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REVIEW

Antioxidant vitamins and mineral supplementation, life span expansion and cancer incidence: a critical commentary

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Abstract
Purpose Experimental evidence indicates a strong connection between oxidative damage, cancer, and aging. Epidemiological observations suggest that a diet rich in fruits and vegetables is associated with lower incidence of some cancers and longer life expectancy; since fruits and vegetables contain natural antioxidants, a considerable effort has been dedicated to understanding their effects in experimental studies and in human trials.
Results A: Effects of antioxidant-containing food and supplements on oxidation damage in humans. Intervention trials employing a variety of biomarkers have shown either a slight decrease in oxidation damage or no effect. B: Effects of selected antioxidants on mortality and cancer incidence. β-carotene and α-tocopherol, alone or in combination, increase cardiovascular and all-cause mortality or have no effect. In some studies, β-carotene and retinyl palmitate significantly increase the progression of lung cancer and aggressive prostate cancer. Protection against cardiovascular mortality or no effect of vitamin E has been reported, with an increase of all-cause mortality at dosages greater than 150 IU/day. Selenium showed beneficial effects on gastrointestinal cancer and reduced the risk of lung cancer in populations with lower selenium status. For multivitamin and mineral supplementation, no significant reduction of mortality or cancer incidence was observed, but some reports indicate a possible preventive effect in cervical cancer.
Conclusions The majority of supplementation studies indicate no variation of general mortality and of cancer incidence or a detrimental effect on both. Antioxidant supplements so far tested seem to offer no improvement over a well-balanced diet, possibly because of the choice of the substances tested or of an excessive dosage. However, new natural or synthetic compounds effective in vitro and in experimental studies might still be worth investigating in human trials.

Keywords Free radicals · Antioxidants · Aging · Cancer

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Introduction

The idea that aging could be the result of accumulated cell damage caused by free radicals originated with Harman more than 50 years ago [1] and has been a popular topic of investigation thereafter. Research in the 80s in the laboratory of Bruce Ames and others produced evidence indicating that oxidation damage and aging were strongly connected. A seminal paper, among many of that period, clearly showed that mice, rats, monkeys, and humans have typical metabolic rates that correspond to the level of oxidation damage in their DNA and proteins and correlate robustly and inversely with life span [2]. These observations stimulated a large effort on the basic mechanisms of the aging process, fueled by the hope of discovering natural

compounds or drugs able to decrease oxidation damage in cells and organisms, thereby increasing life span. The possible applications to public health of any discovery in this field seemed staggering.

Oxidative damage to DNA may also be implicated in carcinogenesis, since it causes mutations, and—if these occur in appropriate genes—cell transformation. On the basis of animal and cell experiments and epidemiological results, Peto, Doll, and others [3] first suggested β-carotene to be possibly anti-carcinogenic in humans and suggested supplementation trials with β-carotene. Consequently, many studies were carried out in different countries using carotenoids and various other antioxidants, including long-term clinical trials on all-cause and cancer mortality or with chronic disease incidence as outcome, and in addition, supplementation trials using various biomarkers of oxidation damage.

Evidence from observational epidemiology

Antioxidants and aging

Fruits and vegetables are a fundamental component of a healthy diet. Epidemiological studies have examined whether vegetarians have a longer life span than carnivores. A review of the existing studies on this topic in Europe and North America [4] concluded that most evidences (except for one instance, discussed in Fortes et al. [5]) show that long-term vegetarians have a significant increase in life expectancy over carnivores (average variation: 3.6-years, 95 % confidence limits (CL): 1.4–5.8). Antioxidants might be causally related to this effect, though the connection is clouded by many confounding factors.

Observational studies of body antioxidant levels in relation to mortality, cancer, and heart disease

Epidemiological evidence links a higher consumption of fruits and vegetables with a lower risk of cancer. This connection is fully discussed for all major cancer types in the *WCRF* publications [6, 7] and will not be reviewed again here. According to general scientific wisdom, the protective effect of fruits and vegetables is explained by the presence of antioxidants. Therefore, human population studies in which plasma antioxidant levels were measured are of particular interest, since it is possible to verify whether subjects in the lowest percentiles of concentration of some antioxidants (like vitamin A, C, E and β-carotene) have an increased risk of cancer and/or an altered incidence of cardiovascular accidents. However, most evidence comes from case–control studies, and this type of epidemiological study is prone to artifacts, since a variation of a marker related to dietary intake (e.g., a low plasma level of a specific antioxidant) could be an effect rather than a cause of a specific disease. Prospective studies are more reliable, in which disease incidence can be related to biomarkers measured years previously.

It is not the purpose of this commentary to review the whole, extensive body of literature on this topic. In this section, we will comment only on studies of relatively large population groups (>500), with a long follow-up time (>5 years) and a robust experimental structure; for a more detailed analysis, we refer to the many recently published reviews and meta-analyses on each substance.

One of the first publications on the relation between antioxidant vitamin levels and human health was a 12-year mortality follow-up of 2,974 participants (the "Basel Study", published in 1991), which showed a significant elevation of the relative risk (RR) for lung and bronchial cancer of subjects with low β-carotene plasma levels (<0.23 μmol/L); low plasma levels of β-carotene and vitamin A were associated with an increased risk of all types of cancer (RR: 2.47, $p < 0.01$); the risk of cerebrovascular death was also elevated with low plasma β-carotene and vitamin C (RR: 4.17, $p < 0.01$) [8].

