

**Specificity and sensitivity**

In applying mass-selective detection methods at least to standard mixtures or biological specimens spiked with the compound in question, the concentrations were found to be high enough to enable the characteristic mass fragments to be identified. This differs from other methods, such as HPLC-UV/VIS, ECD (electron capture detection), or use of fluorescence detectors, in which peak identity cannot be confirmed with sufficient certainty. Antibody-based assays are also subject to failures in specificity due to cross-reactions with other matrix compounds. For all these methods, confirmation of their results by use of MS-based techniques is necessary. MS-based systems also have their problems in connection with concentrations close to the detection limit, however, where the characteristic fragments may be absent.

Sensitivity is a major issue with regard to analytical methods for determining polyphenols in biological specimens. In epidemiological studies in particular, in which the sample volumes available are usually very small, the degree of sensitivity a method provides can be decisive for whether it can be used or not (along with other factors, such as analytical time and costs). Detection limits very close to 1 nmol/l of polyphenols have been found for techniques involving HPLC-ECD, HPLC-MS-MS, and TR-FIA (immunoassay). The sensitivity achievable with HPLC-MS, LC-MS, GC-MS and HPLC with use of fluorometric detection is slightly lower.

**Reproducibility, validity and reliability**

For each well-developed method, satisfactory figures concerning the analytical precision and accuracy are available. The higher values here are those for the MS-based techniques (coefficient of variation < 5–7%, recovery 90–105%), the immuno-assays being located at the other end of the scale, having coefficients of variation close to or above 10% and recovery rates frequently at < 90%. However, working at the detection limit of a method is always a challenge, and reports on the quality of the method in question are usually obtained clearly above the detection limit. As already mentioned, MS-based systems also have their problems when the concentrations involved are very low. To the best of our knowledge, except for some few small studies [14], no systematic findings on repeated within-subject samples or on losses during sample storage are available.
References

3.3. Anticarcinogenic compounds of olive oil and related biomarkers

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Institute of Biological Research and Biotechnology, The National Hellenic Research Foundation, Athens, Greece

Olive oil is an important ingredient of the Mediterranean diet. Epidemiological studies demonstrate rather conclusively that populations within Europe consuming this diet have a particularly low incidence of a number of common cancers [1,2]. A plethora of minor constituents in olive oil have been identified as being effective agents mitigating against the initiation, promotion and progression of multistage carcinogenesis. These include toco-pherol and carotenoid antioxidants, that have been thoroughly studied, a number of simple and bound phenolics (tyrosol, hydroxytyrosol, secoiridoids and lignans), the triterpene hydrocarbon squalene and the phytosterol β-sitosterol [1–3]. The occurrence of these constituents also calls for the development of specific nutritional biomarkers that reflect the nutritional status of these dietary constituents with respect to their intake or metabolism and that can provide information useful for nutritional epidemiology regarding the effects of disease processes that can occur [4]. A brief overview is presented of recent findings concerning the bioavailability of certain minor but important olive oil minor components (polyphenols, lignans, squalene and β-sitosterol), considered as putative nutritional biomarkers in relation to cancer the incidence of cancer.

Phenolic compounds

HPLC chromatography of the methanol extract of virgin olive oil reveals seven major polyphenol peaks corresponding to hydroxytyrosol, tyrosol, oleuropein, the aglycone of ligstroside, two secoiridoids (dialdehydes related to oleuropein and ligstroside but lacking the carboxymethyl group at C4), and a peak containing the lignans (+)-1-acetoxy-pinoresinol and (+)-pinoresinol [1–3] (Table 3.8., Figure 3.2.). Oleuropein and its metabolites tyrosol and hydroxytyrosol, which represent major antioxidants in olive oil, are dose-dependently absorbed in humans after the ingestion of realistic doses of virgin olive oil. When olive oil samples containing increasing amounts of a phenolic extract of olive oil were administered to human volunteers, a dose-dependent decrease in the urinary excretion of the F2-isoprostane 8-iso-PGF2α, a biomarker of in vivo lipid peroxidation processes, was observed. This indicates olive oil phenolics to maintain their antioxidant activities in vivo. It has also been shown that olive oil phenolics are excreted in the urine as glucuronide conjugates and that the urinary free tyrosol concentration is responsive to the dietary intake of virgin olive oil. In addition, a statistically significant negative correlation has been found between homovanillyl alcohol (HValc, a major metabolite of hydroxytyrosol, together with homovanillic acid — HVA) and isoprostane excretion, the excretion of both HValc and HVA also being significantly correlated with the dose of administered hydroxytyrosol. Thus, HValc in urine reflects the in vivo...
Bioactive components in foods: Anticarcinogenic compounds of olive oil and related biomarkers

collection of hydroxytyrosol [5–11]. After the ingestion of olive oil of low phenolic content plasma glutathione peroxidase activity was found to decrease postprandially, but this was not observed after the intake of olive oils of moderate to high phenolic content [12]. An HPLC method for the simultaneous determination of oleuropein and of its metabolites hydroxytyrosol and tyrosol in human plasma has been developed [13,14].

### Table 3.8. Concentrations of the major phenolic compounds found in virgin olive oil

<table>
<thead>
<tr>
<th>Compounds</th>
<th>mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosol</td>
<td>27.45 [2], 2.65–4.75 [3]</td>
</tr>
<tr>
<td>Oleuropein aglycone</td>
<td>103–205 [3]</td>
</tr>
<tr>
<td>Total secoiridoids</td>
<td>27.72 [2]</td>
</tr>
<tr>
<td>Lignans</td>
<td>41.53 [2], 38–65 [3]</td>
</tr>
</tbody>
</table>

References [2] and [3].

![Fig. 3.2. Structures of certain phenolic compounds detected in olive oil. Hydroxytyrosol (A), oleuropein aglycone (B), (+)-pinoresinol (C).](image)

Recent findings suggest that olive oil may also affect the bioavailability of other food bioactive components with a chemopreventive potential. It was observed in this respect, that the concentration in human plasma of lycopene, a biomarker of the intake of tomato-rich food and hypothesized to be responsible for reducing the risk of various
cancers, increased dramatically after the consumption of tomatoes cooked in olive oil, as compared to the consumption of tomatoes cooked without olive oil [15]. The consumption of tomato products prepared together with olive oil, but not with sunflower oil, was found to improve the antioxidant activity of plasma [16].

Lignans are plant compounds metabolized in the gut to produce the phytoestrogens enterolactone and enterodiol. Phytoestrogens have an anticarcinogenic potential through the anti-estrogenic, anti-angiogenic, proapoptotic and anti-oxidant mechanisms established for them [17,18]. Recent findings suggest that enterolactone is more rapidly metabolized in human colon epithelial cells and/or excreted by them than enterodiol is, that the phase II metabolism of enterolactone and enterodiol already may take place during their uptake in the colon, and that the epithelial cells in the colon may be responsible for this metabolism [19]. Mean residence times and elimination half-lives that have been obtained indicate that enterolignans accumulate in the plasma when consumed 2–3 times a day, their reaching a steady state. Plasma enterolignan concentrations can thus be considered to be good biomarkers of dietary lignan exposure and be used to evaluate the effects of lignans [20]. A number of in vitro and animal studies support a role for lignan-rich foods and of purified lignans in the modulation of cancer events in the breast, the prostate and the colon, whereas the findings of epidemiological studies are controversial [18]. Nevertheless, a tendency for a lower risk of breast cancer to be associated with higher plasma concentrations of enterolactone, restricted almost entirely to estrogen-receptor alpha negative breast cancer has been found, suggesting that dietary lignans may be important in the etiology of breast cancer, particularly in premenopausal women [21].

**Squalene**

It has been suggested that the lower risk of cancers of various types associated with high olive oil consumption (as compared with other human foods) may be due to the presence of squalene (reviewed in [1]). This triterpene hydrocarbon is found mainly in nonedible shark liver oil, while virgin olive oil is a major source of phytosqualene, its content ranging from 800 to 12,000 mg/kg. If virgin olive oil were the sole source of dietary fat, the squalene intake would be more than 200 mg/d [22]. Nevertheless, very little is known concerning the postprandial metabolism of squalene. It has been observed that postprandial squalene metabolism is age dependent [23], and that the content of squalene in the whole plasma and in the lipoprotein fractions (where its ratio to cholesterol is highest in the VLDL and the intermediate density lipoproteins [24]) varies directly with the triglyceride content and is increased in hypertriglyceridemia, which expands the plasma pool of this metabolically active hydrocarbon [25]. Experiments in vitro and animal models suggest squalene to play a tumour-inhibiting role, which is most probably based on its strong inhibitory action on the catalytic activity of beta-hydroxy-beta-methylglutaryl-CoA reductase, leading to a reduced farnesyl pyrophosphate availability for prenylation of the ras oncogene [26]. Although animal studies have enhanced our understanding of the possible
action of squalene in decreasing carcinogenesis, one should be cautious in extrapolating findings there to humans, both because of possible species differences and because the long-term effects of greater consumption of squalene are unknown. Several factors must be taken into account when examining the evidence for squalene’s inhibition of carcinogenesis factors, such as the effective dose used and exposure time [27]. At present, therefore, its use as a nutritional biomarker is hardly to be considered.

