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Review Article

Clinical trials of antioxidants as cancer prevention agents: Past, present, and future

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ABSTRACT

The purpose of this review is to summarize the most important human clinical trials of antioxidants as cancer prevention agents conducted to date, provide an overview of currently ongoing studies, and discuss future steps needed to advance research in this field. To date there have been several large (at least 7000 participants) trials testing the efficacy of antioxidant supplements in preventing cancer. The specific agents (diet-derived direct antioxidants and essential components of antioxidant enzymes) tested in those trials included β-carotene, vitamin E, vitamin C, selenium, retinol, zinc, riboflavin, and molybdenum. None of the completed trials produced convincing evidence to justify the use of traditional antioxidant-related vitamins or minerals for cancer prevention. Our search of ongoing trials identified six projects at various stages of completion. Five of those six trials use selenium as the intervention of interest delivered either alone or in combination with other agents. The lack of success to date can be explained by a variety of factors that need to be considered in the next generation research. These factors include lack of good biological rationale for selecting specific agents of interest; limited number of agents tested to date; use of pharmacological, rather than dietary, doses; and insufficient duration of intervention and follow-up. The latter consideration underscores the need for alternative endpoints that are associated with increased risk of neoplasia (i.e., biomarkers of risk), but are detectable prior to tumor occurrence. Although dietary antioxidants are a large and diverse group of compounds, only a small proportion of candidate agents have been tested. In summary, the strategy of focusing on large high-budget studies using cancer incidence as the endpoint and testing a relatively limited number of antioxidant agents has been largely unsuccessful. This lack of success in previous trials should not preclude us from seeking novel ways of preventing cancer by modulating oxidative balance. On the contrary, the well demonstrated mechanistic link between excessive oxidative stress and carcinogenesis underscores the need for new studies. It appears that future large-scale projects should be preceded by smaller, shorter, less expensive biomarker-based studies that can serve as a link from mechanistic and observational research to human cancer prevention trials. These relatively inexpensive studies would provide human experimental evidence for the likely efficacy, optimum dose, and long-term safety of the intervention of interest that would then guide the design of safe, more definitive large-scale trials.

Contents

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Introduction

Cancer causes an estimated one in four deaths in the United States [1] and one in eight deaths worldwide [2]. The global burden of cancer more than doubled during the past 30 years with 2008 estimates of over 12 million new cases and 25 million persons alive with the diagnosis of cancer [3]. There is compelling, albeit indirect, evidence that a large proportion of cancers could be prevented through modifiable lifestyle-related risk factors such as smoking, obesity, physical activity, and diet [4]. Many of these lifestyle-related factors affect carcinogenesis through oxidative stress that occurs as a result of damage induced by reactive oxygen and nitrogen species (RONS), which produce potentially mutagenic DNA damage [5–8]. Recently, the theory of oxidative stress was refined to account for an alternative mechanism—a disruption of thiol-redox circuits, which leads to aberrant cell signaling and dysfunctional redox control without involving RONS-induced macromolecular damage [9,10].

Many of the lifestyle and dietary factors act as potent prooxidants. Inhaled tobacco smoke is considered a powerful exogenous prooxidant since high concentrations of RONS are present in both its tar and gas phases [11]. The direct increase in the oxidative burden of inhaled tobacco smoke can be further enhanced through the secondary oxidative stress due to inflammation [12]. Dietary fat is a well-documented contributor to oxidative stress through increased lipid peroxidation [13,14]. Red meat is rich in fat, and its consumption is hypothesized to intensify oxidative stress via increased intake of heme iron, which catalyzes the oxidation of ascorbate and the production of highly reactive hydroxyl radicals via the Haber-Weiss reaction [15,16]. It is also possible that heme iron may increase the risk of cancer via other mechanisms, such as the activation of redox-sensitive transcription factors including NF-kB, AP-1, and p53 [17], and the endogenous production of carcinogenic *N*-nitroso compounds [18,19].

Lifestyle and especially diet can also serve as important sources of antioxidants. *In vitro* studies demonstrate that certain micronutrients counteract the effects of RONS and oxidative stress-inducing inflammation by various mechanisms, and may reduce DNA oxidation [20] as well as mutagenicity as reflected in the Ames test [21–23] or mutagen sensitivity assays [24]. Animal and *in vitro* studies also demonstrated the effects of dietary antioxidants on the cell cycle in a variety of tissues including the epithelium of the lung, colon, prostate, and breast—the four most common sites of carcinoma in humans [25–31]. These observations led to a conclusion that supplementation with antioxidant micronutrients may help prevent cancer. As a result, the use of antioxidant supplements in various forms and combinations has become widespread; it was reported that about 30% of healthy adults and up to 87% of cancer patients in the developed countries regularly take antioxidant supplements [32,33].

In general, an antioxidant is defined as a compound that "when present at low concentrations compared to that of an oxidizable substrate significantly delays or inhibits oxidation of that substrate" [34]. With respect to their mechanism of action, antioxidants are divided into two major groups: enzymatic and nonenzymatic. For the purposes of cancer chemoprevention much of the emphasis has been on diet-derived compounds that act through nonenzymatic mechanisms [35]; however, enzymatic agents have also received considerable attention because the activity of antioxidant enzymes depends on the intake of trace metals (most notably selenium, molybdenum, copper, and zinc) [36–39].

Despite the pervasive use of antioxidant supplements, most of the claims about their beneficial effects in humans are based on biochemical *in vitro* assays or animal experiments rather than human studies [40,41]. It is important to emphasize, however, that definitive evidence about the effects of agents on human health (whether harmful or beneficial) can only be established from human studies [42]. When the effect in question is claimed to be beneficial (as in the case of antioxidants), the gold standard study is a randomized, controlled trial [43].

The purpose of this article is to describe the state-of-the-science on the preventive effects of various antioxidants in relation to cancer with an emphasis on randomized trials. This subject has been addressed in several recent comprehensive reviews and meta-analyses [33,44–55] evaluating different aspects of research in this area, and, therefore, another review focusing solely on previously accumulated evidence would be somewhat redundant. Moreover, an exhaustive meta-analysis of all possible research questions addressed in previous trials would be difficult to carry out due to the heterogeneity of interventions and doses tested and the multitude of disease outcomes evaluated. For all of the above reasons, we chose an alternative approach that aims to summarize the most important and influential studies conducted in the past, provide an overview of currently ongoing trials, and discuss future steps needed to advance the science of oxidative stress and the use of antioxidants in relation to cancer prevention. As we report our observations, we expect that a summary of previous studies presented in chronological order will provide a contemporary view on how this field of science developed and matured, an appraisal of the ongoing research will offer a preview of the evidence expected in the next few years, and a discussion of the future steps will inform the planning and design of new trials.

Cancer prevention trials of antioxidants reported to date

A summary of 11 large (at least 7000 participants) trials that tested the effects of antioxidant-related supplements (diet-derived direct antioxidants and essential components of antioxidant enzymes) on cancer incidence or mortality is presented in Table 1. Seven of these trials were conducted in North America, three in Europe, and one in China, with the years of publication ranging from 1993 to 2009. The specific agents tested in those studies included, in descending order of frequency: β-carotene (9 trials), vitamin E (8 trials), vitamin C (5 trials), selenium (3 trials), retinol and zinc (2 trials each), and riboflavin and

Table 1
Completed clinical trials of antioxidants as cancer prevention agents (intent-to-treat results either reported or calculated from the data in the original articles).