In a study of 65 rural counties of China, published in 1992, it was also shown that plasma levels of some dietary antioxidants were correlated with cancer mortality. In particular, plasma levels of ascorbic acid were inversely correlated with the frequency of common cancers and low levels of selenium specifically with higher incidence of esophageal and stomach cancer. Higher levels of β-carotene (independently from retinol) had a protective effect, in particular for stomach cancer [9].

Greenberg et al. [10] followed a total of 1,188 men and 532 women for 8.2 years in the USA and reported a lower risk of death from all-causes (RR: 0.52; CL: 0.44–0.87) and from cardiovascular diseases (RR: 0.57; CL: 0.34–0.95) in subjects in the highest quartile of β-carotene who had been enrolled for the prevention of non-melanoma skin cancer.

It was later (1999) shown in an ecological study of 634 men (aged 40–49), sampled randomly from five regions in Japan, that plasma levels of β-carotene and α-tocopherol were inversely correlated with gastric cancer ($r = -0.31$ and -0.89, respectively) [11].

In 2006, partial results were published on a study involving 29,092 Finnish men (α-Tocopherol, β-Carotene Cancer Prevention (ATBC) Study [12]), showing that subjects in the highest quintiles of serum vitamin E had significantly lower risk of total, cause-specific and all-cause mortality, independently of supplementation (RR: 0.82; CL: 0.78–0.86).

Increased serum levels of vitamin C were also negatively correlated with gastric cancer among non-drinkers

and non-smokers in a study of 18,244 older men in Shanghai, China [13].

Among prostate cancer cases in the ATBC Study in Finland, subjects in the highest quintile of α-tocopherol at baseline had improved survival (RR: 0.51; CL: 0.20–0.90) [14]. In the SENECA study [15], after a follow-up of 10 years of 1,168 European elderly men and women, plasma carotene concentrations were associated with a low risk of mortality from cancer (RR: 0.59; CL: 0.44–0.79) or cardiovascular disease with a body mass index <25 (RR: 0.67; CL: 0.49–0.94).

Within the Physicians' Health Study (PHS) in the USA, a nested case–control study involving 1,286 cases and 1,267 controls showed an inverse relationship between high levels of selenium and prostate cancer mortality among subjects without a high-risk genotype (polymorphism of the selenoprotein SEP15 [16]).

A recent population-based study of 1,043 older adults living in Chianti, Italy, established that adults in the lowest quartile of plasma selenium at enrollment had a higher all-cause mortality compared with the highest quartile (RR: 1.60; CL: 1.04–2.47); adults in the highest tertile of plasma carotenoids at enrollment also had lower mortality compared with the lowest tertile (RR: 0.81; CL: 0.65–0.99) [17].

Recently, using data of the Third National Health and Nutrition Examination Survey in the USA, it was shown that among 13,393 participants, those belonging to the lowest plasma carotenoid quartile (<1.01 μmol/L) had significantly higher all-cause mortality (RR: 1.38, CL: 1.15–1.65) compared with the ones in the highest quartile (>1.75 μmol/L) [18].

In conclusion many studies, published over about two decades, conducted in different countries and on various populations, involving relatively large numbers of healthy or disease-carrying individuals, with a follow-up time sufficient to show delayed events, consistently indicated that higher body levels of antioxidant vitamins have a beneficial effect on health and, inversely, that low systemic levels are associated with a higher risk of several chronic diseases and/or mortality. However, it is not clear whether higher levels of antioxidant vitamins are important *per se* or are simply a marker of a well-balanced nutrition and of a healthy life-style. High levels of plasma β-carotene, vitamin C and E are in fact an indicator of the consumption of a diet rich in fruits and vegetables; accordingly, a Norwegian study indicated that plasma concentrations of α-carotene, β-carotene and lutein could be used accurately to assess fruits and vegetable intake [19]. However, it is also possible that healthier diets might contain additional active compounds which might have been disregarded in the studies published so far.

Observational studies based on biomarkers

Do higher levels of antioxidant intake correlate with lower levels of biomarkers of oxidative stress including oxidative damage to DNA? One possible answer to this question derives from the comparison of population groups with different intakes of fruits and vegetables.

Kazimirova et al. [20] compared groups of vegetarians and non-vegetarians, known from previous work to differ in plasma levels of antioxidants. Vegetarians ($n = 24$), with higher mean levels of vitamin C, β-carotene, and vitamin A compared with non-vegetarians ($n = 24$), had significantly ($p = 0.005$) lower levels of combined strand breaks (SBs) and oxidized purines (principally 7,8-dihydro-8-oxoguanine [8-oxoGua], measured with the comet assay plus formamidopyrimidine DNA glycosylase, FPG). The sub-group of lactovegetarians ($n = 11$) had markedly lower levels of oxidized pyrimidines (detected with endonuclease III) ($p = 0.0017$). There were no differences in frequencies of micronuclei or chromosome aberrations. Dhawan et al. [21] reported significantly ($p < 0.001$) lower levels of DNA SBs in vegetarians ($n = 26$) compared with non-vegetarians ($n = 36$). Similar groups were studied by Fenech and Rinaldi [22]; while vegetarians had higher plasma levels of vitamin C and folic acid, they were relatively low in vitamin B12 and had similar levels of vitamin E to non-vegetarians. There were no remarkable differences in micronucleus frequency between the two groups.