Phytosterols

Phytosterols are plant sterols that are structurally similar to cholesterol and that possess anticarcinogenic properties [27]. Together with squalene, they represent markers of cholesterol synthesis and absorption and are transported together with cholesterol in serum lipoproteins [24]. β-Sitosterol, one of the most common phytosterols and the main olive oil sterol [1], together with campesterol are the two predominant phytosterols in the blood. It has been suggested that the high reproducibility and high reliability over time (consistency of the plasma phytosterol level over time) of the plasma measurements of these sterols makes them suitable for clinical and population-based studies of cancer prevention [28]. In recent years, functional foods high in phytosterol-ester content for lowering the cholesterol level have been developed. Although phytosterols act as immune modulators and anticancer agents in vitro [29], the protection (if any) that high concentrations of phytosterol provide against the development of cancer in humans has not been adequately examined, further study of this being needed.

Conclusions

Since the phenolic content of the olive oil consumed may account for the postprandial antioxidant activity in vivo after the ingestion olive oils of moderate to high phenolic content, we suggest that these biomolecules, or certain polyphenol metabolites in human plasma and urine, can serve as practical biomarkers for olive oil consumption and as an alternative biomarker for future epidemiological studies in dietary cancer prevention and health promotion.

References


3.4. Anticarcinogenic effects of glucosinolate breakdown products

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Glucosinolates and their association with cancer chemoprevention

Epidemiological studies have revealed that regular consumption of cruciferous vegetables, such as broccoli, Brussels sprouts, cabbage, cauliflower, kale, swede and turnip, is associated with a reduced incidence of cancer [1,2]. Furthermore, greater health benefit may be obtained from raw as opposed to cooked vegetables [3]. The types of neoplastic disease in man that these vegetables appear to protect against include colorectal cancer [4], lung cancer [5], and possibly prostate cancer [6]. Feeding experiments in animals have also suggested broccoli can protect against liver cancer [7]. Cruciferous vegetables uniquely contain glucosinolates at approximately 20 \( \mu \)mol/g dry mass of vegetable [8,9], and it is thought that these phytochemicals are primarily responsible for the putative cancer chemoprevention conferred by eating diets that contain significant quantities of these vegetables [10,11].

Glucosinolates are substituted \( \beta \)-thioglucoside N-hydroxysulfates, formed by the plant from any one of eight amino acids, namely, alanine, valine, leucine, isoleucine, phenylalanine, methionine, tyrosine and tryptophan [2]. Over 115 naturally occurring glucosinolates have been identified. Each cruciferous vegetable contains a mixture of glucosinolates that varies according to the strain of the plant [8,12–15]. The glucosinolate content is primarily under genetic control, though it can be influenced by environmental factors [16,17]. Much of the diversity amongst glucosinolates arises from the addition of different sized alkyl groups to the side chain of those amino acids, principally valine, phenylalanine and methionine, used in their biosynthesis; this variable elongation of amino acid side chains entails repetitive additions of methyl groups through a series of transamination, condensation, isomerisation and decarboxylation reactions [18]. As shown in Figure 3.3., the synthesis of glucosinolates proceeds through the conversion of elongated amino acids to their oxime derivatives, catalysed by members of the cytochrome P450 (CYP) 79 family [19]. Subsequently, the oxime is metabolised to a thiohydroximate, which is in turn conjugated with glucuronic acid to form a desulfoglucosinolate before finally being sulfated to yield the glucosinolate [2].

The task of establishing a link between the ingestion of particular glucosinolates and their possible health benefits is not straightforward. This endeavour is simplified to some extent by the fact that relatively few glucosinolates are present in the human diet. The most common of these are the methylsulfinylalkyl glucosinolates glucoiberin and glucoraphanin, the olefinic glucosinolates sinigrin, gluconapin, glucobrassicanapin and progoitrin, and the aromatic glucosinolate gluconasturtiin (Table 3.9.) [9,20]. Glucoraphanin has been reported to be abundant in broccoli [9], though certain broccoli...
strains also contain substantial amounts of glucoiberin [21]. Sinigrin has been reported to be the predominant glucosinolate in Brussels sprouts, cabbage, cauliflower and kale [9]; gluconapin is also found in high levels in Brussels sprouts [9]. Substantial amounts of progoitrin are present in many cruciferous vegetables [9]. The aromatic glucosinolate gluconasturtiin is present in watercress. The indolyl glucosinolate glucobrassicin is present in Savoy cabbage, Brussels sprouts and cauliflower [9,22], and whilst not abundant it can elicit distinct pharmacological effects.

Table 3.9. Trivial names of some glucosinolates with the corresponding side-chain (R) composition

<table>
<thead>
<tr>
<th>Name</th>
<th>R side-chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sinigrin</td>
<td>2-Propenyl</td>
</tr>
<tr>
<td>Gluconapin</td>
<td>3-Butenyl</td>
</tr>
<tr>
<td>Glucobrassicin</td>
<td>3-Indolymethyl</td>
</tr>
<tr>
<td>Glucobrassicinapin</td>
<td>4-Pentenyl</td>
</tr>
<tr>
<td>Progoitrin</td>
<td>2-Hydroxy-3-butenyl</td>
</tr>
<tr>
<td>Glucoiberin</td>
<td>3-Methylsulfinylpropyl</td>
</tr>
<tr>
<td>Gluconapoleiferin</td>
<td>2-Hydroxy-4-pentenyl</td>
</tr>
<tr>
<td>Glucocheirolin</td>
<td>3-Methylsulfonylpropyl</td>
</tr>
<tr>
<td>Glucoerucin</td>
<td>4-Methylthiobutyl</td>
</tr>
<tr>
<td>Glucoberteroin</td>
<td>5-Methylthiopentyl</td>
</tr>
</tbody>
</table>

Fig. 3.3. Synthesis of glucosinolates. The R group is derived from the original amino acid and is highly variable.
Production of isothiocyanates, thiocyanates, nitriles, cyano-epithioalkanes and oxazolidine-2-thiones from glucosinolates

Evidence suggests that inhibition of carcinogenesis by glucosinolates is not primarily attributable to this class of compound, but rather it appears to be due to certain of their breakdown products. Hydrolysis of these phytochemicals is catalysed by myrosinase (β-thioglucoside glucohydrolase, EC 3.2.3.147), an enzyme that is physically segregated from glucosinolates within the intact plant by virtue of the fact that it is sequestered in specialised “myrosin” cells [23]. Upon wounding of the vegetable, for example during harvesting, during freeze-thawing, during food preparation, or during chewing whilst eating, myrosinase is released from the “myrosin” cells and is able to hydrolyse glucosinolates within the damaged plant. In addition, myrosinase activity may be present in human colonic microflora, suggesting that glucosinolates can be hydrolysed in the gastrointestinal tract during digestion of food [24,25]. Myrosinase cleaves glucosinolates at the thioglycoside linkage to produce glucose and an unstable aglycone thiohydroximate-O-sulfonate that spontaneously rearranges to yield several breakdown products. The outcome of the reaction with myrosinase depends on the nature of the aglycone, as well as the reaction temperature, the pH and the presence of ferrous ions (Figure 3.4A. and 3.4B.).

![Figure 3.4A. Hydrolysis of glucosinolates. At high or neutral pH the formation of isothiocyanates is favoured while at low pH the formation of nitriles is favoured. Epithiospecifier protein (ESP) in the presence of Fe^{2+} ions interacts with myrosinase to promote the transfer of the sulfur to the alkenyl group from the S-Glucose of the terminally unsaturated glucosinolate, resulting in the formation of an epithioalkane.](image-url)
Bioactive components in foods: Anticarcinogenic effects of glucosinolate breakdown products

ESP — epithiospecifier protein.

Fig. 3.4B. Hydrolysis of sinigrin. Following damage to the plant tissue the glucosinolate sinigrin is hydrolysed by myrosinase resulting in the formation of four distinct compounds. On the right-hand side of the figure, an arrow shows that allyl thiocyanate, formed from sinigrin, can convert spontaneously to form allyl isothiocyanate [26].

The thiohydroximate-O-sulfates formed from methylsulfinylalkyl, olefinic and aromatic glucosinolates undergo a Lossen rearrangement, with the elimination of sulfate, to form their respective isothiocyanates (ITCs), thiocyanates or nitriles [10,23]. Certain thiocyanates that are formed during a Lossen rearrangement, such as allyl-ITC, are unstable and can undergo a relatively slow spontaneous conversion to their respective isothiocyanate [26]. Elemental sulfur is also formed in certain circumstances. At neutral pH, hydrolysis of glucosinolates with aliphatic or aromatic side chains gives rise primarily to isothiocyanates (ITCs). The glucosinolates glucoiberin, gluconapin, glucoraphanin, glucobrassicanapin and sinigrin yield 3-methylsulfanylpropyl-ITC, 3-butenyl-ITC, 4-methylsulfanylbutyl-ITC (sulforaphane), 4-pentenyl-ITC and 2-propenyl-ITC (allyl-ITC), respectively.