Study name, location	Intervention and primary outcome	Study population	References	Cancer sites	RR (95% CI)	Comparison
Physician Health Study (PHS), USA	Intervention: 2×2 design of aspirin (325 mg) and β-carotene (50 mg qOD) or both vs placebo Primary outcome: CVD and total cancer incidence	22,071 healthy male physicians 40–82 years of age followed for up to 13 years	Cook et al. [61]	All sites	1.0 (0.9–1.0)	β-carotene vs placebo
				Prostate	1.0 (0.9–1.1)	β-carotene vs placebo
				Colon	0.9 (0.7–1.2)	β-carotene vs placebo
				Rectum	1.1 (0.7–1.8)	β-carotene vs placebo
				Lung	0.9 (0.7–1.2)	β-carotene vs placebo
				Lymphoma	1.0 (0.8–1.4)	β-carotene vs placebo
				Leukemia	0.8 (0.5–1.2)	β-carotene vs placebo
				Melanoma	0.9 (0.6–1.2)	β-carotene vs placebo
				Bladder	1.5 (1.0–2.2)	β-carotene vs placebo
				Brain	0.8 (0.5–1.3)	β-carotene vs placebo
				Pancreas	1.4 (0.8–2.6)	β-carotene vs placebo
				Stomach	0.9 (0.5–1.8)	β-carotene vs placebo
				Thyroid gland	9.5 (2.2–40.7)	β-carotene vs placebo
Beta-Carotene and Retinol Efficacy Trial (CARET), USA	Intervention: β-carotene (30 mg) plus retinol (25000 IU) vs placebo Primary outcome: lung cancer incidence	18,314 adults at risk for lung cancer: 14,254 smokers + 4060 asbestos workers followed for 4 years	Omenn et al. [64]	Lung	1.36 (1.07–1.73)	intervention vs placebo
Alpha-tocopherol and beta-carotene (ATBC) study, Finland	Intervention: 2×2 design; 50 mg of α-tocopherol (50 mg), or β-carotene (20 mg), or both versus placebo Primary outcome: lung cancer incidence	29,133 male smokers 50–69 years of age followed for 5–8 years	ATBC Group [69], Virtamo et al. [74]	Lung	0.98 (0.81–1.19)	α-tocopherol vs placebo
					1.16 (0.97–1.38)	β-carotene vs placebo
					1.15 (0.96–1.38)	both vs placebo
			Heinonen et al. [70]	Prostate	0.64 (0.44–0.94)	α-tocopherol vs placebo
					1.20 (0.87–1.66)	β-carotene vs placebo
					0.84 (0.59–1.20)	both vs placebo
			Rautalahti et al. [72]	Pancreas	0.96 (0.56–1.67)	α-tocopherol vs placebo
					0.46 (0.23–0.92)	β-carotene vs placebo
					1.00 (0.52–1.73)	both vs placebo
			Albanes et al. [68]	Colon/rectum	0.79 (0.48–1.28)	α-tocopherol vs placebo
					1.06 (0.68–1.66)	β-carotene vs placebo
					0.82 (0.50–1.32)	both vs placebo
			Virtamo et al. [73], Virtamo et al. [74]	Urothelium (bladder, renal pelvis, ureter)	1.27 (0.83–1.96)	α-tocopherol vs placebo
					1.17 (0.75–1.81)	β-carotene vs placebo
					1.14 (0.73–1.78)	both vs placebo
				Kidney (renal cell)	1.00 (0.59–1.71)	α-tocopherol vs placebo
					0.78 (0.44–1.38)	β-carotene vs placebo
					1.01 (0.59–1.71)	both vs placebo
			Malila et al. [71]	Stomach	1.34 (0.78–2.29)	α-tocopherol vs placebo
					1.55 (0.92–2.62)	β-carotene vs placebo
					1.38 (0.81–2.36)	both vs placebo
			Wright et al. [75]	Oral cavity/pharynx	0.84 (0.42–1.66)	α-tocopherol vs placebo
					0.84 (0.42–1.66)	β-carotene vs placebo
					0.95 (0.49–1.84)	both vs placebo
				Esophagus	0.86 (0.29–2.56)	α-tocopherol vs placebo
					0.86 (0.29–2.56)	β-carotene vs placebo
					0.72 (0.23–2.27)	both vs placebo
				Larynx	1.00 (0.51–1.97)	α-tocopherol vs placebo
					0.71 (0.34–1.48)	β-carotene vs placebo
					0.59 (0.27–1.29)	both vs placebo
Linxian Study, China	Intervention: 4×2 design with 8 groups AB, AC, AD, BC, BD, CD, ABCD, or placebo. Supplement A: retinol (5000 IU), zinc (22.5 mg). Supplement B: riboflavin (3.2 mg) Supplement C: Vit. C (120 mg), molybdenum (30 μg). Supplement D: β-carotene (15 mg), selenium (50 μg), α-tocopherol (30 mg) Primary outcome: gastric and esophageal cancer mortality	29,584 adults ages 40–69 from four communes in Linxian county followed for 6 years	Blot et al. [84], Qiao et al. [85]	Esophagus	0.97 (0.81–1.17)	A vs no A
					0.90 (0.75–1.08	B vs no B
					1.06 (0.88–1.28)	C vs no C
					1.00 (0.84–1.21)	D vs no D
				Stomach	1.05 (0.86–1.27)	A vs no A
					1.08 (0.89–1.31)	B vs no B
					1.06 (0.87–1.28)	C vs no C
					0.81 (0.66–0.98)	D vs no D
			Kamangar et al. [86]	Lung	0.82 (0.59–1.14)	A vs no A
					1.16 (0.84–1.60)	B vs no B

					1.01 (0.73–1.39)	C vs no C
					0.98 (0.71–1.35)	D vs no D
			Qu et al. [87]	Liver	1.19 (0.87–1.64)*	A vs no A
					1.16 (0.84–1.59)*	B vs no B
					1.19 (0.87–1.64)*	C vs no C
					1.22 (0.89–1.68)*	D vs no D
Women's Health Study (WHS), USA	Intervention: 2×2×2 design of vit. E (600 IU), β-carotene (50 mg qOD), aspirin (100 mg): each alone, 3 mixtures of 2 agents, and all 3 vs placebo Primary outcome: CVD and total cancer incidence	39,876 women aged 45 years or older followed for 10 years; β-carotene was stopped after a median of 2.1 years	Lee et al. [89] Lee et al. [90]	All sites	1.03 (0.89–1.18)	β-carotene vs placebo
					1.01 (0.93–1.16)	vit. E vs placebo
				Breast	1.00 (0.90–1.12)	vit. E vs placebo
				Lung	1.09 (0.83–1.44)	vit. E vs placebo
				Colon	1.00 (0.77–1.31)	vit. E vs placebo
Health Outcomes Prevention Evaluation (HOPE) and HOPE the Ongoing Outcomes (HOPE-TOO) studies, International	Intervention: vitamin E (400 IU) vs placebo Primary outcome: CVD and cancer incidence	HOPE: 9541 adults 55+ years of age with CVD or diabetes followed for a mean of 4.5 years. HOPE-TOO: 7030 HOPE subjects followed for 7 years.	Lonn et al. [93]	Prostate	0.98(0.76–1.26)	vit. E vs placebo
				Lung	0.72(0.53–0.98)	vit. E vs placebo
				Oral cavity/pharynx	0.50(0.24–1.18)	vit. E vs placebo
				Colon/rectum	1.22(0.86–1.73)	vit. E vs placebo
				Breast	0.86(0.50–1.47)	vit. E vs placebo
				Melanoma	0.84(0.42–1.66)	vit. E vs placebo
Heart Protection Study (HPS), United Kingdom	Intervention: Vit. E (600 mg) vit. (250 mg) and β-carotene (20 mg) vs placebo Primary outcome: CVD incidence	20,536 adults of 40–80 years of age with vascular disease, or diabetes followed for 5 years.	HPS Group [94]	All sites	0.98 (0.89–1.08)	intervention vs placebo
				Lung	1.13 (0.91–1.42)*	intervention vs placebo
				Stomach	1.32 (0.91–1.90)*	intervention vs placebo
				Prostate	0.90 (0.72–1.13)*	intervention vs placebo
Supplémentation en Vitamines et Minéraux Antioxydants (SU.VI.MAX) study, France	Intervention: vit. C (120 mg), α-tocopherol (30 mg), β-carotene (6 mg), selenium (100 μg), zinc (20 mg) vs placebo. Primary outcome: CVD and cancer incidence	13 017 adults (7876 women aged 35–60 years and 5141 men aged 45–60 years) followed for a median of 7.5 years	Hercberg et al. [96] Meyer et al. [98]	All sites	0.91 (0.77–1.07)*	intervention vs placebo
				Prostate	0.88 (0.60–1.29)	intervention vs placebo
Women's Antioxidant Cardiovascular Study (WASC), USA	Intervention: 2×2×2 design of vitamin C (500 mg/day), vit. E (600 IU qOD), β-carotene (50 mg qOD) each alone, 3 mixtures of 2 agents, and all 3 vs placebo. Primary outcome: CVD incidence	7627 women at least 40 years of age selected from 8171 participants in the CVD study if they were cancer-free. Average follow-up 9.4 years	Lin et al. [100]	All sites	1.11 (0.95–1.30)	any vit. C vs placebo
					0.93 (0.79–1.09)	any vit. E vs placebo
					1.00 (0.85–1.17)	any β–carotene vs placebo
				Breast	1.11 (0.87–1.41)	any vit C vs placebo
					0.98 (0.77–1.25)	any vit. E vs placebo
					1.01 (0.79–1.30)	any β-carotene vs placebo
				Lung	1.84 (1.14–2.97)	any vit. C vs placebo
					1.25 (0.79–1.97)	any vit. E vs placebo
					1.26 (0.80–1.99)	any β-carotene vs placebo
				Colon/rectum	0.76 (0.42–1.38)	any vit. C vs placebo
					0.63 (0.34–1.15)	any vit. E vs placebo
					1.32 (0.73–2.39)	any β-carotene vs placebo
Physician Health Study II (PHS II), USA	Intervention: 2×2×2×2 design of vit E (400 IU) vitamin C (500 mg), multivitamin (Centrum Silver) and β-carotene (50 mg) vs placebo. The β-carotene intervention was stopped after 4 years) Primary outcome: CVD and cancer incidence	14,641 male physicians aged 50 years or older, including 1307 men with a history of prior cancer followed for a mean of 8 years	Gaziano et al. [103]	All sites	1.02 (0.91–1.15)	vit. E vs placebo
					1.00(0.89–1.12)	vit. C vs placebo
					1.03 (0.91–1.16)	both vs placebo
				Prostate	1.04 (0.88–1.23)	vit. E vs placebo
					1.10 (0.93–1.30)	vit. C vs placebo
					0.97 (0.82–1.15)	both vs placebo
				Colon/rectum	0.93 (0.61–1.42)	vit. E vs placebo
					0.93 (0.61–1.41)	vit. C vs placebo
					0.73 (0.47–1.14)	both vs placebo
				Lung	0.66 (0.38–1.13)	vit. E vs placebo
					0.71 (0.42–1.22)	vit. C vs placebo
					0.84 (0.51–1.40)	both vs placebo
Selenium and Vitamin E Cancer Prevention Trial (SELECT), USA	Intervention: 2×2 design of selenium (200 μg), vit E (400 IU), or both vs placebo Primary outcome: prostate cancer incidence	35,533 men age 50 years or older with serum PSA ≤4 ng/mL and negative DRE followed for 7–12 years	Lippman et al. [106]	Prostate	1.13 (0.99–1.29)¶	vit. E vs placebo
					1.04 (0.90–1.18)¶	selenium vs placebo
					1.05 (0.91–1.20)¶	both vs placebo
				Lung	1.00 (0.64–1.55)¶	vit. E vs placebo
					1.12 (0.73–1.72)¶	selenium vs placebo
					1.16 (0.76–1.78)¶	both vs placebo
				Colon/rectum	1.09 (0.69–1.73)¶	vit. E vs placebo
					1.05 (0.66–1.67)¶	selenium vs placebo
					1.28 (0.82–2.00)¶	both vs placebo

* Intent-to-treat RR calculated based on data provided in the original article.
¶ Indicates 99% confidence intervals.

molybdenum (both in the same trial). The design features of these trials and their main findings, focusing primarily on the intent-to-treat analyses, are presented in chronological order in the sections below. The doses of antioxidants used in those trails are compared to the reference daily intakes (RDIs) as reported by the National Academies Institute of Medicine [56,57]. The results for each study are expressed as rate or risk ratios (RR) along with corresponding 95 or 99% confidence intervals (CI). If the intent-to-treat results were not reported, the RRs were calculated using the data provided in the original articles. The trials are presented in chronological order based on the year of study initiation (Table 1).