DNA strand breaks, oxidized purines or pyrimidines, conjugated dienes of fatty acids and carbonylated proteins were measured in young women aged 20–30 (46 vegetarians, 48 non-vegetarians) versus older women aged 60–70 (33 vegetarians, 34 non-vegetarians) [23]. In younger subjects, no differences in oxidative damage parameters or plasma β-carotene or vitamin C were observed between vegetarian and non-vegetarians. However, in older vegetarians significantly decreased DNA oxidation and lipid peroxidation were reported, with higher values of plasma vitamin C and β-carotene relative to the non-vegetarians ($p < 0.05$). Significant age-dependent increases in oxidative stress parameters were noted only in non-vegetarian ($p < 0.05$) [23]. A recent study of 100 healthy Indian subjects documented that serum malonialdehyde (MDA) levels were significantly increased ($p < 0.001$) in non-vegetarians compared with lacto-vegetarians and lacto-ovo-vegetarians; in this study, non-vegetarians had significantly lower levels of plasma glutathione peroxidase and vitamins A and E compared with lacto-vegetarians and lacto-ovo-vegetarians ($p < 0.001$) [24].

Another approach is to look for correlations between plasma micronutrients and markers of oxidative damage across a normal healthy population. Lymphocyte DNA

damage, in the form of oxidized pyrimidines, was measured with the comet assay in a group of healthy subjects and found to show a significant negative correlation with the concentration of total carotenoids ($p < 0.05$) [25]. Notably, there were no subjects with a high concentration of carotenoids and a high level of DNA base oxidation. It should, however, be noted that the plasma carotenoid concentration is a biomarker of consumption of fruits and vegetables, and so it could be some other component of the foods that is responsible for protecting the DNA. Giovannelli et al. [26] did not find any significant correlation between oxidized purines, measured with the comet assay, and plasma antioxidants (6 carotenoids, retinol, and α- and γ-tocopherol) in a healthy population. Another observational study using the comet assay was carried out by Dusinska et al. [27]: significant negative correlations were found between plasma lycopene and SBs plus oxidized pyrimidines ($r = -0.191$, $p = 0.051$) and SBs plus endonuclease III-sensitive sites ($r = -0.23$ $p = 0.016$) and between vitamin C and SBs plus oxidized purines ($r = -0.23$ $p = 0.016$). Trace elements were also measured; selenium and zinc both have roles in enzymic antioxidant defences, but while selenium showed a negative correlation with SBs + FPG, strand breaks plus FPG-sensitive sites DNA oxidation ($r = -0.75$, $p = 0.029$), zinc was positively correlated ($r = 0.78$, $p = 0.022$).

Experiments on aging

Following the early work in this field, it was shown that increases in 8-oxoGua and protein carbonyl content are associated with diminished life span in houseflies [28] and in mammals [29]; it was also demonstrated that *Drosophila melanogaster* age-resistant strains have enhanced superoxide dismutase (SOD) activity [30]; that human senescent fibroblasts have higher levels of 8-oxoGua in DNA; and that spin-trapping agents delay senescence in vitro [31]. More recently, overexpressing human catalase in transgenic mice, an increased life span and a reduced hydrogen peroxide production was observed [32].

The idea that oxygen damage is associated with cell aging is a sound result confirmed in a variety of living organisms. Later research has not challenged the theory connecting oxidation damage to aging, but has indicated that the process might be regulated by a complex system and not only by the steady state of intracellular oxidation. In fact, some strains of mice deficient in superoxide dismutase and glutathione peroxidase-1 have, as expected, increased oxidative damage, but no reduction in longevity. It is worth noting that tumor burden, but not incidence, is increased in these animals [33]. Recently, it has also been shown that specific mutants of *C. elegans*, characterized by slow metabolism and increased life span, have no increased resistance to oxidative stress or decreased oxidative damage, despite elevated levels of superoxide dismutase [34]. In these organisms, over-expression of SOD-1 seems to increase life span through the activation of longevity-promoting transcription factors [35].

A good example of the complexity of the aging processes is shown by the case of sirtuins. Over-expression of sirtuins was suggested as a mechanism of an increased life span in a variety of organisms. However, according to a recent report, these associations were due to the confounding effect of a varied genetic background. In fact, using more stringent genetic controls, no correlation was observed between over-expression of sir-2.1 and life span extension in *C. elegans* and *D. melanogaster* [36].

Considering results on life span extension by antioxidants and other supplements, despite a large body of published research, only a few controlled studies report convincing evidence. Among these, we can quote experiments on vitamin E in rats [37]; vitamin C and β-carotene in mice [38, 39]; vitamin E in *Paramecium tetraurelia* [40]; butylhydroxyanisole (BHA) in *Callosobruchus maculatus* [41]; the spin-trapping agent N-tert-α-phenyl-butylnitrone and β-catechin in senescence-accelerated mice [42, 43]; antioxidant vitamins in exercising rats [44]; Chinese traditional herbs in *D. melanogaster* [45]; and synthetic superoxide dismutase/catalase-mimetics in *C. elegans* [46].

Intervention studies based on biomarkers

There are many reports of studies in which subjects have been given antioxidant supplements and biomarkers of oxidative stress have been measured. It is helpful first to outline the various approaches taken.

The design of the study can be a single intervention, samples being taken at appropriate intervals over a day or so, or a trial of one to several weeks with (normally) daily administration of antioxidant(s). The simplest design of repeated intervention trial involves taking samples before and after the intervention. However, this is not recommended; a parallel group of matched subjects receiving a placebo should be run alongside the supplemented group to act as a control. The best design is probably the placebo-controlled crossover, in which two matched groups receive either placebo or supplement in a first phase, and then—after a washout period—the roles are reversed in phase two, so that all subjects ultimately have the same treatment.

Subjects can be healthy, or suffering from some disease or at risk of disease. Results with healthy individuals are more generally relevant. On the other hand, it is supposed that larger effects of antioxidants might be expected in subjects suffering from oxidative stress resulting from disease (though the supposition has not been confirmed by results).

