

Review

Vitamin C as an Antioxidant: Evaluation of Its Role in Disease Prevention

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Vitamin C in humans must be ingested for survival. Vitamin C is an electron donor, and this property accounts for all its known functions. As an electron donor, vitamin C is a potent water-soluble antioxidant in humans. Antioxidant effects of vitamin C have been demonstrated in many experiments *in vitro*. Human diseases such as atherosclerosis and cancer might occur in part from oxidant damage to tissues. Oxidation of lipids, proteins and DNA results in specific oxidation products that can be measured in the laboratory. While these biomarkers of oxidation have been measured in humans, such assays have not yet been validated or standardized, and the relationship of oxidant markers to human disease conditions is not clear. Epidemiological studies show that diets high in fruits and vegetables are associated with lower risk of cardiovascular disease, stroke and cancer, and with increased longevity. Whether these protective effects are directly attributable to vitamin C is not known. Intervention studies with vitamin C have shown no change in markers of oxidation or clinical benefit. Dose concentration studies of vitamin C in healthy people showed a sigmoidal relationship between oral dose and plasma and tissue vitamin C concentrations. Hence, optimal dosing is critical to intervention studies using vitamin C. Ideally, future studies of antioxidant actions of vitamin C should target selected patient groups. These groups should be known to have increased oxidative damage as assessed by a reliable biomarker or should have high morbidity and mortality due to diseases thought to be caused or exacerbated by oxidant damage.

Key teaching points:

- Vitamin C is essential for life and is a powerful water-soluble antioxidant.
- Antioxidant actions of vitamin C have been shown by *in vitro* experiments.
- Oxidant damage of biological molecules result in oxidation products that can be measured. These assays have not been fully validated.
- Diet rich in fruits and vegetables are associated with lower risk of cardiovascular disease and cancer. It is not known whether vitamin C contributes to these benefits.
- When vitamin C is given by mouth, the relationship between oral dose and plasma concentration is sigmoidal. Plasma concentrations are tightly controlled and excess vitamin C is excreted.
- Other than preventing scurvy, vitamin C has no proven benefits. In humans, vitamin C treatment has not resulted in changes in biomarkers of oxidation or in clinical outcome.

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Abbreviations: LDL = low density lipoprotein, 8OHdG = 8-hydroxy-2'-deoxyguanosine, SVCT = sodium dependent vitamin C transporter, NO = nitric oxide.

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INTRODUCTION

This review focuses on the actions of vitamin C action as an electron donor (antioxidant) in non-enzymatic reactions and examines whether this action has a role in the prevention of human disease.

PHYSIOLOGY OF VITAMIN C AND LABORATORY STUDIES OF ANTIOXIDANT ACTIONS

Biochemistry

Vitamin C (ascorbic acid) is a six-carbon lactone that is synthesized from glucose in the liver of most mammalian species, but not by humans, non-human primates and guinea pigs. These species do not have the enzyme gulonolactone oxidase, which is essential for synthesis of the ascorbic acid immediate precursor 2-keto-L-gulonolactone. The DNA encoding for gulonolactone oxidase has undergone substantial mutation, resulting in the absence of a functional enzyme [1,2]. Consequently, when humans do not ingest vitamin C in their diets, a deficiency state occurs with a wide spectrum of clinical manifestations. Clinical expression of vitamin C deficiency, scurvy, is a lethal condition unless appropriately treated. Thus, humans must ingest vitamin C to survive.

Vitamin C is an electron donor and therefore a reducing agent. All known physiological and biochemical actions of vitamin C are due to its action as an electron donor. Ascorbic acid donates two electrons from a double bond between the second and third carbons of the 6-carbon molecule. Vitamin C is called an antioxidant because, by donating its electrons, it prevents other compounds from being oxidized. However, by the very nature of this reaction, vitamin C itself is oxidized in the process.

It is noteworthy that when vitamin C donates electrons, they are lost sequentially. The species formed after the loss of one electron is a free radical, semidehydroascorbic acid or ascorbyl radical. As compared to other free radicals (a species with an unpaired electron), ascorbyl radical is relatively stable with a half-life of 10^{-5} seconds and is fairly unreactive. This property explains why ascorbate may be a preferred antioxidant. In simple terms, a reactive and possibly harmful free radical can interact with ascorbate. The reactive free radical is reduced, and the ascorbyl radical formed in its place is less reactive. Reduction of a reactive free radical with formation of a less reactive compound is sometimes called free radical scavenging or quenching. Ascorbate is therefore a good free radical scavenger due to its chemical properties [3,4].

Ascorbyl radical, with its unpaired electron, is not a long-lived compound. Upon loss of a second electron, the compound formed is dehydroascorbic acid. Dehydroascorbic acid stability depends on factors such as temperature and pH, but is often

only minutes [5]. Dehydroascorbic acid may exist in one of several different structural forms [6], but the dominant form *in vivo* has not been elucidated. Formation of both ascorbyl radical and dehydroascorbic acid is mediated by wide variety of oxidants in biological systems discussed below, including molecular oxygen, superoxide, hydroxyl radical, hypochlorous acid, reactive nitrogen species and the trace metals iron and copper.

Once formed, ascorbyl radical and dehydroascorbic acid can be reduced back to ascorbic acid by at least three separate enzyme pathways as well as by reducing compounds in biological systems such as glutathione. In humans, there is only partial reduction back to ascorbic acid; therefore, all the ascorbic acid that is oxidized is not recovered. Some of the dehydroascorbic acid is metabolized by hydrolysis and is lost. If the reduction process were complete, humans would not get scurvy. It is not known what the precise efficiency of the reduction process is *in vivo*, nor what factors regulate the reduction reactions *in vivo*.

If dehydroascorbic acid is not reduced back to ascorbic acid, it is hydrolyzed irreversibly to 2,3 diketogulonic acid. This compound is formed by irreversible rupture of the lactone ring structure that is a part of ascorbic acid, ascorbyl radical, and dehydroascorbic acid. 2,3-diketogulonic acid is further metabolized into xylose, xylonate, lyxonate and oxalate [7]. The formation of oxalate has clinical significance because hyperoxaluria (overexcretion of oxalate) can result in oxalate kidney stones in some people.

Enzymology

Although the focus of this review is on the role of vitamin C as an electron donor in non-enzymatic reactions, enzymatic reactions are briefly described for completeness. In humans, vitamin C acts as an electron donor for eight different enzymes [8]. At least for some of the enzymes, ascorbate adds electrons sequentially, with formation of the ascorbyl radical intermediate. Of the eight enzymes, three participate in collagen hydroxylation [9–11]. These reactions add hydroxyl groups to the amino acids proline or lysine in the collagen molecule, thereby greatly increasing stability of the collagen molecule triple helix structure. Two other vitamin C dependent enzymes are necessary for synthesis of carnitine [12,13]. Carnitine is essential for the transport of fatty acids into mitochondria for ATP generation. The remaining three vitamin C dependent enzymes have the following functions: one participates in the biosynthesis of norepinephrine from dopamine [14,15], one adds amide groups to peptide hormones, greatly increasing their stability [16,17], and one modulates tyrosine metabolism [18,19].

The enzymes with which ascorbic acid acts function as either monooxygenases or dioxygenases and are reviewed in detail elsewhere [8]. Briefly, the monooxygenases incorporate a single oxygen molecule into a dopamine substrate for norepinephrine synthesis or a glycine terminating peptide for amidation of peptide hormones. The dioxygenases incorporate two

oxygen molecules in two different ways. As part of tyrosine metabolism the enzyme 4-hydroxyphenylpyruvate dioxygenase incorporates two oxygen molecules into one single product. The other dioxygenases, functioning in carnitine synthesis and hydroxylation of collagen, incorporate one molecule of oxygen into succinate and one into an enzyme-specific substrate [8].

Vitamin C as an Antioxidant in Human Biology

As alluded to above, vitamin C can be oxidized by many species that have potential to be involved in human diseases [20,21]. The relevant species, which receive electrons and are reduced by vitamin C, can be divided into several classes: 1) Compounds with unpaired electrons (radicals) such as oxygen related radicals (superoxide, hydroxyl radical, peroxy radicals), sulphur radicals and nitrogen-oxygen radicals. With the exception of the sulfur radicals, these compounds are sometimes termed reactive oxygen species and reactive nitrogen species. 2) Compounds that are reactive but are not radicals, including hypochlorous acid, nitrosamines and other nitrosating compounds, nitrous acid related compounds and ozone. 3) Compounds that are formed by reaction with either of the first two classes and then react with vitamin C. An example is formation of the alpha tocopheroxyl radical, which is generated when exogenous radical oxidants interact with alpha tocopherol in low-density lipoprotein (LDL). The tocopheroxyl radical can be reduced by ascorbate back to alpha tocopherol [22]. 4) Transition metal-mediated reactions involving iron and copper. For example, reduction especially of iron by ascorbate can lead to formation of other radicals through Fenton chemistry [23]. On the other hand, reduction of iron could be an endpoint reaction: an example is that reduced iron may be the preferred form for intestinal absorption [24,25].