The Physicians' Health Study (PHS): 1982–1995

The Physicians' Health Study was a randomized, double-blind, placebo-controlled trial with a 2×2 factorial design that tested aspirin and beta carotene in the primary prevention of cardiovascular disease and cancer. In this study 22,071 United States male physicians, 40 to 84 years of age in 1982 and without any history of cancer (except nonmelanoma skin cancer), myocardial infarction, stroke, or transient cerebral ischemia, were randomly assigned to one of four groups: aspirin (325 mg on alternate days), β-carotene (50 mg on alternate days), both active agents, or placebo [58]. Although there is no RDI value for β-carotene [56], the daily dose of 25 mg (i.e., 50 mg every other day) is over 100 higher than the reported median dietary intake of this micronutrient in the general population [59]. The randomized aspirin component of the study was terminated early, on January 25, 1988, on the advice of the external data-monitoring board, because there was a statistically significant 44% reduction in the risk of myocardial infarction in the aspirin group [60]. The randomized β-carotene component continued uninterrupted until its scheduled termination, on December 31, 1995. Following an average of almost 13 years of study, 2667 incident cancers were confirmed, including 1117 prostate, 267 colon, and 178 lung cancers [61]. There were no significant differences between the supplementation and placebo groups for total cancer incidence (RR = 1.0) or for incidence of cancers of the most common sites, including prostate (RR = 1.0), colon (RR = 0.9; 95% CI: 0.7–1.2), and lung (RR = 0.9, 95% CI: 0.7–1.2). In subgroup analyses, total cancer incidence was modestly reduced with supplementation among those aged 70+ years (RR = 0.8; 95% CI: 0.7–1.0), daily drinkers of alcohol (RR = 0.9; 95% CI: 0.8–1.0), and those in the highest BMI quartile (RR = 0.9; 95% CI: 0.7–1.0). Prostate cancer incidence was reduced with supplementation among those in the highest BMI quartile (RR = 0.8; 95% CI: 0.6–1.0), and colon cancer was reduced among daily drinkers of alcohol (RR = 0.5; 95% CI: 0.3–0.8). There was an increased incidence of bladder cancer (RR = 1.5, 95% CI: 1.0–2.2) and thyroid cancer (RR = 9.5; 95% CI: 2.2–40.7) in the supplementation group [61].

Beta-Carotene and Retinol Efficacy Trial (CARET): 1985–1996

The CARET trial began as a study that included 816 men with substantial occupational exposures to asbestos who were randomized to either the combination of 15 mg β-carotene and 25,000 IU retinol daily or placebo (1:1), and 1029 men and women with extensive cigarette smoking histories who were randomly assigned to receive 30 mg β-carotene, 25,000 IU retinol, both, or neither [62,63]. The pilot study participants were transferred to the full-scale CARET regimen of 30 mg β-carotene plus 25,000 IU retinyl palmitate taken daily, and the project was expanded 10-fold at six study centers around the United States [64]. The 25,000 IU of retinol in this study is equivalent to 7500 μg [65] or 8.3 times the RDI of 900 μg/day for vitamin A. The primary intent-to-treat analysis was designed to test for differences between intervention groups in the incidence of lung cancer by use of a stratified, weighted log-rank statistic, with the weight function rising linearly from 0 at the time of randomization to 1.0 at 2 years after randomization and thereafter. The analysis revealed a weighted RR of 1.36 (95% CI: 1.07–1.73) for lung cancer incidence and a weighted RR of 1.59 (95% CI: 1.13–2.23) for lung cancer mortality. These findings prompted a decision by the Steering Committee to stop the intervention in January 1996 [64]. In the subsequent postintervention follow-up through December 31, 2001, the RR of lung cancer for the active intervention group compared with the placebo group was 1.12 with a 95% CI between 0.97 and 1.31 [66].

Alpha-tocopherol and beta-carotene (ATBC) study: 1985–1993

The ATBC study was a randomized, double-blind, placebo-controlled, 2×2 factorial design, primary prevention trial testing the efficacy of α-tocopherol (50 mg/day, or 3.3 times the RDI) and β-carotene (20 mg/day) supplements in reducing the incidence of lung cancer and possibly other malignancies [67]. Between 1985 and 1993, 29,133 eligible male smokers residing in southwestern Finland and aged 50 to 69 years at entry were randomly assigned to receive daily active supplements or placebo capsules for 5 to 8 years (median 6.1 years), accumulating 169,751 years of follow-up. The results of the ATBC study have been reported for various cancer outcomes in several publications [68–75]. The trial was terminated early because there was a statistically significant increase in lung cancer incidence and all-cause mortality among persons receiving β-carotene [69,76]. All other intent-to-treat analyses according to the original 2×2 design indicated essentially null results. The two exceptions were a statistically significant decrease in risk of prostate cancer in the α-tocopherol alone arm and a decrease in risk of pancreatic cancer for the β-carotene alone arm; but these exceptions are thought to be due to chance since none of the analyses showed any discernable pattern. Secondary analyses of the data seemed to suggest that the effect of vitamin E could be modified by other antioxidants (such as vitamin C) and by age, but those results require confirmation [77]. A post-intervention follow-up of study participants found no evidence of any lasting effects of α-tocopherol or β-carotene supplementation [74].

The Linxian Study: 1986–1991

Linxian is a rural county in north-central China known to be among the areas with the highest rates of epithelial malignancies (most notably carcinomas of the stomach and esophagus) in the world [78–80]. These high rates were thought to be attributable to suboptimal nutrition resulting in low circulating levels of retinol, carotenoids, tocopherols, and other vitamins. Based on the observed high incidence of esophageal and gastric cancers and widespread, albeit subclinical, deficiencies of several micronutrients among the population, Linxian was selected as the site for two randomized intervention trials to test whether supplementation with multiple vitamins and minerals might reduce the rates of cancer [81]. The first, much smaller, trial was limited to approximately 3000 subjects with esophageal dysplasia [82,83], and is beyond the scope of this review. The second population-based trial had a larger sample with nearly 30,000 participants [84], and is reviewed below. The intervention for the population-based Linxian trial included four combinations of supplements: Supplement A included retinol palmitate (5000 IU or 1.7 times the RDI) and zinc as 22.65 mg of oxide (twice the RDI); Supplement B included riboflavin (3.2 mg, 2.5 times the RDI) and niacin (40 mg or 2.5 times the RDI); Supplement C included vitamin C as 120 mg of ascorbic acid (1.3 times the RDI), and molybdenum as 30 μg of Mo–yeast complex (2/3 of the current RDI); and Supplement D was a combination of 15 mg β-carotene (roughly 64 times of the median intake in the United States population), selenium as 50 μg of Se yeast (roughly equal the RDI), and 30 mg of α-tocopherol (twice the RDI) [81]. The eight intervention groups in this fractional design study were defined by the following combinations of supplements: AB, AC, AD, BC, BD, CD, ABCD, or placebo. Thus, for example, persons in

group AB received retinol, zinc, riboflavin, and niacin, those in group ABCD received all nine vitamins and minerals, and those in the placebo group received none. This choice of groups resulted in half the participants receiving each of the four factor nutrient combinations. Among those receiving factor D there was a statistically significant reduction in overall mortality (RR = 0.91; 95% CI: 0.84–0.99) and cancer mortality (RR = 0.87; 95% CI: 0.75–1.00). Among site-specific results, the only statistically significant treatment effect was observed for stomach cancer when comparing supplement D to any other type of intervention (RR = 0.81; 95% CI: 0.66–0.98) [85]. There was no evidence of efficacy for any other supplements or for any other cancer mortality endpoints [84–87].

The Women's Health Study (WHS): 1992–2004

The Women's Health Study was a randomized, double-blind, placebo-controlled trial to test the balance of benefits and risks of aspirin (100 mg), α-tocopherol (600 IU, 9 to 13.5 times the RDI depending on the compound [65]), and β-carotene (50 mg)—all given on alternate days using a 2 × 2 × 2 factorial design. The aim of the WHS trial was the primary prevention of cancer and cardiovascular disease [88]. A total of 39,876 female health professionals, aged 45 years or older and without a history of cancer (except nonmelanoma skin cancer), coronary heart disease, or cerebrovascular disease, were randomly assigned to one of the following eight treatment groups: all three active agents, three groups taking two active agents and one placebo, three groups taking one active agent and two placebos, and one group taking all three placebos. The β-carotene component of the trial was terminated early in 1996, primarily because of the adverse or null findings from other studies [89]. Among women assigned to receive β-carotene, there were no statistically significant differences in the incidence of all cancers (RR = 1.03; 95% CI: 0.89–1.18) or any specific cancer sites [89]. A comparison of the vitamin E group to placebo also yielded an essentially null result for all cancers (RR = 1.01; 95% CI: 0.94–1.08), and for cancers of the breast (RR = 1.00; 95% CI: 0.90–1.12), lung (RR = 1.09; 95% CI: 0.83–1.44), and colon (RR = 1.00; 95% CI: 0.77–1.31). Cancer deaths also did not differ significantly among treatment groups [90].