Supplements can be either isolated antioxidants (or mixtures of antioxidants), or foods or drinks rich in antioxidants; the latter approach is more realistic, but less easy to carry out and the outcome is less easy to analyze because of the complexity of the food matrix.

Biomarkers of in vivo oxidative damage include lipid peroxidation products [F2-isoprostanes or malondialdehyde (MDA)] measured in blood or urine; protein carbonyls in plasma; oxidized DNA bases in lymphocytes; and oxidized nucleosides in urine. Questions are frequently raised concerning the validity of these biomarkers. Particular scrutiny was given in the ESCODD project [47] to 8-oxoGua (or its deoxyribonucleoside 8-oxodGuo) measured in the DNA of blood cells, because it was clear that reported levels of this lesion varied by orders of magnitude depending on the method used. Chromatographic methods are prone to oxidation of DNA during sample preparation, which results in a serious artifact. The comet assay and other methods that rely on release of 8-oxoGua by digestion with FPG seem not to suffer from this problem and give more accurate (though less precise) estimates of DNA damage. Reports of background damage levels exceeding about three 8-oxoGua per million guanine (Gua) in healthy subjects should be considered of doubtful value (and are not included in this review).

A distinct biomarker strategy is the ex vivo approach in which material (lymphocytes, plasma, etc.) is taken from the subjects before and after supplementation and tested for resistance to oxidation. H_2O_2 is a common challenging agent, inducing SBs in cellular DNA, the frequency of which reflects antioxidant status. The rate of oxidation of plasma lipids by ferrous iron and ascorbate is another indicator of susceptibility.

A comprehensive review of studies with biomarkers of DNA oxidation has been published by Møller and Loft [48]. Here, we will present just a few examples of repeated intervention trials.

In one of the first trials to use the comet assay to measure oxidized bases, a daily supplement of vitamins C and E and β-carotene or a placebo was given to smokers and non-smokers (25 in each group). After 20 weeks, significantly ($p < 0.002$) less oxidation of pyrimidines in lymphocyte DNA was seen in the supplemented groups, and their DNA was less damaged by H_2O_2 in vitro [49]. A four-week trial with vitamins C and E or placebo resulted in significant decreases in endonuclease III- and FPG-sensitive sites when vitamin C was administered in a slow release form, but not as a standard tablet [50]. In a crossover designed trial with different 'doses' of kiwifruit, significant decreases occurred in oxidized purines and pyrimidines in lymphocyte DNA, together with increases in resistance to H_2O_2 ($p < 0.01$) [51].

In another study, 246 women were randomly assigned to receive either 3.6 or 9.2 servings of fruits and vegetables per day for 2 weeks and a significant reduction in the urinary excretion of 8-iso-PGF2-α was observed in subjects on the high fruits and vegetables diet ($p < 0.01$) [52]. Purine oxidation in lymphocyte DNA was reported to be significantly decreased after one kiwifruit per day ($p < 0.01$); pyrimidine oxidation decreased after two fruits per day ($p < 0.05$) [53]. Consuming a portion of steamed broccoli (250 g/day) was recently associated with increased protection against H_2O_2-induced DNA SBs and lower oxidized DNA bases in lymphocytes from 27 young healthy smokers [54].

In contrast, a diet containing 600 g/day of fruits and vegetables (or the equivalent in antioxidants) [55] showed no effect; 2 months of dietary supplementation with vitamin C and vitamin E had also no significant effect on oxidative DNA damage measured as urinary 8-oxodGuo in non-smoking adults [56]. Various dosages of α-tocopherol for 8 weeks followed by an 8-week washout period had no effect on urinary lipid peroxidation in healthy adults [57].

There are some examples of trials with patients/stressed subjects: Vitamin C and E supplementation for 6 weeks in a small group of children with familial hypercholesterolemia restored endothelial function but not biomarkers of oxidative stress (antibodies to oxidized LDL, F2-isoprostanes, urinary 8-oxodGuo) [58]. In postmenopausal breast cancer survivors from the Women's Healthy Eating and Living Study, a significant inverse association was found between total plasma carotenoid concentrations and urinary 8-oxodGuo and lipid peroxidation, ($p = 0.001$) but no protection by carotenoid supplementation [59]. A decrease in plasma F2-isoprostanes was found in 396 obese non-smokers who received vitamin C, vitamin E or placebo for 2 months, but only when baseline F2-isoprostane levels were >50 μg/mL ($p = 0.01$) [60]. Type 2 diabetes patients, given a flavonol (quercetin)-rich diet for 2 weeks in a crossover design, showed an increase in resistance to H_2O_2 attack on DNA ($p = 0.037$), but no effect on endogenous oxidized pyrimidines [61]. In a trial of 37 healthy, non-smoking postmenopausal women, randomly assigned to consume mixed carotenoids (beta-carotene, lutein, and lycopene; 4 mg each), 12 mg of a single carotenoid (β-carotene, lutein or lycopene), or placebo for 56 days, all carotenoid-supplemented groups showed significantly lower endogenous DNA damage (DNA breaks measured by the comet assay) than at baseline ($p < 0.01$), but there was no protection against H_2O_2-induced breaks [62].

Recently, 49 overweight, postmenopausal women were assigned to consume 2, 5, or 10 servings of fresh vegetables for 3 weeks with a 4-week washout period. Plasma carotenoids increased with number of servings, (overall $p < 0.001$); however, urinary concentrations of 8-isoprostane F2α, hexanoyl lysine, and serum C-reactive protein (all indicators of oxidative stress) were not affected [63].