At low pH, the thiohydroximate-O-sulfates formed by myrosinase from glucosinolates with a side chain containing a double bond (e.g. sinigrin, gluconapin and glucobrassicanapin) may, in the presence of an epithiospecifier protein (ESP) and ferrous ions, give rise to a cyano-epithioalkane [27]. In this case, ESP interacts with myrosinase to promote sulfur transfer from the S-glycosyl unit to the alkenyl chain derived from the amino acid part of the aglycone [28]. Thus, at pH 4 and in the presence of Fe$^{2+}$ ions,
myrosinase and ESP convert sinigrin to 1-cyano-2,3-epithiopropane [29]. Gluconapin can similarly be converted by the combined actions of myrosinase and ESP to 1-cyano-3,4-epithiobutane [30,31]. Likewise, glucobrassicanapin can be hydrolysed to 1-cyano-4,5-epithiopentane [31]. Progoitrin, a (2R)-hydroxy-3-butenyl glucosinolate, is also converted in the presence of myrosinase, ESP and Fe$^{2+}$ ions to an epithionitrile [32] and in the case of epi-progoitrin ((2S)-hydroxy-3-butenyl glucosinolate), it can be hydrolysed by myrosinase to crambene (1-cyano-2-hydroxy-3-butene) [33,34]. Two cDNAs for ESP have recently been cloned from Arabidopsis and broccoli and the purified proteins characterized following their heterologous expression in E. coli [35,36].

If the aglycone generated by myrosinase is from a glucosinolate with a side chain lacking a double bond, the sulfur atom may be lost and a nitrile formed [37–39]. This reaction may involve ESP, and is diminished by heating [40]. A few glucosinolates produce thiocyanates though the mechanism involved is unclear [23]. Upon hydrolysis by myrosinase, those aglycones from glucosinolates that contain β-hydroxylated side-chains form oxazolidine-2-thiones, as a consequence of spontaneous cyclization. Examples of these include progoitrin, glucoconringiin and gluconapoleiferin [2].

**Production of indoles from glucosinolates**

The indolyl glucosinolates glucobrassicin and neoglucobrassicin are synthesised by the plant from tryptophan. The best studied of these is glucobrassicin. At neutral pH, hydrolysis of glucobrassicin by myrosinase does not generate an ITC, but rather gives rise to indole-3-carbinol and a thiocyanate ion (Figure 3.5); this reaction probably proceeds

![Fig. 3.5. Production of indoles from glucosinolates. At neutral pH the hydrolysis of glucobrassicin by myrosinase leads to the formation of an unstable isothiocyanate intermediate which degrades to form indole-3-carbinol and a thiocyanate ion.](image-url)
through a Lossen rearrangement generating an unstable ITC intermediate [22]. At acidic pH, hydrolysis of glucobrassicin yields indole-3-acetonitrile, hydrogen sulfide and elemental sulfur [22]. In the acidic environment of the stomach, indole-3-carbinol condenses to form various compounds including indolo\[3,2-b\]carbazole and 3,3′-diindolylmethane, both of which have potent pharmacological effects [41]. It can also combine with ascorbic acid to form ascorbigen [42] (Figure 3.6.).

![Structures of indoles produced from glucosinolates — 3,3′-Diindolylmethane (DIM), indolo\[3,2-b\]carbazole (ICZ) and ascorbigen (ASG).](image)

**Chemopreventive mechanisms stimulated by glucosinolate hydrolysis products**

In view of the diverse spectrum of chemicals generated from glucosinolates by the actions of myrosinase and ESP, it is not surprising that a number of distinct cancer chemopreventive mechanisms have been proposed to account for the putative anti-cancer properties of cruciferous vegetables. These include induction of antioxidant and detoxifying genes, inhibition of CYP enzyme activity, cell cycle arrest, and stimulation of apoptosis. There is a dose-dependency in these responses: generally, induction of cytoprotective genes and inhibition of CYP activity occurs at relatively low concentrations of phytochemical, whereas activation of cell cycle arrest and apoptosis occurs at higher levels of phytochemical [43,44]. A major problem exists in interpreting experiments utilizing vegetable extracts because of uncertainty about attributing biological effects to specific phytochemicals. A challenge in evaluating the literature arises from the emphasis placed on certain glucosinolate breakdown products and the dearth of data relating to others. Thus, there is a relative abundance of information about isothiocyanates and indoles, when compared with the data about thiocyanates, nitriles, cyanoepithioalkanes, and oxazolidine-2-thiones.

**Induction of gene expression mediated by Nrf2**

Isothiocyanates increase the expression of antioxidant and detoxication proteins, such as glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1), both *in vivo* and *in vitro* [45,46]. Agents such as ITC that increase GST and NQO1
enzymes without increasing arylhydrocarbon hydroxylase activity, catalysed by the phase I drug-metabolising enzyme cytochrome P450 (CYP) 1A1, are sometimes called mono-functional inducers [47]. Sulforaphane, 3-methylsulfinylpropyl-ITC, allyl-ITC, 7-methylsulfinylheptyl-ITC, 8-methylsulfinyloctyl-ITC, benzyl-ITC and phenethyl-ITC, induce NQO1 in the mouse Hepa-1c1c7 hepatoma cell line [48,49] (Figure 3.7.). Many of these compounds induce GST P1-1 in the rat liver RL-34 cells [50]. The mouse nqo1 gene contains a functional antioxidant response element (ARE) in its 5′-upstream region [51], as does the rat GSTP1 gene (called GPEI) [52]. The induction of these two genes by ITCs is mediated by the Nrf2 (Nuclear Factor-Erythroid 2 p45-related factor 2) bZIP (basic-region leucine zipper) transcription factor that is recruited to the ARE as a heterodimer with small Maf protein [51]. Indeed, as far as is known, all genes that are induced by ITCs contain an ARE in their promoters and are regulated by Nrf2.

Fig. 3.7. Structures of isothiocyanates which induce NQO1: 1) Allyl-ITC; 2) Phenethyl-ITC; 3) Benzyl-ITC; 4) 3-methylsulfinylpropyl-ITC; 5) 7-methylsulfinylheptyl-ITC; 6) 8-methylsulfinyloctyl-ITC.

Examination of nrf2−/− and wild-type mice, suggests AREs are present in the promoters of at least 100 genes [53–55]. The battery of genes regulated by Nrf2 includes those encoding aldo-keto reductase (AKR), carboxyl esterase, ferritin, glutamate cysteine ligase catalytic (GCLC) and modifier (GCLM) subunits, GST, heme oxygenase 1, NQO1, metallothionein, microsomal epoxide hydrolase, multidrug resistance-associated protein, thioredoxin, thioredoxin reductase, UDP-glucuronosyl transferase. Many of these genes are induced by sulforaphane in vivo in an Nrf2-dependent fashion in the stomach, small intestine and liver of rodents [53,54,55–58]. Importantly, feeding broccoli seed to mice increased the levels of GCLC, GST and NQO1 in the gastrointestinal tract in an Nrf2-dependent fashion [21]. It is thought that ITCs possess the ability to induce ARE-driven gene expression because they are thiol-active [59]. Through this characteristic, ITCs modify cysteine residues in the Cullin 3:Rbx1 E3 ubiquitin ligase substrate adaptor Keap1, leading to its inhibition and inability to serve as a substrate adaptor.
required for the ubiquitination of Nrf2 under homeostatic conditions [60–62]. Consistent with this view, exposure of RL-34 cells or HepG2 cells to sulforaphane causes stabilisation and rapid accumulation of Nrf2 [63,64]. Surprisingly, allyl-ITC does not increase Nrf2 stability [64] and there may therefore be other factors involved in enzyme induction by these phytochemicals. Treatment of cells with benzyl-ITC causes a rapid increase in the level of reactive oxygen species, and this may also contribute to gene induction [46].

It has been found that administration of cranbene to Fischer 344 rats causes an elevation in hepatic GST and NQO1 enzyme activities, but not CYP1A1, suggesting it is a mono-functional inducer [65]. By comparison with sulforaphane, cranbene was found to be an approximately equally potent inducer in the rat [66]. However, it was not a particularly effective inducer of NQO1 activity in Hepa-1c1c7 cells suggesting that the relatively high potency of induction observed in vivo is due to bio-transformation of cranbene to a thiol-active metabolite. The identity of this metabolite is not known.

The indole-containing glucobrassicin breakdown products can activate gene expression by several mechanisms. Indole-3-carbinol is a modest inducer of Nrf2-dependent ARE-driven gene expression in the liver and small intestine of mice [52,67]. Although indole-3-acetonitrile has not been studied in the Nrf2 knockout mouse, it is a good inducer of GST enzyme activity in mouse liver and small intestine [68], a fact that implies it works through Nrf2.