Health Outcomes Prevention Evaluation (HOPE) and HOPE the Ongoing Outcomes (HOPE-TOO) studies: 1993–2003

The HOPE investigators enrolled a total of 2545 women and 6996 men 55 years of age or older known to be at high risk for cardiovascular events because of having cardiovascular disease or diabetes. Patients were recruited from December 1993 to June 1995 at 129 centers in Canada, 27 centers in the United States, 76 centers in 14 western European countries, 30 centers in Argentina and Brazil, and 5 centers in Mexico [91]. Participants were randomly assigned according to a 2 × 2 factorial design to receive either 400 IU of RRR-α-tocopheryl acetate daily (18 times the RDI) or matching placebo and either an angiotensin-converting-enzyme inhibitor (ramipril) or matching placebo for a mean of 4.5 years. Cancer incidence was listed among the secondary outcomes [92]. At the conclusion of the HOPE trial in April 1999, all study centers were invited to participate in a trial extension (HOPE-TOO), which was conducted between April 1999 and May 2003. Of the initial 267 centers, 174 agreed to participate in the HOPE-TOO trial. These 174 centers had originally randomized 7030 patients [93]. After a median 7.0 years of follow-up for the entire study population (HOPE and HOPE-TOO) and a median 7.2 years for patients at centers continuing in the trial extension, there was no overall effect of vitamin E on cancer incidence. There was also no evidence that vitamin E prevented most site-specific cancers. The only exception was a decrease in lung cancer (RR = 0.72; 95% CI: 0.53–0.96), a finding attributed to multiple hypothesis testing [93].

Heart Protection Study (HPS): 1994–2001

The primary purpose of the HPS study was to test the efficacy of cholesterol-lowering medications with and without antioxidant vitamins on cardiovascular disease risk using a 2 × 2 factorial design [94]. In the vitamin supplementation part of the trial the 20,536 participants from 69 United Kingdom hospitals were randomly allocated to receive 600 mg of all-*rac*-α-tocopherol (18 times the RDI), 250 mg of vitamin C (2.8 times the RDI), and 20 mg of β-carotene daily versus matching placebo. In the interval between randomization (July, 1994–May, 1997) and the final follow-up (May–October, 2001) new primary cancers (excluding nonmelanoma skin cancer) were diagnosed in 800 (7.8%) of the participants allocated to vitamins compared with 817 (8.0%) of those allocated to placebo (RR = 0.98; 95% CI: 0·89–1.08). The intent-to-treat analyses by organ systems (e.g., gastrointestinal or respiratory) demonstrated no statistically significant effect of the intervention. Similar analyses by specific cancer sites revealed no significant differences between the treatment groups [94].

Supplémentation en Vitamines et Minéraux Antioxydants (SU.VI.MAX) study: 1994–2002

The primary objective of the SU.VI.MAX study was to test the efficacy of a combination of antioxidants at nutritional, rather than pharmacologic, doses as preventive agents against cancer and ischemic heart disease [95]. The study population included 7876, 35- to 60-year-old French women, and 5141, 45- to 60-year-old French men. The daily multivitamin and mineral supplement included 6 mg of β-carotene, 120 mg of vitamin C (1.3 times the RDI), 30 mg of α-tocopherol (twice the RDI), 100 μg of selenium-enriched yeast (twice the RDI), and 20 mg of zinc gluconate (twice the RDI). These doses were designed to supply one to three times the French recommended daily allowances [95]. No overall differences in total cancer incidence were detected between the treatment groups. However, a statistically significant protective effect of antioxidants in reducing total cancer incidence was observed among men (RR = 0.69; 95% CI: 0.53–0.91), but not among women (RR = 1.04; 95% CI: 0.85–1.29); *P* for interaction = 0.04 [96]. This difference was apparently driven by lower incidence of digestive, respiratory, and skin cancers [97], although the quantitative results for those cancer sites were not reported. The differential effect in men and women motivated a reevaluation of the data on prostate cancer [98]. There was a small, not statistically significant, reduction in prostate cancer incidence associated with the supplementation (RR = 0.88; 95% CI: 0.60–1.29); however, there was a stronger statistically significant reduction (RR = 0.52; 95% CI: 0.29–0.92) among men with a normal PSA at baseline [98].

Women's Antioxidant Cardiovascular Study (WASC): 1995–2005

The WACS was a randomized, double-blind, placebo-controlled trial to test the efficacy of vitamin C (500 mg/day, 5.5 times the RDI), vitamin E (600 IU of natural *d*-α-tocopherol acetate every other day or 13.5 times the RDI), and β-carotene (50 mg every other day) in preventing vascular events among women with a history of CVD or three or more cardiovascular risk factors [99]. A total of 8171 women were randomized to the above interventions according to a 2 × 2 × 2 factorial design. In a secondary analysis of cancer endpoints, participants with a history of malignancy were excluded leaving 7627 women (93.3%) eligible for a substudy of cancer incidence and mortality [100]. Following an average of 9.4 years of treatment there were no statistically significant effects of any antioxidant on total cancer incidence; the RRs (95% CIs) were 1.11 (0.95–1.30) in the vitamin C group, 0.93 (0.79–1.09) in the vitamin E group, and 1.00 (0.85–1.17) in the β-carotene group. Similarly, relative to the placebo group, the RRs (95% CIs) for cancer mortality were 1.28 (95% CI: 0.95–1.73), 0.87 (95%: 0.65–1.17), and 0.84 (95% CI: 0.62–1.13) for vitamin C, vitamin E, and β-carotene

groups, respectively. Combined use of the three antioxidants also had no effect on overall cancer incidence or mortality [100]. Statistically significant site-specific results included a reduction in non-Hodgkin lymphoma risk in the β-carotene group (RR = 0.46; 95% CI: 0.22–0.97), and an increased incidence of lung cancer in the vitamin C group (RR = 1.84; 95% CI: 1.14–2.97). There were no statistically significant interactions [100].

Physician Health Study II (PHS II): 1997–2007

The Physicians' Health Study II (PHS II) was a randomized, double-blind, placebo-controlled 2 × 2 × 2 × 2 factorial trial to test the efficacy of alternate day β-carotene (50 mg), alternate day vitamin E (400 IU synthetic α-tocopherol 6 times the RDI), daily vitamin C (500 mg or 5.5 times the RDI,), and a daily multivitamin (Centrum Silver) in preventing prostate cancer, cardiovascular disease, cataracts, and macular degeneration [101]. The study recruited two types of participants: 7641 subjects who completed the PHS I trial and 7000 physicians who were recruited *de novo*. The β-carotene intervention was stopped early, whereas the vitamin E, vitamin C, and multivitamin components were continued [102]. During a mean follow-up of 8.0 years, there were 1008 confirmed incident cases of prostate cancer and 1943 total cancers. Compared with placebo, the prostate cancer rate ratios (95% CIs) were 0.97 (0.85–1.09) for vitamin E and 1.02 (0.90–1.15) for vitamin C. Neither vitamin E nor vitamin C had a significant effect on the incidence of total or site-specific cancers [103].

Selenium and Vitamin E Cancer Prevention Trial (SELECT): 2001–2008

SELECT was a randomized trial of selenium (200 μg/day of L-selenomethionine or 3.6 times the RDI), synthetic vitamin E (all-*rac*-α-tocopheryl acetate400 IU/day, 12 times the RDI), or both as chemoprevention agents against prostate cancer. The choice of selenium as the agent of interest was primarily motivated by the secondary results of a previously conducted smaller trial of selenium (200 μg/day of selenized yeast) as a preventive agent against skin cancer among persons with a history of basal cell carcinoma, which reported a significant reduction in prostate cancer incidence in the intervention group [104,105]. The addition of vitamin E was motivated by the results of the ATBC study which found a decrease in prostate cancer incidence in the group that received α-tocopherol (50 mg/day) alone [74]; however, the dose of vitamin E used in SELECT was 3.6 times higher assuming the conversion of 1 mg = 1 IU × 0.45 [65]. The eligible men recruited for SELECT included African Americans 50 years of age or older or men of other racial groups at least 55 years of age with no prior prostate cancer diagnosis, a serum PSA of 4 ng/mL or less, and a negative digital rectal examination. After a median follow-up of 5.5 years the rate ratios for prostate cancer were 1.13 (99% CI: 0.95–1.35) for vitamin E, 1.04 (99% CI: 0.87–1.24) for selenium, and 1.05 (99% CI: 0.88–1.25) for both agents combined. There were no significant differences between any of the intervention groups and placebo for any other cancer sites [106].