Although a few years have elapsed, we may still conclude this section with the comments of Møller and Loft [48]. They reviewed repeated dose intervention trials measuring a variety of biomarkers of oxidative damage to DNA. Half of these were classified as having good design and realistic baseline values for DNA oxidation. None of the studies reported an increase in DNA damage, though many showed no effect. The reasons for null effects remain obscure; but Møller and Loft emphasized that 'reduced levels of oxidatively damaged DNA in white blood cells… were reported in far more studies than expected by chance alone.'

Intervention studies with clinical endpoints

The possibility that the administration of antioxidant vitamins might decrease cell oxidative damage, thereby decreasing the incidence of degenerative diseases and cancer, was investigated with enthusiasm in the 90s and motivated many clinical studies. In fact, experimental work indicated a tumor-preventive effect of some antioxidants in animal models (see the review by Moon, [64]). An article published at the end of the century [65] listed about 50 promising compounds for future clinical trials on the basis of the literature published at that time.

A detailed review of this field is not the scope of the present commentary. However, it is evident that epidemiological considerations had their place in the choice of chemo-preventive compounds. Since a high consumption of fruits and vegetables was documented to affect common forms of cancers, it was reasonable to assume that antioxidant vitamins abundant in vegetables and fruits would have an effect on their own and that their chronic administration as supplements might result into a chemo-preventive effect in clinical trials [66].

Reviewing this material and the many review articles published in that period, it is hard to ignore the widespread assumption that natural antioxidants and vitamins had low inherent toxicity; therefore, clinical trials seemed to have few risks and possible big gains. In trials aimed at inhibiting cancer, also all-cause mortality and cardiovascular mortality was often recorded. These additional data are clearly of interest for the evaluation of the overall health effects of these supplementations.

Out of the large body of literature reporting results on intervention studies with clinically relevant endpoints, we selected for the present critical evaluation only data obtained from relatively large clinical trials (>1,000), with a long follow-up (>3 years), published up to 2011. We examined cardiovascular and all-cause mortality and cancer incidence/mortality as endpoints. Our aim was not to review in detail all the existing literature, a task accomplished by many available reviews and meta-analyses, but to comment on the most relevant information and suggest some general explanations for the results obtained.

Effects on mortality

α-tocopherol and β-carotene

One of the first studies involved 1,720 subjects in a program for skin cancer prevention (SCPS) in the USA; it was published in 1996 [10]. β-carotene (50 mg) was administered daily for 4.3 years and after a follow-up of 8.2 years no variation was reported for all-cause mortality (RR: 1.03; CL: 0.82–1.30). Similar results were reported in 1996 by the Physicians' Health Study (PHS) [67], with the same dosage of β-carotene administered to 22,071 subjects on alternate days for 12.9 years, with a follow-up of 6.1 years (RR: 1.01; CL:0.93–1.1).

On the contrary, a clear detrimental effect was reported in the ATBC study in Finland, involving 1,862 males with previous myocardial infarction and 1,057 with previous stroke, administered α-tocopherol (50 mg) and/or β-carotene (20 mg) daily for 5.3 years [68, 69]. In this study the RR for total cardiovascular mortality with α-tocopherol was 1.33 (CL: 0.86–2.05) and for the combined treatment 1.58 (CL: 1.05–2.4). The same group reported [70] an increased risk for all-cause mortality on 29,133 male smokers with the same combined treatment for 6.1 years and a follow-up of 12.1 years (RR: 1.5; CL: 1–2.25).

The CARET study in the USA (2004), administering β-carotene (30 mg) and retinyl palmitate (25,000 IU) daily, singly or combined, for 6.1 years, ended up after 12.1 years recording a non-significant variation in all-cause mortality (RR: 1.08; CL: 0.99–1.17) [71].

It is also worth citing a large a cluster trial in Bangladesh published recently on 125,257 pregnant and postpartum women, in which retinol (7 mg) and β-carotene (42 mg) were administered, reporting no significant variation in maternal and newborn mortality (retinol: 1.15; CL: 0.75–1.76 and β-Carotene: 1.21; CL: 0.81–1.81) [72].

A clear protection against cardiovascular mortality was observed in the early CHAOS study in the UK (1996), on 2,002 subjects administered 400–800 IU vitamin E daily for 366 or 731 days and followed for 510 days (RR: 0.53; CL: 0.34–0.83) [73]. This study created strong expectations on the preventive effects of vitamin E in cardiovascular mortality, but unfortunately later studies did not confirm this favorable effect: the GISSI in Italy (1999), administering 300 mg vitamin E for 3.5 years to 2,830 infarction patients, reported a RR of 0.86; CL: 0.72–1.82) [74]; the HOPE study in Europe and the Americas (published in 2000) on 9,541 subjects administered 400 mg vitamin E for 4.5 years, followed for 6 years, also did not show effects on mortality (RR: 1.05; CL: 0.9–1.22) [75]; all-cause

mortality was not varied in a large USA study (WHS) published in 2005 on 38,876 women administered vitamin E (400 IU) for 4.5 years (RR: 1.04; CL: 0.93–1.12) [76].

In 2005, a meta-analysis was published of 19 clinical trials of vitamin E supplementation, ranging from 16.5 to 2,000 IU/day, in which a relationship between the dosage of vitamin E administered and all-cause mortality was apparent, with significant increased risk with dosages greater than 150 IU/day [77].