Detection of Vitamin C Action as an Antioxidant: Biomarkers of Oxidative Reactions

The oxidants just described can react with three general classes of biomolecules. We have categorized them roughly in the order in which they are found, from the outer envelope of the cell, to the interior of the cell: lipid, protein and DNA. If ascorbate is present, it can modify the reactions and their products. For each biomolecule class we will discuss here principles of the oxidant-mediated reactions and reaction products that can be measured and the potential effects of ascorbate. In a subsequent section we will discuss applications of the measurements to *in vitro* and *in vivo* experiments and the limitations of the measurements.

For lipids, membrane lipids and lipids in circulating lipoproteins such as low-density lipoprotein (LDL) can interact with reactive oxygen species resulting in lipid peroxidation. Once lipid peroxides form, they can react with oxygen to form highly reactive peroxy radicals. Continued formation of lipid hydroperoxides can result, a process termed radical propagation. Ascorbate can reduce the initiating reactive oxygen species so

that initial or continued lipid peroxidation is inhibited. Markers of lipid peroxidation include measurement of thiobarbituric acid reactive substances (TBARS) and F₂-isoprostanes and their metabolites. TBARS are believed to represent production of malondialdehyde, a peroxidation product of polyunsaturated fatty acids. F₂-isoprostanes and their metabolites are relatively stable products of radical mediated peroxidation of arachidonic acid and may be the most reliable markers of lipid peroxidation [26].

A related means to assess lipid oxidation is *ex vivo* oxidation of LDL. The principles supporting use of this measurement are those of the oxidative modification hypothesis [27–29]. This hypothesis, although unproven, is a widely accepted model of atherogenesis in humans and is based on oxidative modification of LDL as an initiating event in atherosclerosis. The major carrier of cholesterol and triglycerides in plasma is low-density lipoprotein (LDL). LDL can infiltrate the intimal layer of arteries and undergo oxidation locally, although the mechanism of oxidation is not fully understood. Oxidized LDL activates adhesion factor expression in endothelial cells. This induces monocytes to adhere to endothelium, where they are activated to differentiate into macrophages, in part via cytokines also induced by oxidized LDL. Macrophages accumulate oxidized LDL and remain in the vascular wall, developing into foam cells and subsequently into fatty streaks, the telltale lesion of atherosclerosis. In theory, the susceptibility of LDL to oxidation *in vivo* can be ascertained by *ex vivo* oxidation, in which LDL isolated from animals or humans is oxidized *in vitro* by added oxidants. If ascorbate reduces either initiating oxidants or oxidized intermediates, LDL oxidation should be decreased.

Proteins also undergo oxidation by several mechanisms [30,31]. A peptide chain can be cleaved by oxidants, or specific amino acids can be oxidized. The two amino acids most prone to oxidative attack are probably cysteine and methionine. Other amino acids involved include arginine, proline, threonine, tyrosine, histidine, tryptophan, valine and lysine. As occurs in lipids, radical propagation can occur in proteins, with formation of additional reactive species [32]. By reducing the radical initiators, ascorbate can prevent protein or amino acid oxidation and radical propagation. Protein oxidation most commonly is measured by detection of modified groups (carbonyl groups) or the oxidized amino acids themselves. Sugars and their oxidized products can also react with lysine moieties to form advanced glycation endproducts, although other substrates contribute to these products, such as amino groups on phospholipids. Ascorbate itself is proposed to be a substrate for some advanced glycation endproducts via oxidation and glyoxal formation, especially in the aging lens [33].

Oxidative processes can affect DNA indirectly through protein oxidation or lipid oxidation or directly by oxidation of DNA [21,34,35]. Indirect mechanisms leading to DNA damage include protein oxidation, which could alter repair enzymes and DNA polymerases. When reactive oxygen species interact with lipids, resulting lipid peroxidation products might then subsequently react with DNA, inducing mutations [36]. Similarly,

reactive nitrogen species can also damage proteins needed for oxidant defense or DNA repair or induce lipid peroxidation resulting in further cell damage to lipids, protein or DNA [37,38]. The most important mechanisms of DNA damage, however, are believed to involve direct attack of oxidants on individual nucleotides in DNA [34]. Guanine is the DNA base most susceptible to oxidative attack. When this occurs, there is formation of the nucleotide oxidation product 8 hydroxyguanine (abbreviated 8OHG or 8-oxoG) and its nucleoside derivative 8-hydroxy-2'-deoxyguanosine (abbreviated 8OHdG or 8-oxodG). Both of these compounds can be measured directly or by derivatization [34]. DNA can also be damaged by reactive nitrogen species, some of which can be derived from nitrosamines [37,38]. For example, nitric oxide radicals and related compounds can cause DNA strand breaks and point mutations [37–40]. Ascorbate should be able to diminish DNA damage by reducing radical species directly, decreasing formation of reactive species such as lipid hydroperoxides or preventing radical attack on proteins that repair DNA. Ascorbate as an antioxidant can prevent nitrosamine formation, so subsequent formation of some reactive nitrogen species is prevented. Once nitrosamines give rise to reactive nitrogen species, prevention of mutagenic activity by ascorbate is less effective in prevention of DNA damage [38].

Thus ascorbate reduces a variety of oxidant species; reactions giving rise to these species might occur in many cell compartments influencing lipids, proteins and DNA, and some of these reaction products can be quantitated, with and without ascorbate. Are these reactions relevant to humans? The answers depend on the range of ascorbate concentrations achieved in humans, the influence of the relevant ascorbate concentrations on relevant biomarker measurements as determined by experiments *in vitro* in animals and in humans, whether biomarker measurements are related to outcome and whether ascorbate influences outcomes predicted by biomarkers. These issues will be discussed in turn below.

Dietary Availability

To address ascorbate concentrations found in humans, it is necessary to describe ascorbate availability. Humans can obtain ascorbate only exogenously. Humans consume vitamin C by mouth with subsequent gastrointestinal absorption and distribution or receive it parenterally. Although ascorbate is added to enteral and parenteral formulations, we will focus here on ascorbate found in foods and supplements.

Vitamin C is mainly found in fruits and vegetables [41] (Table 1). Rich fruit sources include cantaloupe, grapefruit, honeydew, kiwi, mango, orange, papaya, strawberries, tangelo, tangerine and watermelon. Fruit juices containing vitamin C in abundance include grapefruit and orange juices. Several fruit juices are fortified with vitamin C, including apple, cranberry and grape juices. Rich vegetable sources of vitamin C include asparagus, broccoli, brussels sprouts, cabbage, cauliflower,

Table 1. Food Sources of Vitamin C

| Source (Portion Size) | Vitamin C, mg |
|--------------------------------------|---------------|
| Fruit | |
| Cantaloupe (1/4 Medium) | 60 |
| Fresh grapefruit (1/2 fruit) | 40 |
| Honeydew Melon (1/8 Medium) | 40 |
| Kiwi (1 Medium) | 75 |
| Mango (1 Cup, sliced) | 45 |
| Orange (1 Medium) | 70 |
| Papaya (1 Cup, cubes) | 85 |
| Strawberries (1 Cup, sliced) | 95 |
| Tangerines or tangelos (1 Medium) | 25 |
| Watermelon (1 Cup) | 15 |
| Juice | |
| Grapefruit (1/2 Cup) | 35 |
| Orange (1/2 Cup) | 50 |
| Fortified Juice | |
| Apple (1/2 Cup) | 50 |
| Cranberry juice cocktail (1/2 Cup) | 45 |
| Grape (1/2 Cup) | 120 |
| Vegetables | |
| Asparagus, cooked (1/2 Cup) | 10 |
| Broccoli, cooked (1/2 Cup) | 60 |
| Brussels sprouts, cooked (1/2 Cup) | 50 |
| Cabbage | |
| Red, raw, chopped (1/2 Cup) | 20 |
| Red, cooked (1/2 Cup) | 25 |
| Raw, chopped (1/2 Cup) | 10 |
| Cooked (1/2 Cup) | 15 |
| Cauliflower, raw or cooked (1/2 Cup) | 25 |
| Kale, cooked (1 cup) | 55 |
| Mustard greens, cooked (1 cup) | 35 |
| Pepper, red or green | |
| Raw (1/2 Cup) | 65 |
| Cooked (1/2 Cup) | 50 |
| Plantains, sliced, cooked (1 Cup) | 15 |
| Potato, baked (1 Medium) | 25 |
| Snow peas | |
| Fresh, cooked (1/2 Cup) | 40 |
| Frozen, cooked (1/2 Cup) | 20 |
| Sweet potato | |
| Baked (1 Medium) | 30 |
| Vacuum Can (1 Cup) | 50 |
| Canned, syrup-pack (1 Cup) | 20 |
| Tomato | |
| Raw (1/2 Cup) | 15 |
| Canned (1/2 Cup) | 35 |
| Juice (6 fluid oz) | 35 |