### Induction of gene expression mediated by AhR

The major effect that indole-3-carbinol has on gene expression occurs because it can condense in acid conditions to form indolo[3,2-b]carbazole and 3,3′-diindolylmethane. Both these condensation products induce CYP1A1 genes via the xenobiotic response element (XRE) in their promoter regions because they are ligands for the Ah receptor. Examination of the dose of indole required to double XRE-driven reporter gene expression shows that indolo[3,2-b]carbazole is a much more potent inducing agent than 3,3′-diindolylmethane, indole-3-carbinol or ascorbigen [43,69]. Amongst genes for drug-metabolising enzymes, mouse, rat and human CYP1A1 are prototypic XRE-regulated genes. This cytochrome has O-deethylase activity towards ethoxyresorufin, and there is abundant evidence from enzyme assays, western and northern blotting that CYP1A1 is inducible by indolo[3,2-b]carbazole and 3,3′-diindolylmethane [2]. The promoters of other genes including rat and human NQO1, rat ALDH-3, rat GSTA2, rat UGT1A1, and rat UGT1A6 contain an XRE, as does the mouse BAX gene, and it is therefore anticipated that activation of AhR by glucobrassicin-derived indoles will influence significantly the metabolism of xenobiotics (for a review, see [70]). Interestingly, the mouse nrf2 gene promoter also contains an XRE [71], indicating that AhR ligands could lead to an increased production of Nrf2 mRNA; this may not necessarily lead to an increase in Nrf2 protein levels. Whether indolo[3,2-b]carbazole and 3,3′-diindolylmethane can increase the stability of Nrf2 protein is not known, but unless they are metabolised by CYPs, and thereby generate reactive oxygen species, this seems unlikely [72].
In addition to induction of CYP1A1 by indoles, other cytochromes are inducible, such as CYP1B1 and CYP19 [73], as are the drug metabolising enzymes AKR, GST T1-1, sulfotransferase and UGT1 [74]. Furthermore, 3,3′-diindolylmethane can induce expression of the transcription factors ATF3, c-Jun and NF-IL6 as well as genes involved in cell growth, such as growth arrest and DNA damage (GADD) GADD34, GADD45 and GADD153 [75,76]. Also, p21 is induced by indole-3-carbinole [77]. It is not however clear whether the genes for ATF3, c-Jun, NF-IL6 and the GADD proteins are regulated through XREs and whether the process is mediated by AhR.

It has been argued that induction of CYP1A1 by indoles is potentially deleterious to the cell because the cytochrome can activate polycyclic aromatic hydrocarbons to ultimate carcinogens. This viewpoint is probably an oversimplification and does not take into account the multiple changes in gene expression that indoles affect. Bonnesen et al [43] have reported that treatment of human colon LS-174 cells with indolo[3,2-b]carbazole before exposure to benzo[a]pyrene provides a small measure of protection against DNA damage as measured by the comet assay. Most importantly, prior treatment of the LS-174 cells with both indolo[3,2-b]carbazole and sulforaphane before exposure to benzo[a]pyrene was found to confer substantial protection against genotoxicity, and this protection was greater than was achieved by either phytochemical alone [43].

**Inhibition of carcinogen activation by glucosinolate breakdown products**

Certain isothiocyanates can block the activation of several carcinogens to their ultimate carcinogenic forms. Tumorigenesis caused by the carcinogens 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and N-nitrosobis-(2-oxopropyl)amine can be prevented by phenethyl-ITC through a process that involves inhibition of activation of pro-carcinogens by CYP isoenzymes [78,79]. Inhibition of CYP can also be achieved by sulforaphane [80,81]. In the case of the nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, the ITCs with highest lipophilicity and low reactivity of their NCS group had the greatest ability to inhibit lung tumorigenesis [82].

**Inhibition of histone deacetylase by isothiocyanates**

The isothiocyanate sulforaphane has been shown to inhibit histone deacetylase (HDAC) [83,84]. This function is likely to alter gene expression substantially. It may also have profound implications for cell fate as a change in the balance between histone acetyl transferase (HAT) and HDAC could alter tumourigenesis. Indeed, recognition of this possibility has lead to considerable recent interest in the ability of HDAC inhibitors to act as both chemopreventive and chemotherapeutic agents.

Within the cell, DNA is tightly coiled around an octamer of histone proteins in a structure known as a nucleosome, the basic structural unit of chromatin. Each of the histone proteins contains an evolutionary conserved amino tail protruding from
the nucleosome, which can determine the accessibility of the DNA to transcription factors. The tail is also subject to many post-translational modifications including acetylation. The addition of an acetyl group to the histone tail results in a conformational change which enables the tail to move away from the DNA, allowing transcription factors access to the regulatory regions of genes. Conversely, removal of acetyl groups causes the tail to wrap tightly around the DNA thereby preventing interaction with the transcription machinery. The addition and removal of the acetyl groups is carried out by HAT and HDAC, respectively. In pre-cancerous and cancerous cells, tumour suppressor genes are associated with deacetylated histones resulting in the inactivation of these genes. Inhibition of HDAC may prevent the removal of acetyl moieties from histones thus allowing transcription of the tumour suppressor genes.

Sulforaphane has been shown to diminish HDAC activity with a concomitant rise in histone acetylation in prostate cancer cells [84], human embryonic kidney cells [83] and human colorectal cancer cells [83]. The link between inhibition of HDAC activity and the resultant increase in transcription of tumour suppressor genes has been reported for p21 [84,85], p53 [86] and Bax [84,85]. Equally, mammalian HDAC is capable of down-regulating p53 function, by deacetylation of histones associated with the p53 gene, resulting in a reduction in its transcriptional activity. In addition, sulforaphane has been found to cause a G2/M phase delay with an increase in apoptotic cell fraction in a time- and dose-dependent fashion [87].

Thus the ability of sulforaphane, along with other dietary HDAC inhibitors such as diallyl disulfide [88], to affect chemoprevention via its ability to alter chromatin structure is of acute importance to the welfare of the cell.

**Stimulation of cell cycle arrest and apoptosis by isothiocyanates**

Many of the naturally occurring ITCs can suppress the growth of cultured tumor cells by modulating multiple targets that influence cell cycle arrest, apoptosis and differentiation [89]. However, the majority of the studies into mechanisms by which this class of chemical inhibit cell growth have focussed on sulforaphane and phenethyl-ITC. For example, ApcMin/+ mice treated with sulforaphane at either 300 ppm or 600 ppm in their diet have been reported to develop fewer and smaller polyps in their small intestine than ApcMin/+ mice on a control diet; this was associated with a higher level of apoptosis and lower proliferation in animals on the ITC containing diet [90].

In human PC-3 prostate cancer cells, treatment with sulforaphane or phenethyl-ITC causes an arrest in G2/M phase of the cell cycle that is associated with a decrease in levels of cyclin B1 and cell division cycle (Cdc) 25B and Cdc25C proteins [91,92]. The loss of Cdc25C was reported to be due to proteasomal activity, and was accompanied by its translocation from the nucleus to the cytoplasm [92]. Relocation of Cdc25C was found to be controlled by its phosphorylation at Ser-216, mediated through activation of checkpoint kinase 2 (Chk2) [84]. Cell proliferation by ITCs may also be achieved by disrupting cytoskeletal structure and tubulin polymerisation [93,94].
Administration of ITCs to cells at growth suppressive concentrations results in the rapid generation of reactive oxygen species (ROS), within 1 h of exposure, which appears to be necessary for cell death [92,95]. The generation of ROS by ITCs is accompanied by depletion of intracellular GSH and is achieved through the rapid export of ITC-glutathione and ITC-cysteinylglycine conjugates via MRP1 and Pgp-1 efflux pumps [96]. Consistent with the view that production of ROS is necessary for apoptosis, overexpression of catalase suppresses ITC-initiated programmed cell death, as does pre-treatment with N-acetylcysteine [92]. Furthermore, addition of GSH subsequent to ITC treatment can block apoptosis [44,97]. Treatment with ITCs leads to a loss of mitochondrial membrane potential and release of cytochrome c from mitochondria [98]. There is evidence that ITCs can activate both the intrinsic and extrinsic caspase cascades, though this may be cell specific. For example, in PC-3 cells sulforaphane can increase Fas protein levels and activate caspase-8 whilst simultaneously targeting mitochondria and activating caspase-9 [92]. In human bladder cancer UM-UC-3 cells, benzyl-ITC and phenethyl-ITC are more effective at activating caspase-9 than caspase-8 [99]. By contrast, in human leukaemia HL60 cells, caspase-8 plays a major role in apoptosis stimulated by phenethyl-ITC [100]. The levels of pro-apoptotic proteins Bak and Bax, which neutralize the antiapoptotic effects of Bcl-2, are increased by phenethyl-ITC and sulforaphane in PC-3 prostate cancer cells, and this may lead to induction of Apaf-1 [91,92,101]. Furthermore, the pro-apoptotic proteins Bok and Bim EL are also induced by ITCs, and this is thought to amplify the effects of Bak and Bax [92]. Besides increasing the levels of these pro-apoptotic proteins, ITCs down-regulate the anti-apoptotic proteins Mcl-1 and Bcl-xL, though the effect is cell-specific [101]. Various ITCs have been shown to activate c-Jun N-terminal kinase (JNK) [102,103], and this is mediated by extracellular signal-regulated kinases, ERK1/2 [104]. The use of inhibitors indicated that JNK is essential for phenethyl-ITC to cause cytochrome c release and caspase-3 activation in human HT-29 colon adenocarcinoma cells [105]. However, the mechanism by which JNK activates caspases remains unclear.

