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A (all-trans-retinol and its esters) level was originally determined by bioefficiency assay, a technique that was later superseded by various chromatographic and fluorescent techniques.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.

### 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(^a)</td>
<td>20</td>
<td>[4]</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>&lt;10</td>
<td>2(^a)</td>
<td>50–100(^a)</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>

\(^a\) 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 freezving 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].

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 (52 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 interday 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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 erythematosis 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-\(\alpha\)-tocopherol, 182 mg/d dl-\(\alpha\)-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 1 h 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 be 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 30% 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 \( F_{2\alpha} \) 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* (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_{2\alpha} ) EIA</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>

* Estimates in the author’s laboratory with use of standard semiautomated equipment.
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 2.5. Summary of effects of vitamin C on plasma lipid oxidation markers**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDA</th>
<th>LDL oxidation</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 preceeding 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].

**Fig. 2.3.** Structures of tocopherols and tocotrienols and their relative potency as vitamin E in the rat (TE; dl-α-tocopherol equivalents). Redrawn from [100].
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), *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-α-tocopherol or a placebo for a 6-month period a decrease in *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-α-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, 30 mg beta-carotene and 100 µg 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*-α-tocopherol,
182 mg/d \textit{dl}-\textalpha\text{-tocopherol} alone, or placebo, no effect on plasma antioxidant capacity was observed at 12 or at 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}-\textalpha\text{-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 \textalpha\text{-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}-\textalpha\text{-tocopherol}, 182 mg/d \textit{dl}-\textalpha\text{-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 RRR-\textalpha\text{-tocopherol} and \textit{all-rac}-\textalpha\text{-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}-\textalpha\text{-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}-\textalpha\text{-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 ultra-marathon runners were given either a combination of 300 mg/d \( \alpha \)-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 \( \alpha \)-tocopherol (400 mg/d), vitamin C (500 mg/d) together with \( \alpha \)-tocopherol (290 mg/d) and \( \gamma \)-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 \( \gamma \)-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 \( \alpha \)-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**


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

Steffen Loft1, Peter Møller1, Marcus S. Cook2, Rafał Różalski3, Ryszard Oliński3
1 University of Copenhagen, Denmark
2 University of Leicester, UK
3 Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University, Poland

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 tumours 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^a</th>
<th>Age (yr)^b</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>Multi-vitamin (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^c 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^d 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>
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<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>
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<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^e 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^e 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^e 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^e 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&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Age&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Effect</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antioxidant rich diet or tablets&lt;sup&gt;i&lt;/sup&gt; 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&lt;sup&gt;i&lt;/sup&gt; 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 (^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 \( \beta \)-carotene (6 mg), \( \alpha \)-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 \( \beta \)-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 \( \beta \)-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 C, 60 mg vitamin E, and 15 mg \( \beta \)-carotene.

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

\(^j\) Containing vitamin A (240 mg), \( \beta \)-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 23.5 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
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: \(\alpha\)-carotene (1.4 mg), \(\beta\)-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

Jakob Linseisen and Sascha Abbas
DKFZ, Cancer Epidemiology, Heidelberg, Germany

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>4–400</td>
<td>≤5</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>
</tr>
<tr>
<td>RIA, IDS</td>
<td>Serum or plasma, 50 µL</td>
<td>Two-step reagent extraction</td>
<td>6–360</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>6.3–250</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>17.5–300</td>
<td>18</td>
<td>6.6</td>
<td>11.2</td>
<td>75 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>15–150</td>
<td>4.0</td>
<td>5.2</td>
<td>8.4</td>
<td>20 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>8–312</td>
<td>2.5</td>
<td>9.9</td>
<td>14</td>
<td>1 h, 10 min</td>
<td>The laboratory must have an HPLC unit with a silica column</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)D$_2$ as well as 25-(OH)D$_3$. The HPLC method enables 25-(OH)D$_2$ and 25-(OH)D$_3$ to be quantified whereas there are concerns regarding the detection of 25-(OH)D$_2$ with other assays (Nichols procedure and IDS RIA) [9]. However, a subsequent study refuted these findings and also concluded that vitamin D$_2$ is less potent and has a shorter duration of action than vitamin D$_3$ [14]. This suggests that the contribution of 25-(OH)D$_2$ 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)D$_2$ and the monitoring of vitamin D therapy is complicated by the presence of assays that underestimate 25-(OH)D$_2$. 
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

Björn Åkesson and Katharina Bruzelius

Biomedical Nutrition, Pure and Applied Biochemistry, Lund University,
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 2.9. Some major inorganic and organic forms of selenium

<table>
<thead>
<tr>
<th>Organic</th>
<th>Inorganic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenocysteine (Sec)</td>
<td>HSeCH₂CH(NH₃)COOH</td>
</tr>
<tr>
<td>Selenomethionine</td>
<td>CH₃Se(CH₂)₂CH(NH₃)COOH</td>
</tr>
<tr>
<td>Selenite</td>
<td>SeO₃²⁻</td>
</tr>
<tr>
<td>Selenate</td>
<td>SeO₄²⁻</td>
</tr>
</tbody>
</table>

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]).
tion. Several studies have suggested that methylated Se derivatives, such as Se-methyl-selenocysteine 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 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>SelP</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^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].
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 2.11. Plasma concentrations of SeP, selenium and eGSHPx in different studies (from ref. [63]).