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kale, mustard greens, pepper (red or green), plantains, potatoes, snow peas, sweet potatoes and tomatoes and tomato juices. Variables that affect vitamin C content of fruits and vegetables are harvesting season, duration of transport to the marketplace, period of storage and cooking practices.

As a supplement, vitamin C is available in tablet and powder forms in many doses. In addition, vitamin C is included in many multi-vitamin formulations. Vitamin C is commonly combined with other selected vitamins and the resulting complex is collectively sold as an “antioxidant” supplement.

Due to its presence in a variety of fruits and vegetables, vitamin C is clearly available for consumption in all industrialized countries. United States Department of Agriculture and National Cancer Institute guidelines recommend the ingestion of at least five fruits and vegetables daily [42]. If these recommendations are followed, the amount of vitamin C ingested is estimated to be in the 200–300 mg range depending on the specific vitamin C content of the food consumed.

Although vitamin C is readily available in foods, data from the third U.S. National Health and Nutrition Examination Survey (NHANES III Part 1 1988–91) suggest that the median vitamin C consumption from diet in adult males and females is 84 mg and 73 mg daily, respectively [43]. In children, vitamin C ingestion was reported to be below the RDA in 25% of the population for this age group [44]. A survey of Latino children indicated that 85% did not meet the daily recommended intake of fruits and vegetables [45]. However, these data do not include vitamin C consumption from supplements [46]. It is reasonable to estimate that half of the US population does not ingest supplements [46–48]. For those who do ingest them, it is uncertain whether supplements substantially change total vitamin C consumption [46]. We conclude that despite NHANES III data indicating a small increase in the median dietary vitamin C ingestion in the USA, a substantial fraction of the population still ingests vitamin C at or below the Recommended Dietary Allowance [49].

Vitamin C Concentrations in Humans as a Function of Dose

Vitamin C concentrations in plasma are tightly controlled as a function of dose [50,51]. At plasma concentrations less than 4 μM , symptoms of scurvy may occur. Doses of 30 mg daily yield steady-state plasma concentrations of approximately 7 μM for men and 12 μM for women. For both genders, there is a steep sigmoid relationship between dose and plasma concentrations at doses between 30 and 100 mg daily (Figs. 1 and 2). At 100 mg daily steady-state plasma concentrations are slightly less than 60 μM for men and slightly greater than 60 μM for women. However, the dose-concentration curve between 30 and 100 mg daily is shifted to the left for women compared to men. At doses of 200 mg daily and higher, steady-state plasma values for both genders are similar. Plasma is completely saturated at doses of 400 mg daily and higher, producing a steady-state plasma concentration of approximately 80 μM .

Tight control of vitamin C concentrations is mediated by tissue transport, absorption and excretion [50–53]. As representative of tissue transport, vitamin C concentration in relation to dose has been measured in circulating neutrophils, lymphocytes and monocytes. These cells contain 1–4 mM concentrations of vitamin C and saturate at vitamin C doses between 100 and 200 mg daily (Fig. 3). These doses produce plasma concentrations that are similar to those at which maximal velocity is achieved by the vitamin C tissue transporter SVCT2 [54].

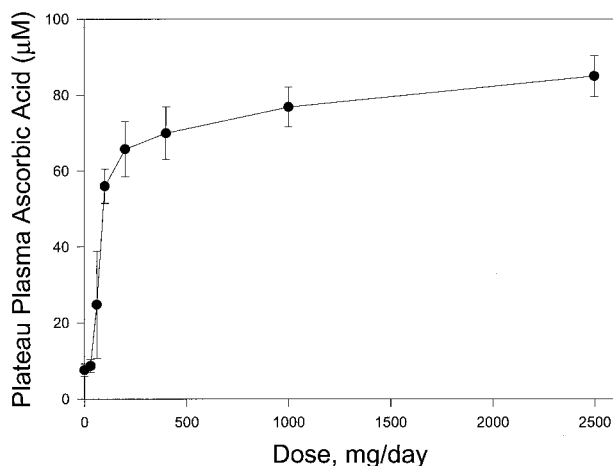


Fig. 1. Steady-state plasma vitamin C concentrations (mean \pm SD) as a function of dose for all doses for seven men. Subjects consumed a vitamin C deficient diet, resulting in plasma and tissue vitamin C depletion. Vitamin C in solution was then administered by mouth at the doses shown until steady state was reached for each dose. Doses through 400 mg daily were received by seven subjects, through 1000 mg daily by six subjects and through 2500 mg daily by three subjects. Reproduced with permission from Proceedings of The National Academy of Sciences, from which details of the study can be obtained [50].

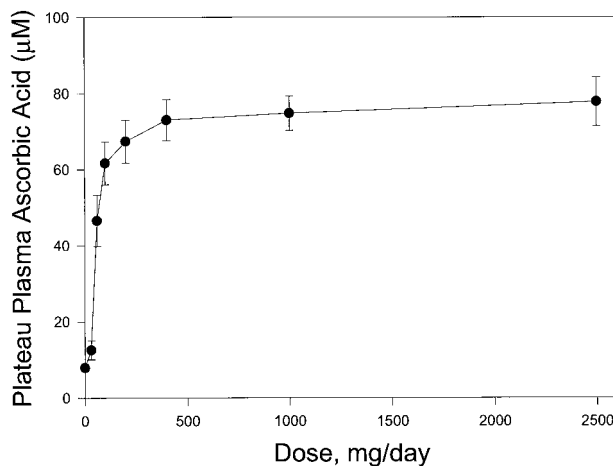


Fig. 2. Steady-state plasma vitamin C concentrations (mean \pm SD) as a function of dose for all doses for 15 women. Subjects consumed a vitamin C deficient diet, resulting in plasma and tissue vitamin C depletion. Vitamin C in solution was then administered by mouth at the doses shown until steady state was reached for each dose. Doses through 200 mg daily were received by 15 subjects, through 1000 mg daily by 13 subjects and through 2500 mg daily by 10 subjects. Reproduced with permission from Proceedings of The National Academy of Sciences, from which details of the study can be obtained [51].

Thus, cells saturate before plasma in men and women. Intestinal absorption of vitamin C at steady-state is inversely related to dose. The higher the dose, the less absorption occurs. For example, at a dose of 30 mg, nearly 90% of the dose is absorbed, while at a dose of 1250 mg less than half of the dose

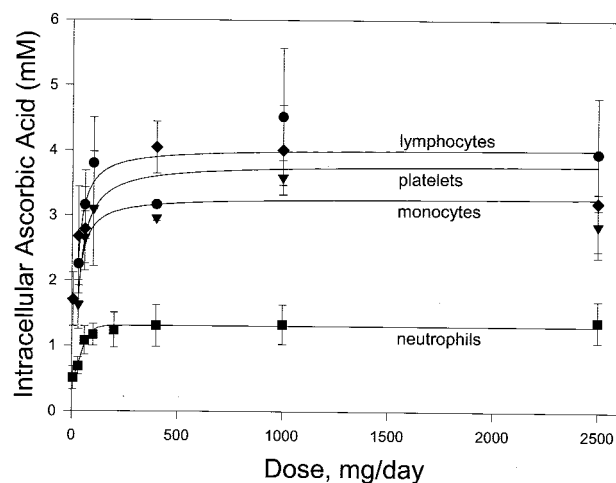


Fig. 3. Intracellular vitamin C concentrations (mean \pm SD) in circulating cells as a function of dose in women. Cells were isolated when steady-state was achieved for each dose. For neutrophils, samples were available from 13 women at doses 0–200 mg daily; from 11 women at doses of 400 and 100 mg daily, and from 10 women at 2500 mg daily. For lymphocytes, monocytes and platelets, samples were available from 13 women at 30 mg daily; from 12 women at 60 mg daily; from six women at 100 mg daily; from two women at 400 and 1000 mg daily; from nine women at 2500 mg daily. Reproduced with permission from Proceedings of The National Academy of Sciences, from which details of the study can be obtained [51].