Ongoing trials of antioxidants as cancer prevention agents

The search for ongoing trials testing the effectiveness of antioxidants as cancer prevention agents was conducted by searching the www.clinicaltrials.gov database maintained by the National Institutes of Health (NIH) and the National Cancer Institute's Physician Data Query (PDQ) for clinical trials available at http://www.cancer.gov/clinicaltrials. This search identified six projects at various stages of completion (Table 2). Five of those six trials are testing the effects of selenium alone or in combination with other agents. Unlike previously completed studies (reviewed above) most of the ongoing trials are taking place outside of the United States, and all but one study include less than 1000 participants. The design, intervention details, and anticipated timelines of these ongoing trials are summarized below.

Selenium in the Prevention of Cancer: 1999–

This is a randomized, double-blind, placebo-controlled, three-arm parallel group trial conducted by the University of Surrey in the United Kingdom. The goal of the trial is to test the efficacy of three doses of selenium in preventing all types of cancer. The treatment, three doses of selenium (100 μg/day, 200 μg/day, 300 μg/day, and placebo) is supposed to continue for up to 2 years. The participants in this trial are men and women (projected $n = 510$) 60 to 74 years of age with no history of cancer (except nonmelanoma skin cancer). The trial appears to have been closed to recruitment; however, no information is available regarding its anticipated completion. The reports in the literature are limited to noncancer outcomes such as plasma homocysteine [107] and thyroid function [108].

Selenium in Preventing Cancer in Patients with Neoplasia of the Prostate: 2002–

This clinical trial, conducted through the network of Community Clinical Oncology Programs in the United States (projected $n = 465$), was scheduled to be completed in 2009 and is testing the efficacy of selenium in preventing prostate cancer among patients with biopsy-confirmed high-grade prostatic intraepithelial neoplasia (HGPIN) [109]. HGPIN is the precancerous condition widely used to determine which patients may be at risk for having carcinoma on repeat biopsy [110]. For this reason, HGPIN is viewed as a useful target/marker for chemoprevention trials [111]. The eligibility criteria for this ongoing trial include a biopsy showing HGPIN, but no cancer; a serum PSA of 10 ng/mL or lower; and no use of finasteride or selenium supplements [109]. All patients are randomly assigned to receive 200 μg/day of selenium or placebo for 3 years. During the first 2 years postrandomization each patient is scheduled to undergo a semiannual evaluation that includes a PSA test, a digital rectal examination, and a query about symptoms, adverse events, and cancer diagnoses. Similar evaluations are then conducted annually for 8 years. A follow-up biopsy is performed at 3 years postrandomization.

Vitamin E, Selenium, and Soy Protein in Preventing Cancer in Patients with High-Grade Prostate Neoplasia: 2003–

A total of 306 Canadian men with histologically confirmed HGPIN, no prior prostate cancer, and no evidence of prostate cancer on at least two biopsies performed within the past 18 months were randomized to one of two treatment arms: a combination of oral soy protein (40 g/daily), vitamin E (800 IU/daily), and selenium (200 μg/daily) versus placebo. In both arms, treatment continued for 3 years in the absence of invasive biopsy-documented prostate cancer or unacceptable toxicity. Patients were followed every 3 months for a year and then every 6 months for 2 years. According to the latest update in the National Cancer Institute's Physician Data Query the trial is complete. To date no results have been published in a peer-reviewed journal; however, early findings from the trial were presented at the 2009 meeting of the American Urological Association. As reported in the abstract, the hazard ratio for whether the nutritional supplement reduced the incidence of prostate cancer was 1.03 (95% CI 0.67–1.60). The Gleason score distribution (a measure of prostate cancer grade) was similar among cases in both treatment groups [112].

Bangladesh Vitamin E and Selenium Trial (BEST): 2006–

Arsenic exposure is an important public health problem in Bangladesh, where between 35 and 77 million people are exposed through contaminated tube wells [113]. One of the known manifestations of arsenic toxicity is the development of keratotic skin lesions that are associated with a very high risk of basal and squamous cell skin cancers and possibly other malignancies [114]. For this reason the arsenic-

Table 2
Ongoing clinical trials of antioxidants as cancer prevention agents.

Study name, location	Intervention and primary outcome	Study population and follow-up	Date of first report*	Status at most recent update	Anticipated completion
Selenium in the Prevention of Cancer, United Kingdom	Intervention: Three nonspecified doses of selenium (low, medium and high) vs placebo Primary outcome: total cancer incidence	510 cancer-free persons stratified by age (60–64, 65–69, and 70–74 years). Treatment for up to 2 years. Follow-up not reported	October 1, 1999	Study recruitment completed	Not reported
Selenium in Preventing Cancer in Patients With Neoplasia of the Prostate, USA	Intervention: Selenium (200 μg) vs placebo Primary outcome: prostate cancer incidence	465 men age 40+ years with high-grade prostatic intraepithelial neoplasia (HGPIN) and no cancer followed every 6 months for 2 years, then annually for 8 years. Repeat biopsy at 3 years.	February 14, 2002	Study recruitment completed	2009
Vitamin E, Selenium, and Soy Protein in Preventing Cancer in Patients With High-Grade Prostate Neoplasia, Canada	Intervention: nonspecified doses of vit E, selenium, and soy protein isolate vs placebo Primary outcome: prostate cancer incidence	306 men of unspecified age with HGPIN and no cancer followed every 3 months for 1 year, then every 6 months for 2 years.	July 8, 2003	Study completed	Not reported
Bangladesh Vitamin E and Selenium Trial (BEST), Bangladesh	Intervention: 2×2 design of selenium (200 μg), vit E (100 mg). or both vs placebo Primary outcome: skin cancer incidence	5000 individuals with manifest arsenic skin lesions aged 25 to 65 years.	October 24, 2006	Study recruitment completed	November 2010
Dietary Bioflavonoid Supplementation for the Prevention of Neoplasia Recurrence, Germany	Intervention: apigenin (20 mg) + epigallocathechin (20 mg) vs placebo Primary outcome: recurrence of colorectal neoplasia	382 patients 50–74 years of age with recent resection of colorectal cancer followed for 3 years.	January 24, 2008	Recruitment not begun	December 2011
Selenium in Preventing Prostate Cancer, USA	Intervention: Two doses of selenium (200 or 400 μg) vs placebo Primary outcome: prostate cancer incidence	700 men ≤79 years of age with clinical suspicion of prostate cancer but negative prostate biopsy. Treatment for up to 57 months. Follow-up not reported.	September 16, 2009	Recruitment not begun	December 2010

*As recorded in www.clinicaltrials.gov.

exposed population of Bangladesh is thought to benefit from skin cancer prevention interventions [115,116]. The Bangladesh Vitamin E and Selenium Trial (BEST) ($n=5000$) is a 2×2 factorial design trial to test the efficacy of selenium (200 μg, about 4 times the RDI) and vitamin E (100 mg, about 6.7 times the RDI), alone and in combination, versus placebo over 5 years in reducing the incidence of skin cancer. Secondary outcomes include mortality and skin dysplasia.

Dietary Bioflavonoid Supplementation for the Prevention of Neoplasia Recurrence: 2008–

The aim of this trial, which is scheduled to begin in early 2011 in Germany, is to test the efficacy of dietary supplementation with bioflavonoids over 3 years in preventing the recurrence of colonic neoplasms among persons who underwent surgical resection of colorectal cancer. Only patients with pathologically proven stage 2 or stage 3 colorectal cancers (without adjuvant chemotherapy or after completion of adjuvant chemotherapy) and a time interval within 3–12 months after surgery are considered for inclusion. The active intervention in this randomized, double-blind, placebo-controlled trial (projected $n=382$) is a commercially available preparation (Flavo-Natin) which contains a mixture of two flavonoids: apigenin (20 mg) from chamomile, and epigallocathechin gallate (20 mg) from green tea, together with vitamins C, B6, B12, and folic acid (www.koehler-pharma.de/060_prod/flavo-natin.php). Adherence to treatment will be assessed by measuring serum concentrations of apigenin and epigallocathechin. The primary outcome measures of this trial are the recurrence rate of colon neoplasia and overall survival.

Selenium in Preventing Prostate Cancer: 2009–

This ongoing multicenter trial (target $n=700$) conducted by the University of Arizona is testing the efficacy of two doses (200 and 400 μg) of selenized yeast (roughly 4 times and 8 times the RDI, respectively) relative to placebo in preventing prostate cancer. Treatment is scheduled to continue for up to 57 months in the absence of unacceptable toxicity or a diagnosis of prostate cancer. Criteria for inclusion into the study are the presence of clinical findings (e.g., PSA and digital rectal examination) that would justify a biopsy of the prostate plus a history of a negative prostate biopsy (for prostate cancer or HGPIN) within the past 12 months. In addition to incident biopsy-confirmed prostate cancer (primary endpoint), other outcomes of interest include the rate of rise in serum PSA levels, and evidence of prostate cancer progression as assessed by levels of serum alkaline phosphatase and chromogranin A.

Discussion of future studies

There appears to be growing consensus that the new generation of cancer prevention trials must take into account two critical methodological issues that were not considered in previous studies of antioxidant supplements. The methodological issues fall into two broad categories: choice of endpoint and duration, and selection of intervention and dose [117–119]. The two issues and the related considerations for future research are discussed below.

Choice of endpoints and trial duration

It is likely that the relatively short duration of treatment used in previous trials was insufficient for evaluating cancer incidence. To illustrate, a 2003 publication based on the Cancer Prevention Study II (CPS II) Nutrition Cohort, which was recruited and is followed by the American Cancer Society, reported that recent multivitamin use was not associated with risk of colorectal cancer, whereas regular multivitamin use 10 years before enrolment was associated with lower risk. Moreover, regular multivitamin users 10 years before

enrolment were at similarly lower risk whether they were still taking multivitamins at enrollment or had stopped [120]. It is also important to point out that, in contrast to studies of clinical endpoints, trials of antioxidant supplements that used markers of cell proliferation as the endpoints of interest were successful [121–123]. These results are consistent with the hypothesis that the effects of recent antioxidant use may only be detectable at a subclinical (molecular) level, whereas a reduction in risk of clinically detectable disease requires prolonged exposures that may not be achievable in a traditional length cancer prevention trial.