**Stimulation of cell cycle arrest and apoptosis by indoles**

Treatment of human MCF-7 breast cancer cells with 100 µM indole-3-carbinol inhibits proliferation through affecting a G1 cell cycle arrest [106]. This may in part be due to 3,3′-diindolylmethane rather than indole-3-carbinol as significant quantities of the indole spontaneously condense to the dimer in culture conditions [107]. Cell cycle arrest at G1 occurs as a consequence of indole-3-carbinol inhibiting both cyclin-dependent kinase (CDK) 2 and CDK6. In the case of CDK6, expression of the gene is reduced because indole-3-carbinol attenuates recruitment of the Sp1 transcription factor to the CDK6 promoter [108]. Furthermore, in HaCaT keratinocytes, treatment with 400 µM indole-3-carbinol induces the CDK4/6 inhibitor p15INK4b mRNA and protein causing hypophosphorylation of Rb protein [109]. It therefore appears that cyclin D-CDK6 activity can be inhibited by dual mechanisms. In the case of CDK2, indole-3-carbinol has been
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reported to decrease the kinase activity in MCF-7 cells and inhibit phosphorylation of Rb protein [77]. The reduction in CDK2 activity is attributed to a selective alteration in the size of the complex in which it is contained, from an active form within a 90 kDa complex to a lower activity form within a 200 kDa complex [110]; the 90 kDa and 200 kDa complexes include forms of cyclin E that differ in size, and the larger complex also contains an additional 75 kDa cyclin E immunoreactive protein. Furthermore, the reduction in CDK2 activity is accompanied by redistribution of the kinase in the 200 kDa complex from the nucleus to the cytoplasm, suggesting that indole-3-carbinol can influence the nucleocytoplasmic shuffling of the kinase [110].

In cells that contain wild-type p53, such as MCF-10A, treatment with 300 µM indole-3-carbinol or 30 µM 3,3′-diindolylmethane has been found to result in activation of the ATM signalling pathway, an increase in p53 protein levels, induction of p21 [111]. These changes result in prevention of the CDK2-mediated G1/S transition [111].

Indole-3-carbinol, but not 3,3′-diindolylmethane, was found to inhibit the expression of the androgen receptor in human lymph node carcinoma of prostate (LNCaP) cells as well as the probable downstream target gene prostate specific antigen [112]. It is possible that down-regulation of this receptor represents an antiproliferative mechanism in prostate cells.

Indoles can affect apoptosis in breast and prostate cancer cells. Treatment of PC-3 prostate cancer cells with 60 µM indole-3-carbinol inhibits the EGF-induced autophosphorylation of PI3K and Akt [113]. Thus, the Akt/PI3K cell survival pathway appears to be targeted by indole-3-carbinol. Also, nuclear translocation of NF-κB is inhibited by 3,3′-diindolylmethane through a reduction in phosphorylation of IκBα [114,115].

In HCT-116 human colon cells, indole-3-carbinol can induce nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1), a TGF-β family member associated with pro-apoptotic activities [116]. This may also mediate the anti-tumour effects of indoles.

**Concluding comments**

It is becoming clear that glucosinolate breakdown products can influence the initiation and progression of carcinogenesis. They also appear to influence apoptotic responses to chemotherapeutic agents, such as tamoxifen [77]. A major impediment to our understanding of the chemopreventative mechanisms stimulated by glucosinolates is that relatively little is known about the biological effects of glucosinolate breakdown products other than isothiocyanates and the indole-containing derivatives. Specifically, there are few data about chemopreventative activities of thiocyanates, nitriles, cyano-epithioalkanes and oxazolidine-2-thiones. It is unclear whether formation of thiocyanates, nitriles, cyano-epithioalkanes and oxazolidine-2-thiones from glucosinolates, at the expense of forming isothiocyanates, is undesirable from a cancer chemoprevention perspective. It is unclear whether the activity of ESP, which reduces the formation of isothiocyanates from glucosinolates, is undesirable. If so, ESP should possibly
be eliminated by genetic means from commercial crops. Furthermore, relatively little is known about the pharmacokinetic properties of glucosinolate breakdown products in the human, and without this information it is difficult to relate responses of cells in culture to certain concentrations of phytochemical to what happens in the *in vivo* situation. These are areas that warrant further examination.

Mammalian cells display marked dose responsiveness to phytochemicals: at low doses of phytochemical, cytoprotective adaptive responses are activated, whereas at higher doses cell cycle arrest and apoptosis occurs. It is presently unclear how these different types of response are co-ordinated by the cell and how decisions about whether adaptation, growth arrest or apoptosis is chosen as the appropriate response are determined. Identification of mechanisms that control such outcomes will be useful.

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3.5. Combined action of different dietary compounds preventive of cancer

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Introduction

The relatively consistent epidemiological finding that the consumption of whole foods of different types such as fruits, vegetables and whole grains is strongly associated with reduced risk of cancer and of other chronic diseases has led to the hypothesis that particular phytochemicals are responsible for the preventive effects observed. In the research conducted, numerous bioactive compounds have been isolated and identified, and their potential health-promoting effects evaluated extensively, both \textit{in vitro} and \textit{in vivo}. One of the key problems of research in this field, however, is that purified phytochemicals do not necessarily have the same beneficial health effect as these compounds do when their source is a food or even a complete diet. There is a growing body of evidence that the actions of phytochemicals administered as dietary supplements fail to provide the health benefits that have been observed for diets rich in fruits, vegetables, whole grains, and the like. Although relatively high doses of single bioactive agents may show potent anticarcinogenic effects, the cancer-preventive effects that certain whole foods and diets are shown to have can perhaps better be explained in terms of the chemopreventive properties that interactions between the different dietary ingredients involved create. In this chapter, evidence that bioactive compounds act synergistically is reviewed.

Evaluating the effects of phytochemicals that act synergistically

Carcinogenesis is an extremely complex multistep process in which numerous molecular mechanisms play an important role. Cancer-preventive dietary compounds may interfere at a variety of different levels with these processes. Table 3.10. summarizes various mechanisms by which phytochemicals can modulate the risk of cancer [1,2]. The combinations of phytochemicals that natural foods contain can reduce the risk of cancer by affecting different overlapping and complementary mechanisms. Isolated and purified compounds, in contrast, may lose their biological activity or fail to behave in the same way as in the complex matrix that the original item of food represents. This can be illustrated by the effects of increased intake of carotenoids and vitamin C in diets high in fruits and green and yellow vegetables, which are believed to have cancer-preventive effects. The intended positive effects of an increased intake of \(\beta\)-carotene or vitamin C as dietary supplements, in contrast, are far from certain. Some studies show no reduced incidence of cancer as a result of taking supplements of vitamin C [3] or \(\beta\)-carotene [4], and there are even reports of increased occurrence of lung cancer in smokers receiving dietary \(\beta\)-carotene supplements [5,6]. In addition to characterizing the chemopreventive effects of individual compounds, therefore, evaluation of the effects of synergistically acting phytochemicals is needed.
Table 3.10. Proposed mechanisms by which dietary phytochemicals can prevent cancer

<table>
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<tr>
<td>Antioxidant activity</td>
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<td>Scavenging of free radicals and reduction of oxidative stress</td>
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<td>Inhibition of cell proliferation</td>
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<td>Induction of cell differentiation</td>
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<td>Inhibition of oncogene expression</td>
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<td>Induction of tumour-suppressor gene expression</td>
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<td>Induction of cell-cycle arrest</td>
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<td>Induction of apoptosis</td>
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<td>Inhibition of signal transduction pathways</td>
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<td>Enzyme induction and enhancing detoxification</td>
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<td>Phase II enzymes</td>
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<td>Glutathione peroxidase</td>
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<tr>
<td>Catalase</td>
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<td>Superoxide dismutase</td>
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<td>Enzyme inhibition</td>
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<td>Phase I enzyme (blocking the activation of carcinogens)</td>
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<td>Cyclooxygenase-2</td>
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<td>Inducible nitric oxide synthase</td>
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<tr>
<td>Xanthine oxidase</td>
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<tr>
<td>Enhancement of immune functions and surveillance</td>
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<tr>
<td>Antiangiogenesis</td>
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<tr>
<td>Inhibition of cell adhesion and invasion</td>
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<tr>
<td>Inhibition of nitrosation and nitration</td>
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<td>Prevention of DNA adduct formation or DNA intercalation</td>
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<td>Regulation of steroid hormone metabolism</td>
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<td>Regulation of estrogen metabolism</td>
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<td>Antibacterial and antiviral effects</td>
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Modified from Liu et al. [2].

**Synergistic effects of combinations of various polyphenols**

A number of studies report enhanced chemopreventive effects of mixtures of polyphenols from green tea or other dietary sources. Table 3.11. presents a selection of relevant studies concerning such synergistic effects. Suganuma et al. [7] reported that the effects of tritium-labelled epigallocatechin gallate (EGCG) being incorporated into human lung cancer cells were enhanced by epicatechin (EC), another green tea polyphenol, one without a galloyl moiety. Epicatechin was found to promote the occurrence
of EGCG-induced apoptosis and to inhibit not only growth of PC-9 lung tumour cells but also the release of tumour necrosis factor-α. These effects, induced by a tea polyphenol having a galloyl moiety, were also enhanced in a dose-dependent way by EC. The study showed that the synergistic effects of two green tea polyphenols could result in the tea as a whole becoming a more efficient anticarcinogenic mixture than if supplementation of EGCG alone had been provided.