is absorbed. Although the fraction of the dose absorbed declines as doses increase above 50 mg, absorption still occurs. Tight control of plasma concentrations is then mediated by renal excretion. In the kidney, vitamin C is filtered through the glomeruli and reabsorbed in the proximal tubule by vitamin C transporter SVCT1 [55]. When the transport reabsorption mechanism approaches maximal velocity, additional vitamin C cannot be absorbed and is lost in urine. The dose at which reabsorption saturates is the threshold dose for vitamin C excretion and occurs at vitamin C doses between 60 and 100 mg daily. At doses of 500 mg and above, the entire absorbed dose is excreted. Vitamin C reabsorption and excretion by the kidney play a key part in tight control of vitamin C plasma and tissue concentrations in healthy humans, and this control is lost in patients with end stage renal disease.

In Vitro and Animal Studies of Antioxidant Effects of Vitamin C

Vitamin C and Lipid Oxidation. In vitro experiments have assessed effects of ascorbate on *ex vivo* LDL oxidation. In these experiments, LDL is isolated from plasma and then subjected to oxidation conditions, usually in the presence of a transition metal (copper or iron). Oxidation products are measured, most commonly conjugated dienes and lipid hydroperoxides. Delay in oxidation by ascorbate is measured as lag time and change in rate of lipid peroxidation. To assess effects of ascorbate the

vitamin is added exogenously because it is lost during LDL isolation. There is no question that *ex vivo* oxidation of LDL is decreased by ascorbate added at physiologic plasma concentrations [56–61]. Unfortunately, there are difficulties interpreting these findings. It is uncertain, and perhaps unlikely, whether transition metals used to induce oxidation *in vitro* are actually present *in vivo* for the required times and at the required concentrations. Binding proteins for iron and copper should bind both metals avidly *in vivo*, so that they would be unavailable to induce oxidation. It is also not clear whether *ex vivo* oxidation of LDL accurately mirrors *in vivo* events [62,63]. For example, vitamin E doses that inhibit LDL oxidation *ex vivo* are not effective *in vivo*, in hypercholesterolemic animals [64].

Lipids in isolated plasma have been oxidized artificially, and the effect of ascorbate was measured. A variety of oxidizing agents have been used [23]. Endogenous and exogenous vitamin C decreased lipid oxidation as measured by lipid hydroperoxide and F_2 -isoprostanes formation [23,65–67]. The latter measurement is important, because unlike most other measurements F_2 -isoprostanes represent specific and stable markers of *in vivo* lipid peroxidation, although these compounds can be technically challenging to measure properly [26]. In some of these experiments physiologic concentrations of vitamin C were studied because the effects of endogenous plasma vitamin C concentrations were assessed, although effects of high unphysiologic concentrations were also reported. A difficulty in interpretation is that the physiologic meaning is uncertain when exogenous oxidizing agents are studied, such as copper or 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH). In smokers, who have the oxidant stress of cigarette smoke, isoprostanes were also decreased as discussed below [68].

In other more recent studies antibodies have been used to quantitate oxidized LDL *in vitro* and *in vivo*. In one approach lipid hydroperoxides were used to generate an antigenic protein epitope that could be detected immunologically. Lipid peroxide-modified serum albumin was injected into rabbits, and a polyclonal antibody was generated [69]. This antibody specifically recognized at least three lipid peroxide-modified proteins, including oxidized LDL. This antibody has been used to detect oxidatively modified proteins, such as those in atherosclerotic lesions in cholesterol fed monkeys [69], cardiac proteins in rat hearts subject to ischemia and reperfusion *ex vivo* [70] and plasma of women with endometriosis [71]. In another approach, a monoclonal antibody against an epitope in oxidized LDL has been used to identify patients with coronary artery disease [72]. Circulating autoantibodies in humans against malondialdehyde-modified LDL have also been described [73]. It is not known whether ascorbate *in vivo* decreases formation of these autoantibodies nor whether ascorbate decreases oxidatively modified proteins detected by antibodies specific for oxidized LDL [74]. Such information would provide strong evidence that ascorbate is a protective antioxidant *in vivo*.

Studies in animals addressed whether vitamin C protects against lipid peroxidation, either when endogenous lipid peroxidation was measured or lipid peroxidation was induced by administration of an exogenous oxidizing agent. When endogenous lipid peroxidation was measured, it was decreased by vitamin C in most [75–77] but not all reports [78]. There are also many studies of exogenously induced lipid peroxidation. For example, animals that do not synthesize vitamin C, such as guinea pigs and osteodystrophy syndrome (ODS) rats, were protected by vitamin C from oxidant stress mediated by carbon tetrachloride [79] and endotoxin [80]. Rats exposed to cigarette smoke were also protected by ascorbate [81]. However, when alloxan was the oxidant stress agent, rats had increased oxidant stress when supplemented with vitamin C [82]. Ascorbate was protective when it was administered before the oxidant stress agent paraquat, but the vitamin accelerated oxidant stress when it was administered after paraquat [83]. Such data suggest that ascorbate can act as an antioxidant or prooxidant, dependent on its concentrations and those of the administered oxidizing agent. In many studies lipid peroxidation in plasma or tissues was assessed by thiobarbituric acid reactive substances as a proxy for malondialdehyde or by exhaled pentane or ethane levels. As noted above, thiobarbituric acid reactive substances are not specific indicators of malondialdehyde, so that the meaning of the measurement is not always clear. More importantly, ascorbate or the oxidant stresses were sometimes administered to animals in pharmacologic doses. Hence their physiologic relevance is uncertain.

Dietary antioxidants can decrease atherosclerosis in LDL-receptor deficient mice, cholesterol fed rabbits and cholesterol fed primates [27,84,85]. For example, animals can be fed high cholesterol diets with induction of atherosclerosis, which was decreased by antioxidants including vitamins C and E and probucol [63,86]. The effect of vitamin C as an antioxidant alone was not determined. These experiments may not reflect physiologic conditions because of the amounts of antioxidants administered and the amounts of cholesterol and fat in the diets. Also, in mice no correlation was observed between the lag phase of LDL oxidation and lesion size in individual animals, suggesting that there are limitations to measuring LDL oxidation *ex vivo* [63].

Vitamin C and Protein Oxidation. *In vitro* experiments have quantitated effects of ascorbate on protein oxidation. The most commonly used and best-studied technique is measurement of protein carbonyls. Advantages are that assays are relatively simple and inexpensive. Disadvantages are that the measurement does not reflect a specific pathway of protein oxidation and may represent uncharacterized products. There are a variety of pathways that can result in carbonyl formation, and some may not reflect direct oxidative modification. Carbonyls are generally more difficult to induce compared to other derivatives, particularly those of methionine and cysteine [31]. Although these two amino acids are quite susceptible to oxidative damage, modifications to them may not alter overall

protein function. Newer techniques to assess protein oxidation utilize mass spectrometry and detect modified tyrosines, including di-tyrosine, nitro-tyrosine, chloro-tyrosine, and tyrosine isomers [62]. These techniques may be more specific and sensitive, but are expensive, require special sample handling and processing and sophisticated instruments and are not as well characterized [87]. As for lipids, there is impressive evidence with isolated proteins that free radical species, particularly reactive oxygen species, are damaging. Reactive oxygen species cause changes in proteins with respect to catalytic activity, other functional activity, heat stability and susceptibility to proteolysis [30,31,88,89]. The issues remain whether these changes are relevant to conditions *in vivo*, given the extraordinary complexity of oxidant and antioxidant chemistry. For example, it is not clear that reactions that generate protein carbonyls with isolated proteins occur *in vivo*. Indeed, some *in vitro* experiments with human plasma show that protein carbonyls are not affected by ascorbate in the presence of oxidant stress [90,91]. In other experiments where protein carbonyls were generated in human plasma, the induction conditions may not be physiologically or clinically relevant [92]. The other side of the coin can be seen in experiments using proteins isolated from human lens [93,94]. Ascorbate accelerated formation of advanced glycation end products through ascorbylation of protein; the ascorbylated proteins bound copper, and the copper protein complexes generated free radicals. These findings suggest that ascorbate could actually accelerate protein damage, although it is unclear whether the experimental systems mimic *in vivo* physiology.