As it takes 10–30 years for a normal epithelium to undergo sufficient molecular changes to produce a clinically detectable neoplastic lesion [117], definitive clinical trials of cancer prevention may, in some cases, be prohibitively expensive, logistically problematic (e.g., because of inability to prevent treatment arm convergence due to treatment drop-out in the active treatment arm and treatment drop-in in the control group over prolonged intervention durations), or not sufficiently informative if they test recent, but not long-term, exposures [124]. A useful alternative to the clinical endpoints is the use of biomarkers of cancer risk. The relevant biomarkers available as endpoints for trials of antioxidants can be divided into three categories: specific markers of carcinogenesis, cell cycle markers, and markers of oxidative stress/inflammation.

The use of specific markers of carcinogenesis is exemplified by studies that use biopsies of rectal mucosa to identify indicators of risk for colorectal neoplasia (Table 3). Rectal tissue biopsies as the source of biomarkers provide an excellent opportunity for research because rectal tissue biopsies are easily accessible, do not require preparatory cleansing of the colon, are painless, and pose virtually no risk [125]. Moreover, most biomarkers obtained from the rectal tissue have been shown to be representative of the processes elsewhere in the lower gastrointestinal tract [126–130].

As described in considerable detail by Vogelstein, Kinzler and others colorectal carcinogenesis is a multistep process involving genetic alterations of *APC*, *K-ras*, a tumor suppressor gene on chromosome 18q, and *p53* [131–133]. There are at least two not necessarily entirely mutually exclusive major pathways driving this process: the "APC Pathway" and the "Mismatch Repair (MMR) Pathway" [132–134]. Each pathway has its own specific biomarkers detectable in colorectal tissue [125,130,135–137] and each pathway affects the cell cycle as reflected in increases in proliferation and decreases in differentiation and apoptosis, for which several markers are available. For example, an informative long-term indicator of proliferation is hTERT, a catalytic subunit of telomerase [138], and a marker of a cell that can no longer proliferate and is differentiated is p21; both of these markers can be measured in tissue biopsy specimens [139–141]. Detection of expression of inhibitors (bcl-2) and promoters (bax) of apoptosis can be readily demonstrated in variety of tissues [142–144], most notably in the in crypts of the normal colon mucosa [139,145–150].

Of relevance to oxidative stress (presumably the main target of antioxidant agents) are findings from a series of biochemical and pharmacological experiments, which suggested that p53 activity acts through a three-step process: (1) the transcriptional induction of redox-related genes, (2) the formation of reactive oxygen species, and (3) the oxidative degradation of mitochondrial components, culminating in cell death [151]. Also, a relatively recently described mechanism by which cells regulate their lifecycle is autophagy, an evolutionary conserved process of degrading and recycling long-lived proteins and organelles [152]. In addition to its housekeeping function, autophagy is also involved in regulating cell growth and response to oxidative stress and increased generation of RONS [153]. The oxidative stress-linked oncosuppressor p53 has also been shown to promote autophagy [154]. A promising biomarker of autophagy that could be used in future clinical trials is beclin1, which was found to exhibit allelic loss in human cancers of the breast, ovaries, and prostate [155]. Oxidative stress both causes and is caused by inflammation [156]. A key molecular link between inflammation and tumor development is nuclear factor-kappa B (NF-κB), which is activated by many proinflammatory cytokines, including interleukin-1-beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNFα). These markers are found in cancer patients and in persons diagnosed with precancerous conditions [157–160].

The cytokine-initiated activation of NF-κB leads to further downstream induction of key enzymes responsible for the biosynthesis of prostaglandins [161], which are usually detectable in urine specimens. The main precursor of prostaglandins is arachidonic acid (AA). The conversion of arachidonic acid (AA) into biologically active prostanoids/eicosanoids and leukotrienes is regulated by several enzymes that are detectable in colorectal mucosa [162]. Prostaglandin synthases 1 and 2 (PTGS1 and PTGS2), also known as cyclooxygenases 1 and 2 (COX-1 and COX-2), are the key enzymes in prostaglandin biosynthesis that convert arachidonic acid into prostaglandin H2 (PGH2) [161]. PGH2 undergoes further downstream conversion into prostaglandin E2 (PGE2), which suppresses apoptosis in human tissue by increasing levels of the antiapoptotic protein bcl-2, and reducing levels of the proapoptotic protein bax [163].

According to the prevailing view, oxidative stress occurs as a result of damage induced by reactive oxygen and nitrogen species [5–7,164–168]. Available tissue biomarkers of RONS-induced macromolecular damage include (*E*)-4-hydroxy-2-nonenal (4-HNE), which is an end product of lipid peroxidation [169], and 8-hydroxydeoxyguanosine (8-OH-dG), which serves as a marker of DNA damage. 8-OH-dG can also be detected in blood and urine samples [170], whereas blood or urinary F2-isoprostanes serve as systemic counterparts of tissue 4-HNE [171–173]. While F2-isoprostanes and 8-OH-dG provide useful information about RONS-mediated oxidative stress, they do not allow evaluation of nonradical oxidation.

The above limitation can be addressed through the use of recently proposed biomarkers that reflect both free radical and nonradical oxidative stress mechanisms. The initial discovery and development of these novel biomarkers was based on the observation that sulfur-containing amino acids and peptides, notably cysteine and cysteine-containing tripeptide glutathione, undergo reversible oxidation-reduction (redox) changes under physiologic conditions. The redox states of glutathione/glutathione disulfide (GSH/GSSG) and cysteine/cystine (Cys/CySS) are oxidized in association with several known oxidative stress-related exposures, health conditions, and measures of physiologic function, including age [174], cigarette smoking [175], type 2 diabetes [176], atherosclerosis [177], and apoptosis of colorectal epithelial cells [178].

The biomarkers of redox state have also been tested in clinical trials. In an ancillary analysis of the Age-Related Eye Disease Study (AREDS), which tested the efficacy of antioxidant supplementation in slowing the progression of age-related macular degeneration, plasma

Table 3
Examples of biomarkers of colorectal carcinogenesis, cell cycle, oxidative balance, and inflammation in various samples.

Biological mechanism	Colorectal tissue	Blood	Urine
Carcinogenesis			
a. APC pathway	APC, E-cadherin, β-catenin		
b. MMR pathway	MLH1, MSH2		
Cell cycle			
a. proliferation	hTERT		
b. differentiation	p21		
c. apoptosis	bax/bcl-2		
Inflammation	COX-2, IL-1β, IL-6, TNFα, NF-κB	IL-1β, IL-6 TNFα, CRP	PGE-M, TNFα, IL-1β, IL-6
Oxidative balance	8-OH-dG, 4-HNE GSH/GSSG, CyS/CySS	F2-IP, 8-OH-dG GSH/GSSG, CyS/CySS	F2-IP, 8-OH-dG

GSH, GSSG, Cys, and CySS were measured, and redox potentials of GSH/GSSG (Eh GSH) and Cys/CySS (Eh Cys) were calculated [179]. At the first blood draw, the means for the antioxidant group and no-antioxidant group were not significantly different with respect to any of the metabolites or redox potentials. However, at the second draw, mean Cys was significantly higher and mean Eh Cys was significantly lower in the antioxidant group.

In another recent randomized, double-blind, placebo-controlled clinical trial, the effects of an antioxidant micronutrient combination (800 mg *dl*-α-tocopherol acetate, 24 mg β-carotene, 1.0 g vitamin C, 200 μg L-selenomethionine, 7.2 mg riboflavin, 80 mg niacin, 60 mg zinc, 5 mg manganese) given daily over 4 months were assessed with respect to a variety of biomarkers, including CySS. There was a statistically significant decrease in plasma CySS in the active treatment group relative to the placebo group, a finding that (along with a decrease in F2-isoprostanes) was particularly pronounced among nonsmokers [180]. Although the above-noted biomarkers are central to our understanding of the possible anticancer effects of antioxidant nutrients there are additional pathways that need to be considered and assessed. One of the most important defensive signaling mechanisms is the NF-E2-related factor 2 (Nrf2) and antioxidant response element (ARE) pathway [181]. The Nrf2–ARE pathway regulates a variety of processes, including detoxication of electrophiles and reactive oxygen species as well as the removal or repair of some of their damage products [182,183]. Based on animal experiments it appears that the beneficial effects of polyphenols, such a curcumin and epigallocathechin, involve activation of the Nrf2–ARE pathway [184,185]; however, human data in support of these observations are not available.