<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</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(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 intakec</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).

a GSHPx activity, U/l.
b GSHPx activity, mU/mg protein.
c 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.

**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].](image)

**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 α1-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 β-carotene, α-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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Vitamins and selenium: Selenium and cancer — selenoproteins as biomarkers


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Vitamins and selenium: Selenium and cancer — selenoproteins as biomarkers

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

Jolanta Gromadzińska, Edyta Reszka, Wojciech Wąsowicz
Department of Toxicology and Carcinogenesis, Nofer Institute of Occupational Medicine, Łódź, Poland

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
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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 (in vitro)</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 (in vitro)</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>NF-κB</td>
<td>I</td>
</tr>
<tr>
<td>CH₃SeCN, p-XSC, Se-methylselenocysteine,</td>
<td>DNA synthesis (in vitro)</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</td>
<td>PKC, PKA</td>
<td>I</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 \( \kappa B \) (NF-\( \kappa B \)) and the activation of AP-1 [39]. NF-\( \kappa B \) 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-\( \kappa B \) 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-\( \kappa B \) activation involves changes in protein conformation and in the binding of numerous genes, such as cytokines, for example, to the promoter regions. Reactive oxygen species (ROS) activates the phosphorylation of one of the NF-\( \kappa B \) subunits, I-\( \kappa B \), directly. GSH-Px overexpression inhibits the translocation of another NF-\( \kappa B \) subunit and reduces the phosphorylation of I-\( \kappa B \) [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-\( \kappa B \) 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-\( \kappa B \) to DNA is influenced by Se, which modifies the structure of Cys in the regulatory subunit of NF-\( \kappa B \) 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-\( \kappa B \) 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-\( \kappa B \)
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-$$\kappa$$B, 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 methylselenol 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
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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 cata-
lyzed 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 de-
toxification [48] and it modulates expression of the enzymes involved in phase I detoxi-
fication. In vitro studies of a mammary cell line exposed to DMBA showed 1,4-phenylene-
bis(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)²</td>
<td>153 (61.0 (13.5) µg/l)³</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)²</td>
<td>145 (57.8 (16.9) µg/l)³</td>
<td>0.41 (0.17–0.94)</td>
<td>0.46</td>
</tr>
<tr>
<td>Ratnasinge 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>Hartman et al. [95]</td>
<td>NCC</td>
<td>Toenail</td>
<td>Male</td>
<td>Finland</td>
<td>5–8</td>
<td>250 (0.537 (0.134) mg/kg)</td>
<td>250 (0.550 (0.129) mg/kg)</td>
<td>0.20 (0.09–0.44)</td>
<td>NA</td>
</tr>
<tr>
<td>Van den Brandt et al. [87]</td>
<td>cohort</td>
<td>Toenail</td>
<td>All</td>
<td>The Netherlands</td>
<td>3.3</td>
<td>317 (0.529 (0.206) µg/g male; 0.537 (0.080) µg/g female)²</td>
<td>2459 (0.547 (0.126) µg/g male; 0.575 (0.109) µg/g female)³</td>
<td>0.50 (0.30–0.81)</td>
<td>0.006</td>
</tr>
<tr>
<td>Hu et al. [96]</td>
<td>CC</td>
<td>Diet</td>
<td>All</td>
<td>China</td>
<td>—</td>
<td>227 (33.12) µg/day</td>
<td>227 (33.07 µg/day)</td>
<td>1.30 (0.70–2.20)</td>
<td>0.48</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; ² data from 189 sets; ³ data from 91 individuals; ⁴ data from 177 individuals; ⁵ male subjects randomized in the earliest in the trial; ⁶ male subjects randomized in the 5th year; ⁷ 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>NCC</td>
<td>Serum</td>
<td>Finland</td>
<td>8–12</td>
<td>46 (59.6 (19.4) µg/l)³</td>
<td>46 (58.3 (14.8) µg/l)³</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)³</td>
<td>456 (114.3 (20.4) µg/l)³</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. [101]</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)³</td>
<td>249 (131.6 µg/l)³</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)³</td>
<td>577 (0.108 (0.018) µg/g)³</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>

³ Nested case-control; ³ 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)*</td>
<td>29 (64.0 (18.2) µg/l)*</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)*</td>
<td>48 (65.3 (15.3) µg/l)*</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)</td>
<td>276 (130.3 (17.8) µg/l)</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) Rectal 76 (0.593 (0.411) 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>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)) Rectal 36 (0.578 (0.091))</td>
<td>1246 (0.575 (0.108))</td>
<td>0.77 (0.41–1.45)</td>
<td>0.733</td>
</tr>
</tbody>
</table>

* Nested case-control; b data from 32 sets; c 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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