As for isolated proteins, oxidative modification and loss of function have been described in animals and insects [31]. For example, oxidative modification in animal models may be relevant to aging [30,95,96], to diabetes in primates [97] and to cataract formation [98,99]. With respect to cataracts, vitamin C in animals might be protective [100,101]. Protein carbonyls have been demonstrated *in vivo* in a variety of disease states in humans, as discussed below, but their meaning is uncertain.

Vitamin C and DNA Oxidation. DNA oxidation has been reported both to be prevented and accelerated in the presence of vitamin C in *in vitro* experiments with DNA, nuclei and cells. For example, in experiments where damage was prevented, the initiators of DNA damage included metal ions, UV light and hydrogen peroxide [102–104]. Sometimes such conditions may not reflect *in vivo* physiology. In other experiments ascorbate has accelerated DNA damage [105]. It is not clear whether some of these measurements reflect unanticipated oxidant effects of trace metals. For experiments with isolated DNA and cells, non-physiologic concentrations of ascorbate can be used inadvertently, either above or below the concentrations found in humans. DNA damage occurs and can be measured in animals. For example, spontaneous DNA damage in old rats is estimated at approximately 66,000 adducts per diploid cell [106]. In animal experiments non-physiologic concentrations of ascorbate can be avoided depending on the animal selected and

the amount of ascorbate administered. Guinea pigs, a species unable to synthesize vitamin C, have been assessed for liver DNA damage at the following vitamin C intakes: marginal, sufficient and megadose. No difference in DNA damage was found, with measurements of 8OHdG [107]. When guinea pigs and rats were exposed to corneal ultraviolet light at high doses, vitamin C protected against DNA strand breaks, although the stimulus was not physiologic [108].

A major problem in many DNA oxidation experiments and in the clinical experiments discussed in a later section is how DNA damage was assessed. The most convenient assay detects DNA strand breaks by their ability to relax supercoiled loops in DNA. Cells are embedded in agarose, lysed to form nucleoids and the nucleoids subjected to electrophoresis at high pH. The relaxed DNA extends from the nucleoid like a tail of comet, explaining how the comet assay was named. Although the comet assay is relatively straightforward, it might substantially underestimate DNA base damage and has not been fully and rigorously tested for its ability to quantitatively detect DNA damage with inclusion of appropriate controls and standards [34].

More sophisticated techniques are available to measure DNA damage, including gas chromatography with mass spectrometry and high performance liquid chromatography with electrochemical detection, but these techniques have perils. For analysis DNA first has to be isolated and hydrolyzed, but these procedures have an associated but uncertain oxidation price. DNA oxidative damage might inadvertently occur due to exposure to oxygen, trace metals in reagents, heating, hydrolysis and derivatization. 8OHdG is the most frequently measured DNA oxidation product, but guanine is easily oxidized accidentally prior to or during analysis. 8OHdG also can be inadvertently destroyed before it is measured. Whether 8OHdG is representative of generalized DNA damage and what are the best methods to detect such damage continue to be debated [34].

It is also uncertain what samples are best to measure for DNA damage and what these measurements mean. 8OHdG is believed to be unaffected by diet and not metabolized in humans, so it appears that urine excretion of 8OHdG is a useful measurement [109]. However, as 8OHdG excretion changes, does this reflect changing damage or changing repair or both? It is difficult to compare cell measurements, using variations on the comet assay, and 8OHdG excretion, because of differences in sensitivity. Recent and continued advances in assay techniques may help to solve some of these problems [34,109].

DNA can also be damaged by nitrosamines. Instead of measuring damaged DNA, some nitrosamine compounds can be measured in the presence and absence of reducing agents like ascorbate. For example, ascorbic acid and other antioxidants prevent formation of nitrosamines *in vitro* [110]. However, ascorbic acid can decrease, not affect, or increase nitrosamine formation in experimental animals [111–113].

CLINICAL STUDIES OF ANTIOXIDANT EFFECTS OF VITAMIN C

Role of Oxidant Damage in Human Disease

Oxidant damage might cause or exacerbate common human diseases, such as atherosclerosis [114–116] and type II diabetes mellitus [114,117,118]. It might also have a role in the pathophysiology of type I diabetes mellitus [119], diabetic complications [120], chronic renal failure [121,122], complications of end stage renal disease and hemodialysis [123], rheumatoid arthritis [124], neurodegenerative diseases and pancreatitis [125]. Oxidants are thought to cause further damage to organ systems during acute illnesses such as myocardial infarction, acute pancreatitis, sepsis and inflammatory disorders and play an important role in the long term damage from cigarette smoking.

Proposed Antioxidant Effects of Vitamin C in Experimental Human Studies

A number of studies in the human have attempted to demonstrate the effects of vitamin C on vascular responsiveness, intestinal iron absorption and reduction of harmful oxidants in the stomach. These effects are thought to be mediated by the antioxidant actions of vitamin C. These effects, though best shown in experimental settings, may play a role in vascular disease, hypertension, iron absorption and in the prevention of gastric cancer.

Effects of Vitamin C on Vascular Endothelium. Vitamin C may increase endothelial nitric oxide (NO) by protecting it from oxidation and increasing its synthesis [126,127]. Vitamin C and the other antioxidant vitamin, vitamin E, appear to have beneficial effects on vascular endothelial function in healthy subjects and in patients with cardiovascular disease [128]. However, these effects are modest and difficult to show at physiological vitamin C concentrations. Some evidence suggests that increased vascular oxidative stress contributes to the pathophysiology of endothelial dysfunction and hypertension [116,128,129]. Low plasma vitamin C concentrations have been associated with hypertension and impaired endothelial function. Vitamin C present in fruits and vegetables may protect NO from oxidation and ameliorate endothelial dysfunction. This might account for some of the protective effects of fruits and vegetables on the cardiovascular system.

Pharmacological doses of vitamin C produce vasodilatation in the brachial and coronary arteries [130,131]. In healthy subjects, vitamin C administration restored endothelium-dependent vasodilatation that was impaired by acute hyperglycemia [132]. Thus vitamin C may have favorable effects on vascular dilatation, possibly through its antioxidant effects on NO [133,134], but these findings are not consistent [135]. Moreover, in most studies, the vitamin C-induced effects on

vasodilatation occurred when vitamin C was administered intra-arterially. It should be noted that oral administration of a gram of vitamin C results in steady state plasma concentrations of 70–80 μM , with transient peaks of 120 μM . In contrast, parenteral administration of the same dose produced plasma vitamin C concentrations that were ten times higher. Whether vasodilatation occurs at physiologically relevant concentrations of vitamin C is uncertain [136].

A high vitamin C intake is associated with lower blood pressure [137]. Plasma vitamin C and dietary intake were found to be covariates of blood pressure in the elderly [138]. Some studies show that supplemental vitamin C intake lowered blood pressure [139,140], but these results have to be confirmed with larger well controlled clinical trials. Dietary supplementation with vitamin C also reduces the development of tolerance to transdermal nitrates [141]. In summary, the effects of vitamin C on vessel dilatation is modest at best, and is generally seen at supra physiological plasma vitamin C concentrations. The clinical significance of these findings are not yet clear.

Antioxidant Effects of Vitamin C in the Gastrointestinal Tract. Vitamin C increases iron absorption from the small intestine [142,143] by keeping iron reduced [144]. This effect is seen at vitamin C doses of 20–60 mg, an amount easily found in one meal of healthy diets. While the effects of supplemental vitamin C on increased iron absorption have been shown in many [145,146] but not all studies [147,148], it has only a modest, if any, effect on increasing hemoglobin concentration [146,149,150]. In normal subjects, the concentration of vitamin C in gastric juice is approximately three times higher than that of plasma [151]. Vitamin C content is low in the gastric juice of patients with hypochlorhydria [152], atrophic gastritis and *Helicobacter pylori* infection, conditions associated with gastric cancer. Eradication of the bacteria increases gastric vitamin C secretion [153]. Gastric juice vitamin C concentrations are normal in patients at risk for familial gastric cancer [154]. Vitamin C may also quench reactive oxygen metabolites in the stomach or duodenum and prevent the formation of N-nitroso compounds that are mutagenic. Nitrosamines have been linked to gastric cancer. Formation of nitrosamines in the gastrointestinal tract can be decreased by administration of vitamin C [155]. High dietary vitamin C intake correlates with reduced gastric cancer risk [156]. Although high dietary vitamin C intake correlates with reduced gastric cancer risk [156], it is not certain what confers protection: vitamin C itself or other components of foods, particularly fruits and vegetables, that also happen to contain vitamin C.