**Table 3.11.** Selection of studies of the synergistic effects of mixtures of polyphenols and of combinations of these with other types of phytochemicals

<table>
<thead>
<tr>
<th>Combination of compounds</th>
<th>Synergistic effect</th>
<th>Mechanisms involved</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polyphenol mixtures</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGCG and EC, sulindac or tamoxifen</td>
<td>Inhibition of growth of human lung cancer cells</td>
<td>Enhanced cellular uptake of EGCG, enhanced apoptosis, reduced release of TNF-α</td>
<td>Suganuma et al. [7]</td>
</tr>
<tr>
<td>EGCG and EC</td>
<td>Inhibition of cell growth and induction of apoptosis in gastric carcinoma cells</td>
<td>Increased production of caspases-3, -8 and -9; Extracellular production of oxygen species</td>
<td>Horie et al. [8]</td>
</tr>
<tr>
<td>EGCG, EC, EGC and ECG</td>
<td>Modulation of CYP1A1 expression in human hepatocytes</td>
<td>Antagonism of TCDD-induced transcription of human CYP1A1 via interaction with the Ah-receptor</td>
<td>Williams et al. [10,11]</td>
</tr>
<tr>
<td><strong>Polyphenols and other phytochemicals</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green/black tea and soy (SPC)*</td>
<td>Inhibition of prostate tumours, tumour weight and metastasis</td>
<td>Reduction in serum levels of testosterone and DHT</td>
<td>Zhou et al. [12]</td>
</tr>
<tr>
<td>Green/black tea and soy (SPC)</td>
<td>Inhibition of breast tumour cell growth</td>
<td>Inhibition of tumour angiogenesis, reduced levels of estrogen receptor-α protein and of serum IGF-I.</td>
<td>Zhou et al. [13]</td>
</tr>
<tr>
<td>Green tea infusions and grape or grape skin extracts</td>
<td>Reduced tumour cell growth</td>
<td>Inhibition of tNOX, induction of apoptosis,</td>
<td>Morré and Morré [16]</td>
</tr>
<tr>
<td>Polyphenols, vitamin E, A and β-carotene</td>
<td>Reduced oxidative stress</td>
<td>Reduced formation of lipid hydroperoxides and malondialdehydes; reduced co-oxidation of vitamins E, C and β-carotene</td>
<td>Gorelik et al. [18]</td>
</tr>
<tr>
<td>EGCG, EC, EGC and ECG or gallic acid and α-tocopherol</td>
<td>Reduced oxidative stress in micelles and human LDL</td>
<td>Reduction in α-tocopheryl radicals, trapping of lipid peroxyl radicals and regeneration of vitamin E</td>
<td>Zhou et al. [19]</td>
</tr>
</tbody>
</table>

Zhou et al. [21] Liu et al. [22]
Synergistic effects of green tea catechins, both on the inhibition of cell growth and the induction of apoptosis, were also found in gastric carcinoma cells [8]. Various gastric cell lines were shown to differ in their susceptibility to EGCG treatment. EC alone had virtually no effect on carcinomic cell growth or induction of apoptosis, but it had a significant synergistic effect on the induction of apoptosis when combined with other catechins. After this combined treatment, the activity levels of caspases 3, 8 and 9 became elevated, indicating them to be involved in catechin-induced apoptosis. Interestingly, catalase was found to block the synergistic effects of EC and EGCG, suggesting that the reactive oxidative species and production of hydrogen peroxide play a part in the synergistic mechanisms here [8].

Since the cytochrome P450 (CYP) enzymes are responsible for the metabolism of many environmental carcinogens, the modulation of their expression and activity by phytochemicals is a potential mechanism by which the risk of cancer can be reduced. Some of the cytochrome P450 genes are expressed constitutively, whereas others are inducible by xenobiotic compounds or by phytochemicals. Enzyme induction usually enhances detoxification, but under some circumstances substrates may be activated to become mutagens, carcinogens or cytotoxic substances [9]. The induction of CYP1A enzymes by PAH and by dioxins such as TCDD occurs at the transcription level and is mediated by the cytosolic aryl hydrocarbon receptor (AhR) [9]. Williams et al. [10] demonstrated that complex green tea extracts exert mixed agonist/antagonist activity on the Ah-receptors, whereas EGCG acts as a strict AhR antagonist. The authors concluded that the modulation of human CYP1A1 expression by green tea extracts cannot be attributed to the action of a single tea catechin, being due instead to the effects of the complex mixture involved. Co-treatment of human hepatocytes with TCDD and different tea catechin mixtures inhibited synergistically both TCDD-induced CYP1A-promoter-driven luciferase reporter activity (in the HepG2 cells) and CYP1A1 expression (in the HepG2 cells and the primary human hepatocytes). The optimal degree of synergy was achieved by a combination of the four major tea catechins — EC, EGCG, epigallocatechin (EGC), and epicatechin gallate (ECG) — and it was not found to be improved further by the addition of other compounds [11].

Table 3.11. Selection of studies of the synergistic effects of mixtures of polyphenols and of combinations of these with other types of phytochemicals — cont.

<table>
<thead>
<tr>
<th>Combination of compounds</th>
<th>Synergistic effect</th>
<th>Mechanisms involved</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 red wine polyphenols</td>
<td>Increased antioxidant potential</td>
<td>Regeneration of phenoxy radicals by phenolic compounds</td>
<td>De Beer et al. [25] Jørgensen et al. [27]</td>
</tr>
<tr>
<td>Different types of vegetables (cauliflower, carrots, peas and unions)</td>
<td>Up- and down-regulation of genes involved in carcinogenesis, interpreted mainly in preventive terms</td>
<td>No indication of specific synergetic mechanisms</td>
<td>Van Breda et al. [23,24]</td>
</tr>
</tbody>
</table>

* SPC — Soy Phytochemical Concentrate.
Synergistic effects of polyphenols and other phytochemicals

In two different studies, Zhou et al. investigated with use of mice models the potential synergistic effects of a combination of bioactive tea components and soy phytochemicals on androgen-sensitive human prostate tumours and estrogen-dependent human breast carcinoma [12,13]. A phytochemical soy concentrate (SPC) and green and black tea infusions were employed (Table 3.11.). In further investigations it was shown that bioactive compounds in tea (particularly EGCG [14]) and soy (the soy isoflavone genistein as well as SPC) inhibit growth of prostate cancer and tumour metastasis in vivo [15]. The synergistic inhibition of the progression and metastasis of prostate tumours by use of the combination of green tea and SPC was found to be associated with effective reduction in the serum levels of both testosterone and dihydrotestosterone, the latter a biologically more active metabolite of testosterone and a prerequisite for the development of benign prostatic hyperplasia and prostate cancer [12]. In an immune-deficient mouse model in which MCF-7 human breast cancer cells were implanted, SPC in combination with green tea showed a synergistic inhibition of tumour cell growth. This was accompanied by inhibition of tumour angiogenesis, reduced expression of estrogen-receptor (ER) alpha and reduced serum levels of the insulin-like growth factor (IGF)-I, all of these being factors in breast cancer development. The modulation of these two different mechanisms may be an explanation of the synergistic effects of the combined phytochemicals [13].

A tumour-specific growth protein possessing NADH oxidase activity (tNOX) has emerged as a potential target of the anticancer action of plant polyphenols and flavonoids [16]. NOX proteins are located at the cell surface and are responsible for the increase in cell size that occurs following cell division [17]. Cells in which NOX activity is blocked, such as by phytochemicals, are unable to enlarge. They cease to divide and eventually undergo apoptosis. Recently, Morré and Morré [16] showed there to be an exceptionally strong (10-fold) synergy between grape polyphenols and tea catechins in the inhibition of tNOX. The strongest synergistic activity was found for ethanol extracts of grape skins, whereas no activity was detected for extracts of grape seeds, indicating that the effects were not caused by resveratrol, which is found predominantly in the seeds. These results suggest that more effective cancer prevention and cancer therapy could be achieved by use of combinations of different phytochemicals.

Polyphenols and dietary antioxidant vitamins can also have synergistic inhibitory effects on lipid peroxidation and on the co-oxidation of dietary antioxidants. It was demonstrated with use of simulated stomach fluid that phytochemicals can prevent the build-up of oxidized lipid products (lipid hydroperoxides and malondialdehyde) as well as the destruction of vitamin E and β-carotene (and of vitamin C too, though to a lesser extent) [18]. In the gastric fluid, vitamin C can enhance the activity of polyphenols through a synergistic antioxidant effect. In line with this, the authors suggested that the antioxidant network in the stomach can decrease the level of hydroperoxides and other cytotoxic compounds, increasing at the same time the amounts of vitamin antioxidants reaching the blood system. The authors indicated that the resulting synergistic increase
in the systemic antioxidant effect might also explain the French paradox (the fact that people in France suffer from relatively low incidence of coronary heart disease, despite their unhealthy dietary habits and high consumption of alcohol in the form of red wine) and the beneficial effects of Mediterranean and Japanese diets that contain complex combinations of polyphenols and other antioxidants [18]. By studying the kinetics of the reaction of \( \alpha \)-tocopheroyl radicals with green tea polyphenols by means of stopped-flow electron paramagnetic resonance, Zhou et al. [19] demonstrated clearly that several green tea polyphenols (EC, EGCG, EGC, ECG and gallic acid) can effectively reduce \( \alpha \)-tocopheroxy radicals so as to allow \( \alpha \)-tocopherol to be regenerated. These green tea polyphenols were also found to trap the initiating radicals (ROO•) as well as the propagating lipid peroxy radicals (LOO•). It is particularly the elimination of the pro-oxidant effect of vitamin E (or the so-called tocopherol-mediated peroxidation) that can occur in the absence of other oxidants [20]. This, combined with an \( \alpha \)-tocopherol regenerating reaction involving coexisting antioxidants, plays a major role in enhancing the antioxidant efficiency of vitamin E. These combined effects may also explain the synergistic antioxidant effects of the polyphenols in tea and of the \( \alpha \)-tocopherol in both the micelles and human low-density lipoprotein that members of this same research group have reported [21,22].