While targeted hypothesis-driven studies remain at the center of cancer prevention research, in recent years various types of "-omics" have been added to our continuously expanding tool kit [186]. High-throughput genotyping (genomics) and gene expression analyses (transcriptomics) have allowed more comprehensive evaluation of molecular responses to drugs and nutrients. Since molecular regulation also occurs at three other levels (i.e., translational, posttranslational, and metabolic) [187], the systems biology now includes, in addition to genomics and transcriptomics, the fields of proteomics, which aims to identify and quantify the translated and posttranslationally modified gene products, and metabolomics, which serves as the quantitative cataloguing of the entire range of metabolites [188]. Of particular interest to studies of oxidative stress, is lipomics (or lipidomics) a branch of metabolomics, defined as "the systems-based study of all lipids, the molecules with which they interact and their function within the cell" [189]. The specific studies using various "-omics" techniques in clinical trials are still rare, but it is clear that new data will become increasingly available in the near future. For example, Hoelzl et al. investigated the effects of Brussels sprout consumption on the proteome profile of white blood cells. After the intervention, there was a significant up-regulation of the synthesis of manganese superoxide dismutase and significant down-regulation of the synthesis of heat shock protein [190]. In another study, administration of *N*-acetyl-L-cysteine, a thiol-based antioxidant, decreased strenuous exercise-induced oxidative stress as shown by altered metabolomic profiles of oxidized glutathione (GSSG), reduced glutathione (GSH), 3-methylhistidine, L-carnitine, *O*-acetyl-L-carnitine, and creatine [191].

Additional progress may be achieved using wide-scale high-performance metabolic profiling (HPMP), which can both reflect disease risk and offer target endpoints for future interventions [192]. HPMP uses high-resolution mass spectrometry coupled to liquid chromatography to provide high-throughput analysis of thousands of chemicals in biologic samples. Half of the chemicals in human plasma are unidentified, and these could include currently unrecognized cancer-causing chemicals and cancer-preventive chemicals that only contribute to cancer risk in subsets of the population, i.e., via gene-environment interactions. By having an affordable way to profile thousands of chemicals in large populations, one could use bioinformatics approaches to identify such cancer risk associations with currently unidentified chemicals. Such powerful and affordable analytic procedures also provide means to address the complexity inherent in cancer prevention trials with real food.

Chemical profiles obtained by HPMP of plasma and other biospecimens also contain thousands of chemicals that reflect endogenous metabolism in the individual. Longitudinal changes in these endogenous metabolic profiles provide an additional way to detect gene-environment interactions affecting cancer risk and cancer prevention.

Selection of intervention and dose

Many of the cancer prevention trials reviewed in the previous sections could be criticized for the inappropriate selection of agents and the inappropriate (likely prooxidant) dosages of antioxidant vitamins. For example, data show that β-carotene, an agent that increased lung cancer incidence in smokers and was otherwise proven to be unsuccessful as antineoplastic agent in several previous studies, may act as an antioxidant in some circumstances and as a prooxidant in others [193,194]. Similarly, evidence indicates that administration of α-tocopherol at >400 IU/day may be associated with increased mortality, an observation attributed to prooxidant effects of α-tocopherol at high doses [195]. It is also unclear whether or not supplemental α-tocopherol exhibits the properties that would make it an appropriate chemoprevention agent. For example, plant-derived α-tocopherol represents a mixture of multiple compounds of which only one form (RRR-α-tocopherol) possesses anti-inflammatory properties, which are not exhibited by the synthetic vitamin E stereoisomers [196].

More generally, the view of antioxidants as nutraceuticals or chemical agents that are capable of preventing illness has a place only in the context of *in vivo* pathophysiological processes in which oxidative stress acts as either a cause or a significant contributor to the disease in question [197]. As observed by Halliwell and others, the *in vitro* assays widely used to measure the antioxidant activity of various nutrients or foods often turn out to be biologically irrelevant [34,198,199]. For example, vitamin C, a potent *in vitro* antioxidant, seems to have no effect on several markers of oxidative stress, including DNA base oxidation products in blood cells, 8-OHdG in urine, and F2-isoprostanes in plasma after a large single oral dose [200]. On the other hand, a daily dose of vitamin C over 2 months was reported to produce a statistically significant decrease in plasma F2-isoprostanes [201].

It is important to recognize that the "traditional" antioxidant micronutrients, such as vitamin E, vitamin C, and carotenoids, when consumed as part of the diet do not act in isolation, but as part of a package along with multiple other bioactive phytochemicals with strong antioxidant/anti-inflammatory properties. As shown in Fig. 1, dietary (i.e., nonenzymatic) antioxidants include two diverse and unequal groups: "traditional" antioxidant vitamins and related compounds (e.g., β-carotene and other carotenoids, α-tocopherol, and vitamin C), and phenolics—a large and heterogeneous category of phytochemicals that includes phenolic acids and their derivatives (e.g., curcumin and ellagic acid), flavonoids (e.g., anthocyanins and genistein), stylbenes (e.g., resveratrol), and various coumarins and tannins [202]. This classification excludes components of enzymatic antioxidants (e.g., selenium and molybdenum), and dietary factors that may decrease oxidative stress via anti-inflammatory effects (e.g., ω-3 fatty acids).

Unlike antioxidant vitamins and carotenoids, the phenolic compounds only recently began to attract attention [203], and most of those have not been tested in randomized, placebo-controlled cancer prevention trials. Of particular interest for future research are curcumin, a phenolic acid derivative obtained from the spice turmeric; resveratrol, a polyhydroxylated stilbene found in grapes and concentrated in red wine; and genistein, an isoflavone readily isolated from soy [204]. All three of these compounds have documented antioxidant and anti-

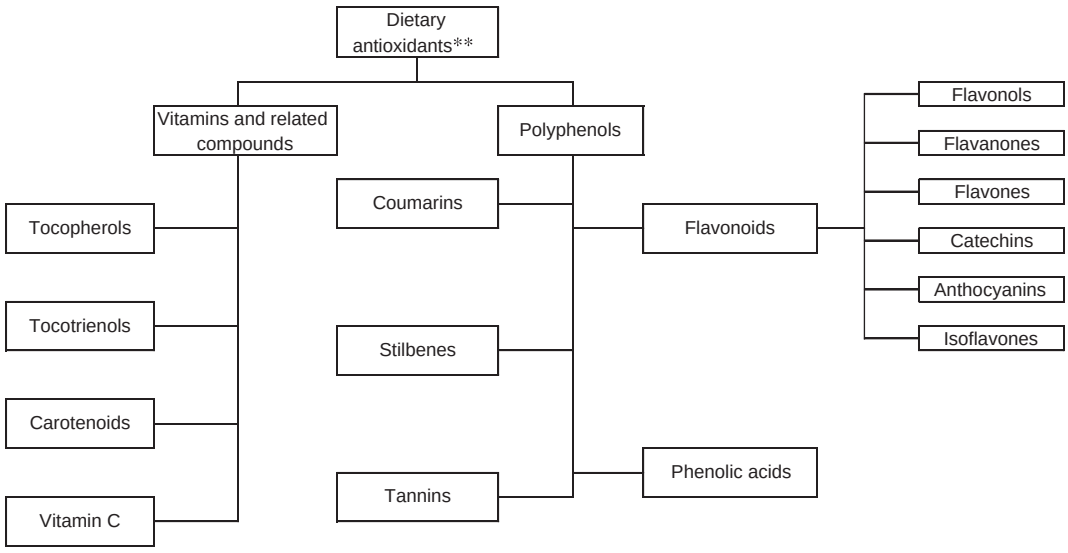

Fig. 1. Classification of dietary antioxidants. Adapted from Sies [35] and Liu [202]. ** This classification excludes components of enzymatic antioxidants (e.g., selenium and molybdenum) and dietary factors (e.g., ω-3 fatty acids) that may decrease oxidative stress via anti-inflammatory effects.

inflammatory properties [20,205,206], are viewed as promising cancer prevention agents based on *in vitro* and *in vivo* animal experiments [207–211], and were found to be safe in preliminary human trials [212–214].

There appears to be mounting evidence that the lack of success in previous trials may stem from the failure to consider interactions among multiple antioxidant agents as well as interactions between supplemental and dietary factors. Both *in vivo* and *in vitro* evidence indicates that antioxidant and anti-inflammatory effects of several micronutrients administered simultaneously may be stronger than the corresponding individual effects of the same micronutrients [215]. Observational epidemiologic studies suggest that beneficial effects of multiple dietary antioxidants combined may be greater than the effect of each of those compounds examined individually [216–219]. For these reasons there is a need to estimate physiologically relevant yet safe doses of multiple antioxidant and anti-inflammatory agents consumed simultaneously. As discussed and illustrated by Meyskens and Szabo (Fig. 2), a change in one or two (or even more) micronutrients may not produce a discernable effect if the biological response depends on multiple factors acting (and perhaps interacting) together [119]. Many of those micronutrients are present in food at physiologically appropriate doses in physiologically important combinations, which cannot be reproduced by using vitamin supplements [202,220].

More recently, attention began to shift from antioxidant compounds delivered as supplements to whole food interventions [221]. The idea of using foods rather than supplements is supported by mechanistic research. For example, Eberhardt et al. measured the antioxidant activity of whole apples by using the total oxygen radical scavenging capacity (TOSC) assay and found that the total antioxidant activity of 1 g of apples with skin was 83.3 TOSC [222]. That is, the antioxidant value of 100 g of apples is equivalent to 1500 mg of vitamin C. Given that the average vitamin C content in fresh apples with skin is only 5.7 mg per 100 g, and that the total antioxidant activity of 0.057 mg vitamin C (in 1 g of whole apples) is only 0.32 TOSC, then almost all of the antioxidant activity in apples is attributable either to phytochemicals other than vitamin C or to the synergistic effect of vitamin C and other antioxidant micronutrients [222]. The findings by Eberhardt et al. are in contrast to those reported by Lotito and Frei, who found high antioxidant capacity of apple polyphenols and apple extracts *in vitro*, but an absence of equivalent *in vivo* antioxidant effects in humans [223]. In a separate experiment by the same authors, consumption of apples and of equivalent amounts of fructose produced similar increases in antioxidant capacity. These findings indicate that increases in plasma antioxidant capacity in humans after apple consumption may be due to the well-known metabolic effects of fructose rather than to the effects of apple-derived antioxidants [224]. Despite the inconsistency of the above findings, the concept of "food synergy" appears to have gained momentum. Data indicate that the health effects of diet are dependent on the absorption of constituents within the foods, the ability of these constituents to survive digestion when ingested as part of a meal, and the extent to which they appear to interact at the cellular level [225,226].