Proposed Antioxidant Role of Vitamin C in Human Disease

Disease Conditions with Low Plasma Vitamin C Concentrations. Many disease conditions that are thought to be caused or exacerbated by oxidant stress are also associated with low plasma and tissue vitamin C concentrations. The most

common prooxidant conditions with low plasma vitamin C concentrations are smoking [157,158] and diabetes mellitus [159–161]. Vitamin C concentrations may also be low in patients with myocardial infarction [162,163], acute pancreatitis [164,165], infections and possibly other disorders. It is however not clear whether low plasma and tissue vitamin C contributes to each of these diseases, is a consequence of the disease process or is merely associated with the disease condition. Some of these conditions are associated with sub-optimal nutrition, and the low vitamin C may simply reflect a poor diet [166]. Further, vitamin C is an unstable compound and is easily oxidized even in blood samples obtained from healthy volunteers. The stability of vitamin C during sample processing, in the presence of oxidants or other substances that may be present in the plasma of these patients, has not been studied. Oxidation of vitamin C in the test tube may produce erroneously low values and could account for some of these findings [167].

Relationship of Vitamin C to Diseases that Result from Putative Oxidant Damage

A number of studies have investigated the effect of vitamin C on chronic diseases. These can be categorized according to whether lipid, protein or DNA is deemed to be the primary target of free radical assault. Although this mechanistic hypothesis may be unduly narrow, it nevertheless provides a conceptual framework to investigate these complex disorders.

Oxidative Damage to Lipids. Reduction in cardiovascular disease by a diet high in fruits and vegetables has been shown in many studies and meta-analyses of epidemiological studies. The presumed mechanism is protection of LDL from oxidation by vitamin C and other dietary antioxidants. However, other dietary factors such as reduction in total fat and caloric intakes may be equally or more important.

A diet rich in fruits and vegetables reduces mortality [168,169], protects against atherosclerosis [170], stroke [171,172] and, to a lesser extent, against coronary artery disease [172–175], though vitamin C itself may not contribute to this protection [176,177]. The use of vitamin C as supplement, often in combination with other antioxidant micronutrients showed either no benefit [176–179] or marginal benefit [180–182]. Systematic reviews of existing literature have concluded that vitamin C may [183] have some protective effect against stroke and a lesser effect against coronary artery disease or that its role is unproven [184,185].

Oxidative Damage to Proteins. Considering the vital role of proteins in the machinery of life, it can be expected that protein oxidation will lead to a wide variety of diseases. Cataract is thought to result, at least in part, from oxidative damage to lens proteins. Studies of cataracts have shown a small [186,187] or no protective effect with a high fruit and vegetable diet or with vitamin C [188,189].

Oxidative Damage to DNA. Mutations are the initiating events in neoplasms. Because of this, DNA oxidation is thought

to increase the incidence of cancers. Epidemiological studies show that a fruit and vegetable rich diet may reduce cancers in general [169,190], in addition to cancers of specific organs, such as stomach cancer [191]. Vitamin C rich food may [192] or may not protect against breast cancer [193]. Vitamin C had no protective effect against basal cell cancer of the skin [194], non Hodgkins lymphoma [195] or colorectal cancer [196]. Vitamin C supplementation did not reduce colorectal adenomas [197] or cancer incidence in the largest such trial so far [198].

Summary of Epidemiological Studies on the Effects of Vitamin C on Human Disease

Studies of the effect of diet on human disease have generally determined food consumption by dietary survey or diet diaries and, occasionally, by direct food measurement. Nutrient concentrations in blood are also measured in some studies. The findings are then correlated with morbidity and mortality. Cross sectional and longitudinal studies show that the occurrence of cardiovascular disease and cancer is inversely related to vitamin C intake and plasma vitamin C concentrations. The main source of vitamin C is fruits and vegetables, and hence plasma vitamin C concentration is a marker of fruit and vegetable intake [199]. Fruits and vegetables also contain other vitamins, antioxidants and myriad other substances whose identity, let alone actions, are unknown. Hence the protective effects seen in these studies are attributable to fruit and vegetable intake and not specifically to vitamin C. Vitamin C may or may not contribute to this protection. Additionally, those who have a high intake of fruits and vegetables differ in many ways from those who have low intake of these foods. In Western countries (where most of these studies have been done), those with a high fruit and vegetable intake tend to be more health conscious, educated and affluent, all of which are independently associated with a lower cardiovascular risk. Perhaps other variables in this population may also be important in disease causation. Therefore, these studies cannot be interpreted to mean that a high intake of vitamin C, by its antioxidant or other actions, has a beneficial effect on morbidity and mortality, nor can they conclusively show that a high vitamin C intake is beneficial. The same applies to smokers and diabetics, who have low plasma vitamin C (which, as noted above, could be to some extent due to measurement artifacts) and also a low fruit and vegetable intake. Subjects who obtain vitamin C by taking it as a supplement also have the same confounding factors of being health conscious and affluent. Additionally, some people taking supplements may be already ill, with illness being the reason for vitamin supplementation. In general, beneficial effects of supplemental vitamin C have been noted in small studies, while large well-controlled and prospective studies have failed to show benefit. Some of the many confounding factors noted above can be controlled for by appropriate statistical treatment of epidemiological study data. Such analysis suggests that vitamin C may have a protective role. However, it cannot

conclusively prove the clinical benefits of vitamin C or its antioxidant actions in humans.

Experimental Studies of Vitamin C in the Human

Dose of Vitamin C at which Clinical Deficiency Occurs.

Very small doses of vitamin C, no more than 10 mg/day in adults, are sufficient to prevent scurvy, a condition that is now rare. At moderately low plasma vitamin C concentrations, no derangements in physiology are discernable, save for fatigue at plasma vitamin C concentration below 20 μM , corresponding to an oral intake of 30–60 mg of vitamin C/day. At higher doses, this symptom disappears. Fatigue is well known to precede clinical scurvy. Whether antioxidant protection accrues at higher doses is unclear. Current recommended dietary intake for vitamin C is 90 mg/day for men and 75 mg/day for women [200].

Clinical Studies of Biomarkers of Oxidation in Relation to Vitamin C

The effect of vitamin C and other putative antioxidants on biomarkers of oxidation have been studied in many pathological states that are thought to result from, or result in oxidant stress. The most commonly used biomarkers of oxidation are protein carbonyls for protein oxidation, 8OHdG for oxidative damage to the DNA and isoprostanes for lipid oxidation.

Lipid. Several studies have evaluated the effect of human disease on plasma and urine isoprostane concentrations [26,201]. They were found to be elevated in atherosclerosis and diabetes [68]. Smokers had much higher concentrations of plasma and urinary isoprostanes and this decreased after smoking abstinence [202]. Isoprostane concentrations have also been reported to be reduced by vitamin C [203–205]. We have shown that isoprostane concentrations do not change in normal women despite changes in steady state plasma vitamin C concentrations from pre-scorbutic concentrations of 8 μM to plasma saturation at about 70 μM [51].

Protein. Oxidative modifications of proteins can be measured by increases in protein carbonyls. Protein oxidation has been demonstrated in several human conditions including diabetes and aging [206]. Studies of vitamin C treatment have shown small reductions in carbonyls but only in subjects with low pretreatment plasma vitamin C concentrations [207].

DNA. The effect of vitamin C on DNA damage has been much discussed but there is no evidence yet of direct benefit from vitamin C [34]. Vitamin C had no effect on placental [208] or urinary 8OHdG [209,210], but reduced it in smokers [211].

Prooxidant Effect of Vitamin C. There are fears that vitamin C may have prooxidant [212] or mutagenic [36] effects. Studies showing these effects have not been reproduced or have used un-physiological doses of vitamin C or artificial conditions. It is not known whether physiological concentrations of vitamin C have prooxidant effects and what their relevance is to clinical practice. The potential toxicity of vitamin C needs further study [21].

Summary of Biomarker Studies. Biomarkers of oxidation may be elevated in many diseases associated with oxidant stress. Studies to date show that vitamin C either has no effect or produces modest reductions in the concentrations of these biomarkers. It is possible that combinations of many antioxidants are more effective. However, a fruit and vegetable concentrate, which should have contained many antioxidants, did not have any effect on markers of oxidation in smokers [213].