Multivitamins and minerals

Controlled trials of with multivitamins and mineral supplementation devoid of apparent bias are comparatively fewer. An early large intervention study (1993) in China, administering various mixes of retinol, zinc, riboflavin, niacin, ascorbic acid, molybdenum, selenium, α-tocopherol, and β-carotene, registered a significant reduction of mortality only for the combination of the last three supplements (50 μg, 30 mg and 15 mg, respectively) in the group of subjects younger than 55 (RR: 0.88; CL: 0.82–0.95) [78].

A study on 4,753 participants with ocular disorders conducted in the USA (AREDS) with vitamin C (500 mg), vitamin E (400 IU), β carotene (15 mg), and zinc oxide (80 mg), supplemented daily, reported in 2004 after 6.5 years of follow-up that only participants randomly assigned to receive zinc had lower mortality (RR: 0.73; CI: 0.61–0.89). No significant effects were reported for the other supplements (RR: 1.15; CL: 0.90–1.48) [79].

A trial in the USA on folic acid (2.5 mg), vitamin B_6 (50 mg), and vitamin B_{12} (1 mg) on 5,442 health professional women at risk [80] reported in 2008 no variation of cardiovascular mortality (RR: 1.01; CL: 0.76–1.35).

Two studies with daily supplementation of selenium (200 μg) in the USA, the first on 1,312 patients with skin carcinoma and the second on 1,004 healthy subjects, reported no significant changes in all-cause mortality (RR: 0.83; CL: 0.63–1.08 and 1.22; CL: 0.76–1.95, respectively) [81, 82].

Finally, in 2010, it was reported that in a large French study involving 12,741 subjects (SUVIMAX), supplementing with ascorbic acid (120 mg), vitamin E (30 mg), β-carotene (6 mg), selenium (100 μg), and zinc (20 mg) for 7 years, with a follow-up of 5 years, there was no significant effect on all-cause mortality (RR: 0.98; CL: (0.75–1.26) [83].

Effects on cancer

β-carotene, retinyl palmitate, vitamin E, vitamin C, and selenium

One of the earliest documented effects of these supplements originates in the Physicians Health Study in the USA. According to a paper published in 1996, β-carotene (50 mg) on alternate days for 12.9 years, with a follow-up of 6.1 years, administered to 22,071 American physicians, did not vary the occurrence of cancer (RR 0.98; CL: 0.91–1.06) [67].

The results of a study in the UK (MRC/BHF), published in 2002, reported that vitamin E (600 mg), vitamin C (250 mg and β-carotene (20 mg), administered daily to 20,536 adults with coronary disease and diabetes for 5 years and a follow-up of 7 years, similarly had no effect on the occurrence of cancer (RR: 0.98; CL: 0.89–1.08) [84].

In the ATBC trial in Finland on 29,133 male smokers, with daily supplements of β-carotene (20 mg), α-tocopherol (50 mg), or both, lung cancer risk was not varied by α-tocopherol (RR: 0.99; CL: 0.87–1.13), but β-carotene significantly increased risk (RR: 1.16, CL: 1.02-1.33); the effect was more evident in heavy smokers (RR:1.25; CL: 1.07–1.46) and heavy drinkers (RR: 1.35; CL: 1.01–1.81) [85, 86]. For prostate cancer incidence ($n = 672$), the RR was 0.88 (CL: 0.76–1.03) for participants receiving α-tocopherol [87–89].

In parallel, the results of the CARET study in the USA were published in 2004; daily supplementation of 18,318 smokers and asbestos-exposed subjects for 10 years with β-carotene (30 mg) and retinyl palmitate (25,000 IU), with a follow-up of 11 years, resulted in a significant increase in lung cancer (RR:1.28; CL: 1.04–1.57). Also an increase of aggressive prostate cancer was reported (RR: 1.52; CL: 1.03–2.24) [89, 90].

On the contrary, the previously quoted WHS group in the USA, supplementing 39,876 women daily for 2.1 years with β-carotene on, published in 2002 that the frequency of total cancer was not altered (in the highest quartile of plasma β-carotene the RR was 1.34 (CL: 0.81–2.22) [91].

A recent meta-analysis [92] considered the effect of β-carotene and related compounds on cancer in 9 randomized trials. No effect of β-carotene supplementation was observed on the incidence of all cancer (RR: 1.01; CL: 0.98–1.04). The incidence of lung and stomach cancers were significantly increased by β-carotene (RR: 1.16; CL: 1.06–1.27 and 1.34; CL: 1.06–1.70, respectively). Fritz et al. [93] analyzed 248 studies on treatment, primary and secondary prevention of lung cancer after supplementation. Although some studies demonstrated significant benefits, there was no overall convincing evidence for the efficacy of vitamin A or related retinoids in the treatment or prevention of lung cancer. Jeon et al. [94] recently reviewed 848 articles for a total of 40,544 participants, of which 20,290 were in β-carotene supplement groups and 20,254 in placebo groups. β-carotene supplements had no significant preventive effect on cancer incidence or mortality.

In conclusion, historic data and recent meta-analyses confirm that β-carotene and related compounds have no positive role in the prevention or treatment of cancer.

No significant protection of vitamin E against cancer was reported by the HOPE study in America and Europe [2000 with vitamin E (400 IU) daily × 4.5 years, follow-up 7 years, with 3,994 subjects in the intervention group and 738 in the passive follow-up; RR for all cancer: 0.94; CL 0.84–1.06] [75].

In 2005, the results of the WHS in the USA, in which Vitamin E (600 IU) was administered daily × 4.5 years to 39,876 women >45 years, similarly reported no effect on cancer incidence (RR: 1.01; CL: 0.94–1.08) [91].