**Synergistic effects of whole foods and complex mixtures of compounds**

In addition to the synergistic effects of several individual compounds on biomarkers of cancer prevention, the synergistic effects of taking whole foods or complex mixtures of compounds have been reported. In two recent animal studies on the effects of vegetable consumption on the modulation of gene expression, it was found that most of the genes that were differentially expressed before and after vegetables were consumed, the changes in gene expression could be interpreted as a cancer preventive effect [23,24]. The effects of consuming one of four different vegetables on gene expression in the colon and the lungs of female C57B16 mice were found to differ from those of consuming a mixture of all four vegetables at once. Consumption of the mixture of the vegetables was able to modulate genes which were not significantly modulated by one of the specific vegetables present in the mixture. On the other hand, the individual vegetables were able to modulate genes which were not significantly modulated by the mixture, indicating that combinations of different foods containing different complex mixtures of phytochemicals can also have an antagonistic effect on gene expression.

Other examples of the assessment of synergistic effects in complex mixtures are to be found in studies aimed at unraveling the antioxidant capacity of red wines. In one study, the Trolox equivalent antioxidant capacity (TEAC) value and phenolic composition were determined for a large number of pinotage wines [25]. When the contributions of the separate phenolic compounds were calculated, it was found that only 11 to 24% of the TEAC values could be explained in terms of the sum of the values for the individual compounds. Taking account of the different mixtures found of the 12 phenolic compounds that were present, and assuming their concentrations to be those typical for red wines,
indicated 16 to 23% of the antioxidant activity to be synergistic. This implies that, in addition to the synergistic effects between the different phenolic compounds, synergistic effects between these and the other wine constituents may have contributed to the TEAC values. The author excludes a potential role of sulphur dioxide in the regeneration of phenolic compounds from their phenoxy radicals as it does not contribute to the total antioxidant potential at the concentrations normally present in red wines [26]. Jørgensen et al. [27] demonstrated, however, the regeneration of quercetin from its phenoxy radical by the presence of (+)-catechin. This suggests that the regeneration of phenolic compounds from phenoxy radicals is a mechanism that can contribute to the synergistic effects observed.

**Other synergistic effects**

In addition to investigating the synergistic effects of various phytochemicals, studying the combined effects of dietary factors and therapeutic compounds may be a promising approach toward optimising pharmacological strategies for the prevention and therapy of cancer [1,28]. Administering multiple agents can increase the efficacy and potency of the chemopreventive measures taken and reduce toxic side-effects. Various combinations of drugs have been proposed for further clinical development based on their synergistic activity as shown *in vitro* or in animal studies. Use of retinoids in combination with such SERMs (selective estrogen receptor modulators) as tamoxifen or raloxifene [29,30] are examples of this. Also, the effects reported by Suganuma et al. [7] of using EGCG to induce apoptosis in human lung cells *in vitro* have been synergistically enhanced by use of cancer preventive agents such as sulindac and tamoxifen. This effect has also been evaluated in an animal study in which treatment of rats with a combination of EGCG and sulindac was found to reduce aberrant crypt formation after their being treated with azoxymethane for colon carcinoma [31]. In that study, EGCG and sulindac were also found to synergistically enhance apoptosis. The results confirm earlier findings showing a combination of phytochemicals and therapeutic agents to provide more effective therapy for cancer than the therapeutic agents alone, its also reducing side effects, particularly those of sulindac, without a loss in treatment potency. A review of the effects of various combinations of chemotherapeutic and chemopreventive approaches in dealing with cancer has appeared recently [28].

**Conclusions**

An increasing number of *in vitro* and *in vivo* studies indicate that combinations of dietary chemopreventive agents can result in a significant level of activity being achieved at concentrations at which any single agent is virtually inactive. The fact that many of these phytochemicals act synergistically may why some food items or diets show cancer preventive effects that cannot be explained on the basis of their separate bioactive ingredients alone. Although our understanding of the molecular mechanism behind the observed combinatorial effects of these chemopreventive agents is still limited, it appears
Bioactive components in foods: Combined action of different dietary compounds preventive of cancer

that many different combinations of complementary modes of action may be involved. There is a clear need of further study of the synergistic effects of dietary phytochemicals. This may contribute to the improvement of cancer treatment and cancer prevention. Especially the development of new dietary supplement regiments, cancer therapies and nutraceuticals can benefit from improved insight into the mechanisms behind the synergistic effects that both natural and synthetic chemopreventive compounds show.

References


3.6. Probiotics and prebiotics — potential anticarcinogenic food components

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Introduction

An example of a functional food, which has been the focus of intense research activity in recent years are probiotics — ‘living micro-organisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition’ [1,2]. Probiotics usually refer to highly selected lactic acid bacteria, e.g. *Lactobacillus* spp. *Bifidobacterium* spp. and *Streptococcus* spp., with defined gut survival properties and associated biological activities and which can be ingested in fermented milk products or as a supplement. The list of healthful effects attributed to probiotic bacteria is extensive [3] and includes: alleviation of lactose intolerance symptoms; serum cholesterol reduction; anticancer effects; alleviating constipation; relieving vaginitis to name but a few. The vast majority of studies on the anticancer effects deal with colorectal cancer (CRC), although there are some on breast [4] and bladder cancer [5].

Mortality from CRC is second only to that of lung cancer in men and breast cancer in women and has shown little sign of decreasing in the last 20–30 years. Diet makes an important contribution to CRC risk [6] implying that risks of CRC are potentially reducible. Evidence from a wide range of sources supports the view that the colonic microflora is involved in the etiology of CRC. This has led to intense interest in factors, such as probiotics, prebiotics (‘a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that have the potential to improve host health’) [3] and synbiotics (combinations of pro- and prebiotics), that can modulate gut microflora and its metabolism. Evidence for protective effects of pro- and prebiotics on cancer is derived from *in vitro* studies, animal models, epidemiology and human intervention studies.

Overall, the supportive evidence is stronger for probiotics than prebiotics (possibly because the latter have only recently come to prominence) and is recently suggestive that synbiotics are more effective than either pro- or prebiotics alone. The evidence from animal studies provides strongest support for anticancer effects and data from human studies (epidemiology and experimental) are limited.

Evaluation of anticarcinogenic dietary effects

Evidence from human studies

Consumption of lactobacilli by healthy volunteers has been shown to reduce the mutagenicity of urine and faeces associated with the ingestion of carcinogens in cooked meat. Administration of *L. acidophilus* to eleven volunteers on a fried meat diet known to increase faecal mutagenicity, resulted in a lower faecal mutagenic activity after 3 days
compared to faecal mutagenic activity after 3 days consumption of ordinary fermented milk (although not significant) [7]. High levels of mutagenicity also appeared in urine on days 2 and 3 of the fried meat and ordinary fermented milk dietary regimen. During \textit{L. acidophilus} administration, the urinary mutagenic activity on days 2 and 3 was significantly lower compared to the ordinary fermented milk period. In most cases, an increase in the number of faecal lactobacilli corresponded to a lower mutagen excretion, particularly in urine. Hayatsu and Hayatsu [8] also demonstrated a marked suppressing effect of orally administered \textit{L. casei} on the urinary mutagenicity arising from ingestion of fried ground beef in the human.

As yet, there are few epidemiological studies addressing the association between fermented dairy products and colorectal cancer. Consumption of large quantities of dairy products such as yoghurt and fermented milk containing \textit{Lactobacillus} or \textit{Bifidobacterium} may be related to a lower incidence of colon cancer [9]. An epidemiological study performed in Finland demonstrated that, despite the high fat intake, colon cancer incidence was lower than in other countries because of the high consumption of milk, yoghurt, and other dairy products [10,11]. In two population-based case-control studies of colon cancer, an inverse association was observed for yoghurt [12] and cultured milk consumption [13], adjusted for potential confounding variables. In another case control study, an inverse relationship for yoghurt consumption with risk of large colon adenomas in men and women was reported [14]. It can also be mentioned that an inverse relationship has been demonstrated between the frequency of consumption of yoghurt and other fermented milk products and breast cancer in women [4,15]. On the other hand, two companion American prospective studies, the 1980–1988 follow-up of the Nurses’ Health Study and the 1986–1990 Health Professionals follow-up study, did not provide evidence that intake of dairy products is associated with a decreased risk of colon cancer [16]. In a cohort study in the Netherlands, it was shown that the intake of fermented dairy products was not significantly associated with colorectal cancer risk in an elderly population with a relatively wide variation in dairy product consumption, although a weak non-significant inverse association with colon cancer was observed [17]. In summary, it would appear that the case control studies indicate protective effects while the prospective studies do not.