Role of Biomarkers of Oxidation in Clinical Studies of Antioxidant Effects of Vitamin C

Clinical studies of antioxidant effects of vitamin C using biomarkers of oxidation have produced conflicting results. Biomarker studies are more likely to show positive results in patient groups with high oxidant stress such as those with diabetes, renal failure or in smokers. This has indeed been the case so far. The clinical significance of changes in biomarker concentrations is not known. Demonstration of a clear relationship between biomarkers and health and disease is essential if such measurements are to be useful [214]. Biomarker assays are constantly evolving, but it is uncertain what the best measures for protein, lipid and DNA oxidation are. For biomarker assays to be widely accepted, they have to fulfill the conditions expected of routinely used clinical assays. Biomarker assay must be accurate and precise, with no artefacts introduced by sample collection and processing. Optimum sampling and storage conditions of blood or urine samples and stability of biomarkers in clinical sample have to be established. Normal ranges in healthy subjects have to be established for each population and laboratory. Where urinary concentrations of biomarkers are measured, the effects of renal threshold and clearance in health and disease have to be established. The chosen biomarker should show a clear association with disease and change with disease severity. In this case, the biomarker will act as a nonspecific indicator of disease, much like fever or erythrocyte sedimentation rate. Although lacking specificity, such a measure nevertheless serves as a useful indicator of organic illness and may serve to monitor disease progression and effects of therapy. If subjects cannot be screened for pre-existing oxidant stress because there is no reliable biomarker, it will remain uncertain whether the correct patient population is being targeted for antioxidant treatment [64]. Ideally, the biomarker should be clearly linked to clinical outcome so that it can be used as a surrogate end point in intervention studies. The magnitude of change in the biomarker, when used as a surrogate end-point, must be meaningful with regard to outcome. Biomarkers currently in use to study oxidative damage to proteins, lipids and DNA do not as yet meet all these criteria. There is an urgent need to establish the clinical validity of biomarker measurements. Antioxidant effects of vitamin C alone or in combination with other antioxidants will be much more easily demonstrated if reliable biomarker assays are available, and if patient groups at high risk of oxidative damage are

maintained at relative extremes with regard to steady-state plasma and tissue vitamin C concentrations.

Problems in Demonstrating Antioxidant Benefit of Vitamin C in Clinical Studies

Despite epidemiological and some experimental studies, it has not been possible to show conclusively that higher than anti-scorbutic intake of vitamin C has antioxidant clinical benefit. This is despite the fact that vitamin C is a powerful antioxidant *in vitro*. It is of course possible that the lack of antioxidant effect of vitamin C in clinical studies is real. It seems more likely that vitamin C has antioxidant or other benefits. Detection of these benefits has remained elusive due to the vicissitudes of experimental design.

Vitamin C may be a weak antioxidant *in vivo*, or its antioxidant actions may have no physiological role, or its role may be small. The oxidative hypothesis is unproven, and oxidative damage may have a smaller role than anticipated in some diseases. Further, antioxidant actions of vitamin C may occur at relatively low plasma vitamin C concentrations. Thus additional clinical benefits that occur at higher vitamin C concentrations may be difficult to demonstrate. Although all these are possible explanations, it seems unlikely that these are the real reasons for the lack of detectable effects of vitamin C in clinical studies.

Many factors may contribute to the failure so far to demonstrate clear antioxidant benefits of vitamin C in clinical studies. The antioxidant actions of vitamin C may be specific to certain reactions or occur only at specific locations. In either case, beneficial effects can be shown only in disorders where such reactions or sites are the focus of disease process. There may be many different antioxidants that are active at the same time. In the face of such redundancy, only multiple antioxidant deficiencies will have detectable clinical effects. Antioxidant deficiency may have to be of long duration for accumulated damage to be noticeable. Antioxidant effects may be of importance only in those with oxidant stress. Thus, normal subjects or those with mild disease may have no need for high antioxidant concentrations. In a way analogous to the effect of acetaminophen on fever, antioxidants may have no effect in the absence of marked oxidant stress. A further problem is presented by the sigmoidal dose concentration curve for vitamin C. Small changes in oral intake of vitamin C produce large changes in plasma vitamin C concentrations. This makes it difficult to conduct controlled studies such that the plasma vitamin C concentrations of the control and study groups differ sufficiently to have physiological meaning.

Recommendations for Future Clinical Studies of Antioxidant Effects of Vitamin C

Despite the above problems, it is possible to design studies that can be reasonably expected to show whether vitamin C has clinically beneficial antioxidant actions. One approach is to study normal volunteers at extremes of plasma vitamin C

concentrations. Each subject will serve as his or her control. Subjects can be depleted of vitamin C by feeding a vitamin C deficient diet but with enough vitamin C given to prevent scurvy. The depleted state can be maintained for a few weeks. Then saturating doses of vitamin C are given, and the saturated state can be maintained as long as necessary. At these extremes of plasma vitamin C concentrations (low of 10 μM , and high of 70 μM), several physiological parameters that may conceivably be related to the antioxidant actions of vitamin C can be measured. A second approach is to study patients who are subject to high oxidant stress and are known to suffer from high morbidity and mortality due to accelerated disease. The commonest such group in the general population are cigarette smokers. A group with an even higher oxidant stress and very high rate of morbid events are those with end stage renal disease [215,216]. These patients may also have low plasma and tissue vitamin C due to loss of vitamin C during dialysis. Targeted studies in such susceptible groups are more likely to show whether vitamin C does have antioxidant actions *in vivo*. The availability of properly validated assays of oxidant damage will make such studies feasible. In addition, clinical studies should also take into consideration the pharmacokinetics of orally administered vitamin C.

Clinical Studies of Health Benefits of Vitamin C

The only proven function of vitamin C is the prevention of scurvy. Intake of as little as 10 mg/day of vitamin C will prevent scurvy. However, the resultant steady state plasma vitamin C concentrations will be less than 10 μM . Five servings of fruits and vegetables contain approximately 200 mg of vitamin C. At this dose, steady state plasma concentrations are about 70 μM . Tissue vitamin C concentrations are higher than that of plasma. Similar to plasma, tissue vitamin C concentrations also change with vitamin C intake. Tissues, however, saturate before plasma, at a vitamin C intake of 100 to 200 mg/day. The accumulated vitamin C in plasma and tissues is much more than that necessary to prevent scurvy and may simply serve as a reservoir of the vitamin. We now know that exquisite mechanisms exist to avidly accumulate and tightly regulate plasma and cellular vitamin C concentrations. When adequate vitamin C is available in the diet, these mechanisms keep plasma vitamin C concentrations at levels that are approximately an order of magnitude higher than that necessary to prevent scurvy. These complex mechanisms, which appear to be well conserved, are likely to subservise some important function. We know that low but non-scorbutic plasma vitamin C concentrations produce fatigue. These facts suggest that large (that is more than the amount needed to prevent scurvy) intake of vitamin C and high plasma and tissue concentrations may have clinical benefits. Similar to the proposed study of its antioxidant benefits, these benefits may be demonstrated in normal volunteers using vitamin C depletion-repletion study design to safely achieve extremes of plasma and tissue vitamin

C concentrations. At these extremes of plasma and tissue vitamin C concentrations, relevant physiological parameters can be measured. Other studies can target clinical consequences of specific enzymatic actions of vitamin C, such as collagen synthesis and consequently, its effects on wound healing, or of neutrophil recycling of vitamin C, and its effects on infection.

CONCLUSIONS

Several lines of evidence suggest that vitamin C is a powerful antioxidant in biological systems *in vitro*. However, its antioxidant role in humans has not been supported by currently available clinical studies. Diets high in fruits and vegetables protect against cardiovascular disease and cancer, but such a protective effect cannot as yet be ascribed to vitamin C. *In vivo* markers of oxidative damage are being developed, and these have yet not shown major changes with vitamin C intake in humans. Future studies of the antioxidant effects of vitamin C should be targeted to patient groups at high risk of oxidant damage and should be designed with attention to the pharmacokinetics of orally administered vitamin C.