The large PHS in the USA [95], administering vitamin C (400 mg) or vitamin E (400 mg) on alternate days to 14,641 physicians, starting in 1997 and completed in 2007, recorded no variation of prostate cancer incidence (RR: 0.97; CL: 0.85–1.09) or total cancer incidence (RR: 1.04; CL: 0.95–1.13) with vitamin E or with vitamin C supplementation (RR for prostate cancer: 1.02 (CL: 0.90–1.15; RR for total cancer: 1.01; CL: 0.92–1.10).

The report of the SELECT study in the USA (2011), administering selenium (200 μg) and vitamin E (400 IU) daily, alone or in combination, to 35,533 older men, showed on the contrary a slight, but significant, increase in the risk of prostate cancer with vitamin E (RR: 1.17; CL: 1004–1.36) and no variation with selenium (RR: 1.09; CL: 0.89–1.22) [96].

Bjelakovic et al. [97] published a meta-analysis on 20 randomized trials (including 211,818 participants), assessing beta-carotene (12 trials), vitamin A (4 trials), vitamin C (8 trials), vitamin E (10 trials), and selenium (9 trials). Antioxidant vitamin supplements were without significant effects on gastrointestinal cancers (RR: 0.94; 95 % CI 0.83–1.06).

Recently, Papaioannou et al. [98] reviewed twelve studies on the effects of vitamin A, C, and E, selenium, and β-carotene antioxidants in the chemoprevention of colorectal cancer and adenomas; in the nine studies comparing antioxidants with controls (recruited subjects: 148,922), there was no difference in the incidence of colorectal cancer (RR: 1.00, CL: 0.88–1.13). Of the 14 combinations of antioxidants employed, one reported a statistically significant increase in relative risk of adenoma after vitamin E (RR 1.74, CL: 1.09–1.79) or vitamin E plus β-carotene (RR 1.63, CL: 1.01–2.63).

Promising results on selenium alone were reported in 2002 by the Nutritional Prevention of Cancer Trial (NPCT) on 1,312 healthy USA subjects, using selenium (200 μg) daily for 4.5 year with a follow-up of 6.4 years. At the end of the treatment, selenium supplementation reduced significantly total cancer (RR: 0.75; CL: 0.58–0.97) and prostate cancer incidence (RR = 0.48; C: 0.28–0.80) [99].

A recent meta-analysis, considering the results of fifteen human studies of supplementation, concluded that selenium may reduce risk of lung cancers in populations with lower baseline selenium status (serum <106 ng/mL); however, an increase of lung cancer risk is observed with higher selenium serum levels (≥121.6 ng/mL). No statistically significant effects were reported for other cancers (RR: 1.51; CL: 0.7–3.24) [100].

According to another analysis of Bjelakovic et al. [97], selenium seemed to show significant beneficial effects on gastrointestinal cancer occurrence (RR: 0.59; CL: 0.46–0.75).

Multivitamins and minerals

During an early study in China (NIT), retinol (5,000 IU), zinc (22.5 mg), (factor A); riboflavin (3.2 mg), niacin (40 mg) (factor B); ascorbic acid (120 mg), molybdenum (30 μg) (factor C), selenium (50 μg), α-tocopherol (30 mg), β-carotene (15 mg) (factor D) were administered to 29,584 adults for the prevention of gastric and esophageal cancer for 6 years. The published report in 1994 showed no statistically significant effects of any treatment combination [101].

The WACS study in the USA, using ascorbic acid (500 mg), vitamin E (600 mg), β-carotene (50 mg), on alternate days on 8,171 health professional women, with a follow-up of 9.4 years, recorded no variation in total cancer with vitamin C (RR: 1.11; CL: 0.95–1.30), vitamin E (RR: 0.93 0.79–1.09) or β-carotene (RR: 1.0; CL: 0.85–1.17) [102]. The same group reported in 2008 that folic acid (2.5 mg), vitamin B6 (50 mg) and vitamin B12 (1 mg) daily, with a follow-up of 7.3 years, administered to 5,442 women, did not vary significantly the risk of total cancer (RR: 0.97; CL: 0.79–1.18) [103].

The previously mentioned SUVIMAX study in France, administering ascorbic acid (120 mg), vitamin E (30 mg), β-carotene (6 mg), selenium (100 μg), and zinc (20 mg) daily for 7 years to 12,741 subjects, with a follow-up of 5 years, registered no variation of total cancer (RR: 0.98; CL: 0.75–1.27) and a significant increase in skin cancer in females (RR:1.7; CL: 1.08–2.67) [83].

Myung and coworkers [104] recently published a meta-analysis on 22 case–control studies for a total of 10,073 participants, showing a significant preventive effect on cervical cancer for subjects supplemented with vitamin B_{12} (RR :0.35; CL 0.19–0.63; $n = 2$), vitamin C (RR 0.67, CL: 0.55–0.82; $n = 8$), vitamin E (RR: 0.56; CL: 0.35–0.88; $n = 10$), and β-carotene (RR: 0.68, CL 0.55–0.84; $n = 9$).

Conclusions

A summary review of the evidence obtained from major clinical trials with antioxidant vitamins and minerals (β-carotene, α-tocopherol, vitamin E, various vitamins and minerals, and selenium) indicates that most studies ended

up with no significant variation in all-cause mortality. All-cause mortality is a robust indicator of effects on human health and is subject to little experimental error. In some supplementation studies, a significant increase in mortality was observed for certain antioxidants. In fact, a meta-analysis of 19 clinical trials of vitamin E supplementation, ranging from 16.5 to 2,000 IU/day, showed a relationship between the dosage of vitamin E administered and all-cause mortality, with significantly increased risk with dosages greater than 150 IU/day [77].