In conclusion, data from human intervention studies are of paramount importance in providing evidence that probiotics, prebiotics or fermented milk consumption are causally related to reduction in cancer risk. Thus, this is an area of high priority for future studies. Presently however the lack of well validated biomarkers for colon cancer limits the relevance of such studies although a wide range of potential biomarkers of risk are under development. Once such markers are available, it will become possible to perform studies in healthy volunteers, at-risk groups and patients. It will be important to define dose and time relationships and it would appear at present, from animal studies, the most profitable approach would be to use combinations of pro- and prebiotics. There will also be, in the near future, the opportunity to exploit genomics and proteomics in investigations of effects of pro/prebiotics on gene expression and post-transcription
events in colonic biopsies and to identify human groups responsive to pro/prebiotic intervention. It will also be particularly important to use data on mechanisms of action to develop hypothesis based intervention studies in humans.

Of relevance here is a clinical trial which is presently ongoing i.e. to examine the effect of a synbiotic preparation on colon cancer risk biomarkers in humans (SYNCAN project, funded by EU, and involving 8 research centres in Europe; http://www.syncan.be). It involves a twelve-week randomised, double blind, placebo controlled trial of a food supplement containing *Lactobacillus GG*, *Bifidobacterium* Bb-12 and Raftilose Synergyl in adenoma patients. In this study, all of the "state of the art" colon cancer risk biomarkers, including colonic mucosal markers, faecal water markers and immunological markers, are being measured. It is hoped that the results of this study will provide much needed information on the cancer protective effects of synbiotics in humans and supply us with additional valuable information on the underlying mechanisms.

**Evidence from laboratory animal studies**

There are several good animal models for colon cancer which have proved useful for identifying dietary factors which may protect us against the development of this tumour. End points used are the tumours themselves or early lesions, such as aberrant crypt foci (ACF). ACF are putative preneoplastic lesions from which adenomas and carcinomas may develop. In recent years, there have been many studies, using these models, which clearly demonstrate a protective effect of dietary supplements of lactic acid bacteria against colon tumour development.

Oral administration of lactic acid bacteria has been shown to effectively reduce DNA damage, induced by chemical carcinogens, in gastric and colonic mucosa in rats. Pool-Zobel et al. [18] reported, using the comet assay, that *Lactobacillus acidophilus*, *L. gasseri*, *L. confusus*, *Streptococcus thermophilus*, *Bifidobacterium breve* and *B. longum* were antigenotoxic toward N’-nitro-N-nitrosoguanidine (MNNG). These bacteria were also protective toward 1,2-dimethylhydrazine (DMH)-induced genotoxicity. Metabolically active *L. acidophilus* cells, as well as an acetone extract of the culture, prevented MNNG-induced DNA damage, while heat-treated *L. acidophilus* was not antigenotoxic. Among different cell fractions from *L. acidophilus*, the peptidoglycan fraction and whole freeze-dried cells were antigenotoxic.

Certain strains of lactic acid bacteria have also been found to prevent putative preneoplastic lesions or tumours induced by carcinogens. Goldin et al. [19] showed that a specific strain of *L. casei* subsp. *rhamnosus* designated GG can interfere with the initiation or early promotional stages of DMH-induced intestinal tumorigenesis and that this effect is most pronounced for animals fed a high-fat diet. Overnight cultures of *L. acidophilus* also inhibited the formation of ACF, induced by azoxymethane (AOM) [20]. Although *B. adolescentis* culture and its supernatant did not show an inhibitory effect in this study [20], feeding of bifidobacteria suppressed the ACF formation induced by AOM [21,22] or DMH [23,24]. Consumption of *B. longum* or inulin was associated with a decrease in AOM-induced colonic small ACF in rats and combined administration
significantly decreased the incidence of large ACF [25]. In addition, it has been reported that colonisation of bacteria with an ability to produce genotoxic compounds and high β-glucuronidase activity enhanced progression of ACF induced by DMH in rats, and that the additional colonisation of *B. breve* reduced the number of ACF with four or more crypts/focus and crypt multiplicity which are reliable predictors of malignancy [26].

Reddy and Rivenson [27] reported that lyophilised cultures of *B. longum* administered in the diet to rats inhibited liver, colon and mammary tumours, induced by the food mutagen 2-amino-3-methyl-3H-imidazo(4,5-f)quinoline (IQ). Goldin and Gorbach [28] showed that dietary supplements of *L. acidophilus* not only suppressed the incidence of DMH-induced colon carcinogenesis but also increased the latency period in rats. Feeding of fermented milk increased the survival rate of rats with chemically induced colon cancer [29]. Dietary administration of a lyophilised culture of *B. longum* resulted in a significant suppression of colon tumour incidence and tumour multiplicity and also reduced tumour volume induced by AOM in rats [30]. Ingestion of *B. longum* also significantly inhibited AOM-induced cell proliferation, ornithine decarboxylase activity and expression of the ras-p21 oncoprotein. Recently, there was a report on the anti-tumourigenic activity of the prebiotic inulin, enriched with oligofructose, in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* in the AOM-induced colon carcinogenesis rat model [31]. The authors concluded that, while a possible protective effect of probiotics was observed, the results indicated that the prebiotic decreased AOM-induced carcinogenesis. The mechanisms by which they act are less clear, but the data presented suggested that they may act through a combination of mechanisms involving an increase in short-chain fatty acid (SCFA) production, lower proliferative activity and a variation in the expression of some enzymes involved in the pathogenesis of colon cancer.

There is additional direct evidence for anti-tumour activities of lactic acid bacteria obtained in studies using pre-implanted tumour cells in animal models. It has been demonstrated that feeding of fermented milk or cultures containing lactic acid bacteria inhibited the growth of tumour cells injected into mice [32,33]. Sekine et al. [34] using whole peptidoglycan isolated from *B. infantis* strain ATCC15697, reported that a single subcutaneous injection significantly suppressed tumour growth. In addition, five intralesional injections resulted in 70% tumour regression in the mice.

More recently, mindful of the fact that the composition and metabolic activities of the intestinal flora of experimental animals are significantly different from those of humans [35], we exploited human flora associated (HFA) mice to test the effects of a probiotic mixture on a parameter of relevance for colon carcinogenesis i.e. DNA adduct formation [36]. Indeed, the results from a previous report from our laboratory, demonstrated that human intestinal microflora had different effects than mouse microflora concerning DNA adduct formation after exposure to mutagens [37]. The probiotic mixture, Biothree®, used in this study contained *Streptococcus faecalis* T-110, *Clostridium butyricum* TO-A and *Bacillus mesentericus* TO-A, which are acid resistant in contrast to most bacteria, which do not survive contact with gastric acid. It has been
Joseph Rafter

reported that *S. faecalis* T-110 and *C. butyricum* TO-A showed strong symbiosis with each other and the growth of enteropathogens (enterotoxigenic *Escherichia coli*, *Salmonella typhimurium*, *Vibrio parahaemolyticus*, *C. difficile* and *C. botulinum*) was inhibited in mixed cultures of *S. faecalis* T-110 and *C. butyricum* TO-A [38]. It has also been reported that *B. mesentericus* TO-A stimulated the growth of *Bifidobacterium* by producing 3,3-dihydroxyazetidine [39,40]. Biothree® is used as a clinical therapy in Japan. It is effective for the improvement of symptoms caused by abnormal intestinal flora, i.e. diarrhoea and constipation. Interestingly, the results of this study demonstrated that the above probiotic mixture had an effect to significantly decrease the DNA adduct formation in the colonic epithelium induced by the food mutagen 2-amino-9H-pyrido[2,3-b]indole (2-amino-alpha-carboline; AAC), given by gavage. Two possible mechanisms may be involved: reduction of direct exposure to AAC and/or induction of DNA repair of the DNA adducts in the colonic epithelium.

**Mechanisms by which probiotic bacteria may be inhibiting colon cancer**

The precise mechanisms by which lactic acid bacteria may inhibit colon cancer are presently unknown. However, such mechanisms might include: alteration of the metabolic activities of intestinal microflora; alteration of physicochemical conditions in the colon; binding and degrading potential carcinogens; quantitative and/or qualitative alterations in the intestinal microflora incriminated in producing putative carcinogen(s) and promoters (e.g. bile acid-metabolizing bacteria); production of antitumourigenic or antimutagenic compounds; enhancing the host's immune response; effects on physiology of the host.

**Conclusion**

Many healthful effects are attributed to the probiotic bacteria and some of these effects have more scientific support than the anticancer effect. The strongest evidence for anticancer effects of probiotics comes from animal studies and evidence from human studies (epidemiology and experimental) is still limited. An important goal for the future should be carefully designed human clinical trials to corroborate the wealth of experimental studies. Also, as mentioned above, there are several possible mechanisms which might explain how lactic acid bacteria might protect against tumour development in the colon. It is possible that different strains target different mechanisms. All of the mechanisms have various degrees of support, mainly originating from *in vitro* and animal experiments and some of them even have some support from human clinical studies. Thus, more work needs to be done to identify the specific strains and strain characteristics responsible for specific antitumour effects and the mechanisms by which these effects are mediated. However, even with the above reservations in mind and mindful of the limited number of human studies available, the use of lactic cultures for human cancer suppression is interesting, holds promise and certainly deserves more scrutiny.
References


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