A good example of a whole food agent is deeply pigmented berries (once a more prominent part of the human diet), which are among the highest ranked fruits with respect to variety, quantity, and quality of antioxidant and anti-inflammatory phenolics, most notably anthocyanins and ellagitannins [227,228]. Experiments that tested whole berry preparations (freeze-dried fruits, extracts, and juices) using *in vitro* models found that the protective effects of berries against DNA damage cannot be explained solely by anthocyanins or any other single category of phenolic compounds [229]. Based on *in vitro* experiments it has been reported that among various berry species certain types of strawberries and, in particular, black raspberries (BRB), produce the most pronounced beneficial proapoptotic effects [230].

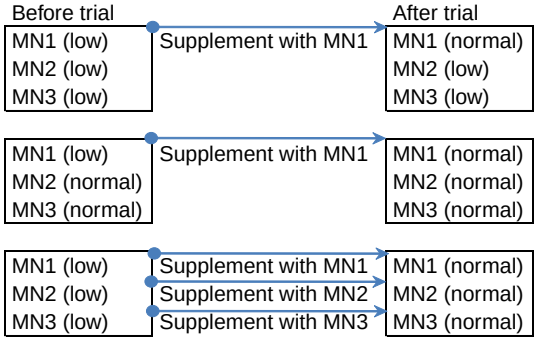

Fig. 2. Hypothetical examples of three dietary micronutrient (MN) trials with different outcomes. Adapted from Meyskens and Szabo [119].

The human data regarding the association between berry, and specifically BRB, consumption and risk of neoplasia are limited, but some preliminary evidence is available. In a pilot, 26-week chemoprevention trial ($n=10$) of 32 or 45 g (women and men, respectively) of freeze-dried BRBs consumed over 26 weeks in patients with Barrett's esophagus, berries were found to reduce the urinary excretion of two markers of oxidative stress, 8-epi-prostaglandin F2α (8-Iso-PGF2) and, to a lesser, more variable extent, 8-hydroxy-2-deoxyguanosine (8-OHdG) [231]. In another recently completed pilot study, 30 subjects with colorectal tumors consumed 20 g of freeze-dried BRB powder in water three times daily (60 g total) until their scheduled surgery date, usually within 2 to 4 weeks. Analyses of posttreatment biopsy specimens demonstrated that proliferation and angiogenesis biomarkers were reduced significantly by the berry treatment, whereas apoptosis was enhanced [232]. In another trial, patients with familial adenomatous polyposis (FAP) who consumed 60 g of BRB powder orally for 9 months had an approximately 50% decrease in the number of rectal polyps [233].

Other clinical trials that tested the efficacy of whole foods in reducing biomarkers of oxidative stress have produced a range of results. On the one hand, there are studies that found statistically significant reductions in markers of oxidative stress, such as F2-isoprostanes, HNE, 8-OH-dG, oxidized low-density lipoprotein, and malondialdehyde, after interventions with various categories of foods, including whole fruit and fruit juices [234–236], almonds and walnuts [237,238], green and black teas [239,240], soya beans and soy protein [241], and wine and raisins [242,243]. On the other hand, there are also studies that found no benefit (at least with respect to biomarkers of oxidative stress) of supplementing regular diet with additional fruit and vegetables [244,245], green tea and green tea extracts [244,245], soymilk [246], tomato drinks [247], and dark chocolate [248]. Unlike trials of berries, these studies are difficult to assess for consistency due to the variability of the selected agents and endpoints, and differences in the duration of the interventions and the study populations.

Summary and conclusions

Despite convincing evidence from *in vitro* experiments and *in vivo* animal studies, human trials that tested "traditional" antioxidant micronutrients as cancer chemoprevention agents have been unsuccessful or even resulted in harm. Based on the available data one has to agree with the previous reviews [32,53,249] that the use of traditional antioxidant vitamins, singly or in limited combinations, at pharmacologic doses, for cancer prevention cannot be justified. The lack of success in these trials and the apparent discrepancies between human experiments and mechanistic/observational data can be explained by a variety of factors, including the lack of sufficient biological rationale for selecting the specific agents of interest, the limited number of agents tested to date, the use of pharmacological (as opposed to dietary) doses that may produce harmful effects, and insufficient duration of the interventions and follow-up.

The latter consideration underscores the need for alternative (intermediate) endpoints (i.e., biomarkers of risk) that are associated with increased risk of neoplasia, but are detectable prior to tumor occurrence [250,251]. These intermediate endpoints should take into account the more modern definition of oxidative stress as "an imbalance in prooxidants and antioxidants with associated disruption of redox circuitry and macromolecular damage" [252]. The biomarkers of disease risk may prove to be the only feasible and informative endpoint for some intervention/cancer endpoint combinations given that clinical cancer development may require several decades of observation. This situation recalls an analogous one with ischemic heart disease 30–35 years ago. With the advent of biological measurements as markers of risk for the disease, including blood pressure, lipid profiles, blood sugar, and anthropometrics, plausible preventive interventions—both lifestyle and pharmacologic—could be readily investigated, response to preventive treatment could be monitored, and subsequently, with individual and population control of the "biomarkers," mortality rates from the disease began a dramatic 67% decline, which continues today [253,254].

Although dietary antioxidants are a large and diverse group of compounds, the trials conducted to date tested only a small proportion of possible candidates (Fig. 1). It appears that the ongoing trials are unlikely to provide very useful new information, because most of them are testing the efficacy of selenium and vitamin E against cancer incidence using similar doses over similar durations as did the previously reported, unsuccessful trials. There is a clear need for randomized, placebo-controlled trials to evaluate relatively novel antioxidant compounds such as resveratrol, curcumin, and genistein, which have not been tested in full-scale human experiments of cancer prevention [204]. Moreover, considering the limitations of supplementation, whole food interventions may prove to be more effective, although clinical trials with whole food interventions may be more difficult to conduct.

Another way to understand the failure of the antioxidant trials is to view them more generally as evidence against the reductionist approach in science, particularly when dealing with multifaceted processes such as oxidative stress and carcinogenesis that involve a variety of modifiable exposures and host-related factors [255,256]. A helpful illustration of this concept is the interaction between antioxidant nutrients and exercise. There is compelling evidence that physical activity may decrease risk and improve the prognosis of several common oxidative stress-related malignancies, including cancers of the colon and rectum, breast, and prostate [257–259]. All of these cancers are also thought to be affected by diet, presumably, at least in part, through the intake of antioxidants [260–263]. It is, therefore, of interest that a bout of physical exercise is known to be followed by increased oxidative stress in humans via increased production of RONS [264–266]. This apparent paradox is explained by the fact that regular exercise leads to increased adaptation to oxidative stress via an increase in antioxidant enzyme activity [267,268]. Studies are needed to examine the mechanisms by which exercise may facilitate cancer prevention and treatment (beyond the well-established benefits of weight control), and to investigate the joint effects of diet and exercise in human carcinogenesis [269,270].

Cancer is a complex disease, and development of a better understanding of cancer systems biology may be needed to effectively design cancer prevention strategies [271]. Genetic and epigenetic variation may mean that different individuals are likely to benefit from different prevention measures. If so, the successes of personalized cancer therapeutics [272] must be extended to personalized cancer prevention [117]. The availability of affordable genomic, proteomic, and metabolomic profiling that is now applicable to large population studies will continue to refine our understanding of individual susceptibility. Moreover, a recently introduced concept of the human "exposome" as a replacement for a single exposure paradigm [273,274] can provide a methodological foundation for future, likely more complex, but also more targeted, trials.

Another important factor that needs to be considered in designing future studies is the dual function of free radicals. While RONS undoubtedly play a critical role in regulating cell growth and differentiation, they also cause cellular damage resulting in the initiation or development of numerous diseases including cancer [275]. Thus, the challenge is to develop interventions that take into account both the beneficial and the harmful effects of free radicals.

In summary, the strategy of focusing on large high-budget studies using cancer incidence as the endpoint and testing a relatively limited number of antioxidant agents has been largely unsuccessful. This lack of success in previous trials should not preclude us from seeking novel ways of preventing cancer by modulating oxidative stress. On the contrary, the well-demonstrated mechanistic link between oxidative stress and carcinogenesis underscores the need for new studies. It

appears that future large-scale projects should be preceded by smaller, shorter, less expensive biomarker-based studies that can serve as a link from mechanistic and observational research to human cancer prevention trials. These relatively inexpensive biomarker-based studies would provide human experimental evidence for the likely efficacy, optimum dose, and long-term safety of the intervention of interest. Assessing long-term safety beyond simple monitoring for acute toxicities can be accomplished by measuring biomarkers of risk for various chronic conditions, such as cardiovascular disease. Also, because of the relatively low cost and short duration of such biomarker-based trials, more interventions can be evaluated quickly and safely. Consider the recently completed SELECT study, which followed its participants for a median of only 5.5 years and was unsuccessful. The cost of the SELECT trial is reported to be over 140 million dollars [117]. These resources would be sufficient to support dozens of the biomarker-based cancer prevention trials that could then be used to inform more definitive, safe, and more likely to succeed large-scale projects.

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