The main conclusions of this commentary regarding the effects of antioxidant vitamins on mortality coincide with the meticulous analysis of the Cochrane collaboration study on antioxidant supplementation [105]. This extensive meta-analysis included evidence published up to 2005 and was based upon forty-seven trials with a low risk of systematic bias, including a total of 180,938 participants. According to their report, antioxidant supplementation significantly increased mortality (RR: 1.05, CL: 1.02–1.08). In the same publication, the analysis of separate effects showed a significantly increased mortality by supplementation with vitamin A (RR: 1.16, CL: 1.10–1.24), β-carotene (RR: 1.07, CL: 1.02–1.11) and vitamin E (RR: 1.04, CL: 1.01–1.07), but no significant benefit or detrimental effect of vitamin C (RR: 1.06, CL: 0.94–1.20) or selenium (RR: 0.90, CL: 0.80–1.01).

Considering the effects of antioxidant supplementation on cancer incidence in clinical trials, it is clear that β-carotene supplementation significantly increased lung cancer incidence and aggressive prostate cancer [85–90].

Selenium on the contrary showed significant beneficial effects on gastrointestinal cancer and reduced the risk of lung cancer in subjects with lower baseline selenium status [99, 100].

For multivitamin and mineral supplements, no reduction in cancer incidence was observed, but some reports, needing to be confirmed, indicate a possible beneficial effect in cervical cancer [104].

As a whole, the evidence available shows sometimes a detrimental but mostly a null effect on mortality and on cancer incidence of supplements with β-carotene, α-tocopherol, vitamin E, various vitamins and minerals with the possible exception of selenium in individuals with low intake.

It is difficult not to regard this enormous amount of work as a disappointment. We can advance a series of hypotheses to explain it.

(A) The dosages of antioxidants in the supplementation regimens were possibly too high. The case is well illustrated by β-carotene. In some trials, initial β-carotene plasma levels were low, given the unbalanced diet of the test populations (such as in Linxian, China, 5.9–6.8 $\mu g \times dL^{-1}$) [101]. In contrast, in countries with adequate or excessive nutrition, the plasma levels of β-carotene were considerably higher (10–34 $\mu g \times dL^{-1}$) [106–108]. Although in these studies, a plasma level-related effect was not detectable or statistically significant, it is possible that a supplementation with additional β-carotene might have exerted a detrimental effect in well-fed populations. This could be the case for β-carotene, vitamin E, and α-tocopherol, which have U-shaped dose–response curves.

(B) The effect of food is complex and some disease-preventive effects of an optimal diet may not be reproduced with selected purified ingredients. Administering supplements instead of improving the diet is a shortcut that might not provide an adequate solution to dietary imbalances.

(C) In different human populations, the association between a low/high level of antioxidant vitamins and the increase/decrease of a specific pathological process might be the indicator of a bad/good nutritional status and not causally connected with antioxidant vitamins themselves.

(D) The choice of specific antioxidant supplements in many intervention studies might not have been ideal. The list of natural antioxidants with possible health-promoting effects is long, and the chosen supplements might not have been the best.

(E) The hypothesis suggesting beneficial effects of increasing *ab-externo* cellular antioxidants might be simplistic. Increasing levels of antioxidants may actually have deleterious effects on cellular functions. For instance, in carcinogenesis, some recent evidence indicates that excessive oxidative damage might limit the growth of cancer cells through an increased apoptotic rate in the tumor or its metastases [109]. Decreasing oxidative damage with externally administered compounds at relatively high dosage may have detrimental effects, possibly increasing the growth of tumors. An increased incidence of cancer decreases life span, given its fundamental role in human mortality. At present, it is generally agreed that intracellular free radicals have important signaling functions, which might be needed for optimal cell homeostasis and cancer control.

(F) Although some antioxidants increase cell survival in vitro, this does not necessarily mean an increase in life span by chronic administration in animals or humans, as demonstrated by the failure of resveratrol administration to extend life span in mice [110].

(G) A preventive effect of antioxidant vitamins in chemically induced tumors of experimental animals may not automatically translate into an effect on spontaneous human cancer.

The contrast between the generally null or deleterious health effects of antioxidant vitamins and the protective effects of antioxidants shown in many studies as a decrease in the level of oxidation damage also demands explanation. At least in healthy individuals, the intrinsic antioxidant defences are sufficient to keep oxidation damage at a very low level, which likely reflects a tolerable, or even desirable balance. After all, as discussed above, evidence is accumulating that we need a certain amount of oxidative stress, since reactive oxygen is involved in various metabolic processes including the inflammatory response and cell signaling. A decrease in DNA oxidation might not have favorable effects if accompanied by imbalances in cell metabolism.

In conclusion, the efforts to find in the antioxidant vitamins the pill of eternal youth, protecting against cancer and cardiovascular diseases, have produced no striking results. Millions of people ingest the antioxidant supplements described in this commentary, convinced that they will improve their health and survival. Often these supplements are consumed at mega-doses, with probably more detrimental effect. As nutrition scientists, we should warn health authorities not to encourage, or even positively to discourage, these practices. An official warning to consumers was recently issued by the Ministry of Health of Italy [111].

At present, more than 1,700 clinical studies with antioxidant vitamins are recorded in PubMed. We hope that new agents will be more successful, but more information about the mechanisms of action and the effective dosages of these new compounds in experimental systems would improve the chances of finding useful human applications and reducing their possible harm.

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