REFERENCES

1. Nishikimi M, Fukuyama R, Minoshima S, Shimizu N, Yagi K: Cloning and chromosomal mapping of the human nonfunctional gene for L-gulonogamma-lactone oxidase, the enzyme for L-ascorbic acid biosynthesis missing in man. *J Biol Chem* 269: 13685–13688, 1994.
2. Nishikimi M, Yagi K: Biochemistry and molecular biology of ascorbic acid biosynthesis. *Subcell Biochem* 25:17–39, 1996.
3. Buettner GR, Moseley PL: EPR spin trapping of free radicals produced by bleomycin and ascorbate. *Free Radic Res Commun* 19:S89–S93, 1993.
4. Bielski BH, Richter HW, Chan PC: Some properties of the ascorbate free radical. *Ann N Y Acad Sci* 258:231–237, 1975.
5. Washko PW, Wang Y, Levine M: Ascorbic acid recycling in human neutrophils. *J Biol Chem* 268:15531–15535, 1993.
6. Tolbert BM, Ward JB: Dehydroascorbic Acid. In Seib PA, Tolbert BM (eds): "Ascorbic Acid: Chemistry, Metabolism, and Uses." Washington, DC: American Chemical Society, pp 101–123, 1982.
7. Lewin S. "Vitamin C: Its Molecular Biology and Medical Potential." London: Academic Press, 1976.
8. Levine M, Rumsey SC, Wang Y, Park JB, Daruwala R. Vitamin C. In Stipanuk MH (ed): "Biochemical and Physiological Aspects of Human Nutrition." Philadelphia: W B Saunders, pp 541–567, 2000.
9. Prockop DJ, Kivirikko KI: Collagens: molecular biology, diseases, and potentials for therapy. *Annu Rev Biochem* 64:403–434, 1995.
10. Peterkofsky B: Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy. *Am J Clin Nutr* 54:1135S–1140S, 1991.
11. Kivirikko KI, Myllyla R: Post-translational processing of procollagens. *Ann N Y Acad Sci* 460:187–201, 1985.

12. Rebouche CJ: Ascorbic acid and carnitine biosynthesis. *Am J Clin Nutr* 54:1147S–1152S, 1991.
13. Dunn WA, Rettura G, Seifter E, Englund S. Carnitine biosynthesis from gamma-butyrobetaine and from exogenous protein-bound 6-N-trimethyl-L-lysine by the perfused guinea pig liver. Effect of ascorbate deficiency on the in situ activity of gamma-butyrobetaine hydroxylase. *J Biol Chem* 259:10764–10770, 1984.
14. Levine M, Dhariwal KR, Washko P, Welch R, Wang YH, Cantilena CC, Yu R: Ascorbic acid and reaction kinetics in situ: a new approach to vitamin requirements. *J Nutr Sci Vitaminol (Tokyo)* Spec No:169–172, 1992.
15. Kaufman S: Dopamine-beta-hydroxylase. *J Psychiatr Res* 11: 303–316, 1974.
16. Eipper BA, Milgram SL, Husten EJ, Yun HY, Mains RE: Peptidylglycine alpha-amidating monooxygenase: a multifunctional protein with catalytic, processing, and routing domains. *Protein Sci* 2:489–497, 1993.
17. Eipper BA, Stoffers DA, Mains RE: The biosynthesis of neuropeptides: peptide alpha-amidation. *Annu Rev Neurosci* 15:57–85, 1992.
18. Englund S, Seifter S: The biochemical functions of ascorbic acid. *Annu Rev Nutr* 6:365–406, 1986.
19. Lindblad B, Lindstedt G, Lindstedt S: The mechanism of enzymic formation of homogentisate from p-hydroxyphenylpyruvate. *J Am Chem Soc* 92:7446–7449, 1970.
20. Buettner GR: The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. *Arch Biochem Biophys* 300:535–543, 1993.
21. Halliwell B: Vitamin C: poison, prophylactic or panacea? *Trends Biochem Sci* 24:255–259, 1999.
22. Neuzil J, Thomas SR, Stocker R: Requirement for, promotion, or inhibition by alpha-tocopherol of radical-induced initiation of plasma lipoprotein lipid peroxidation. *Free Radic Biol Med* 22: 57–71, 1997.
23. Carr A, Frei B: Does vitamin C act as a pro-oxidant under physiological conditions? *Faseb J* 13:1007–1024, 1999.
24. Hallberg L: Iron and vitamins. *Bibl Nutr Dieta* 52:20–29, 1995.
25. Lynch SR: Interaction of iron with other nutrients. *Nutr Rev* 55:102–110, 1997.
26. Morrow JD: The isoprostanes: their quantification as an index of oxidant stress status in vivo. *Drug Metab Rev* 32:377–385, 2000.
27. Steinberg D: Low density lipoprotein oxidation and its pathobiological significance. *J Biol Chem* 272:20963–20966, 1997.
28. Heinecke JW: Oxidants and antioxidants in the pathogenesis of atherosclerosis: implications for the oxidized low density lipoprotein hypothesis. *Atherosclerosis* 141:1–15, 1998.
29. Witztum JL, Steinberg D: The oxidative modification hypothesis of atherosclerosis: does it hold for humans? *Trends Cardiovasc Med* 11:93–102, 2001.
30. Berlett BS, Stadtman ER: Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem* 272:20313–20316, 1997.
31. Shacter E: Quantification and significance of protein oxidation in biological samples. *Drug Metab Rev* 32:307–326, 2000.
32. Stadtman ER, Berlett BS: Reactive oxygen-mediated protein oxidation in aging and disease. *Drug Metab Rev* 30:225–243, 1998.
33. Baynes JW: The role of AGEs in aging: causation or correlation. *Exp Gerontol* 36:1527–1537, 2001.
34. Halliwell B: Why and how should we measure oxidative DNA damage in nutritional studies? How far have we come? *Am J Clin Nutr* 72:1082–1087, 2000.
35. Halliwell B: Vitamin C and genomic stability. *Mutat Res* 475: 29–35, 2001.
36. Lee SH, Oe T, Blair IA: Vitamin C-induced decomposition of lipid hydroperoxides to endogenous genotoxins. *Science* 292: 2083–2086, 2001.
37. Luperchio S, Tamir S, Tannenbaum SR: No-induced oxidative stress and glutathione metabolism in rodent and human cells. *Free Radic Biol Med* 21:513–519, 1996.
38. Odin AP: Vitamins as antimutagens: advantages and some possible mechanisms of antimutagenic action. *Mutat Res* 386:39–67, 1997.
39. Wink DA, Kasprzak KS, Maragos CM, Elespuru RK, Misra M, Dunams TM, Cebula TA, Koch WH, Andrews AW, Allen JS, et al.: DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* 254:1001–1003, 1991.
40. Nguyen T, Brunson D, Crespi CL, Penman BW, Wishnok JS, Tannenbaum SR: DNA damage and mutation in human cells exposed to nitric oxide in vitro. *Proc Natl Acad Sci USA* 89: 3030–3034, 1992.
41. Haytowitz DB: Information from USDA's Nutrient Data Bank. *J Nutr* 125:1952–1955, 1995.
42. Lachance P, Langseth L: The RDA concept: time for a change? *Nutr Rev* 52:266–270, 1994.
43. Life Sciences Research Office: "Third Report on Nutrition Monitoring in the United States." Washington, DC: US Government Printing Office, 1995.
44. Simon JA, Schreiber GB, Crawford PB, Frederick MM, Sabry ZI: Dietary vitamin C and serum lipids in black and white girls. *Epidemiology* 4:537–542, 1993.
45. Basch CE, Zybert P, Shea S: 5-A-DAY: dietary behavior and the fruit and vegetable intake of Latino children. *Am J Public Health* 84:814–818, 1994.
46. Block G, Sinha R, Gridley G: Collection of dietary-supplement data and implications for analysis. *Am J Clin Nutr* 59:232S–239S, 1994.
47. Koplan JP, Annett JL, Layde PM, Rubin GL: Nutrient intake and supplementation in the United States (NHANES II). *Am J Public Health* 76:287–289, 1986.
48. Dickinson VA, Block G, Russek-Cohen E: Supplement use, other dietary and demographic variables, and serum vitamin C in NHANES II. *J Am Coll Nutr* 13:22–32, 1994.
49. Levine M, Rumsey SC, Daruwala R, Park JB, Wang Y: Criteria and recommendations for vitamin C intake. *JAMA* 281:1415–1423, 1999.
50. Levine M, Conry-Cantilena C, Wang Y, Welch RW, Washko PW, Dhariwal KR, Park JB, Lazarev A, Graumlich JF, King J, Cantilena LR: Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. *Proc Natl Acad Sci USA* 93:3704–3709, 1996.
51. Levine M, Wang Y, Padayatty SJ, Morrow J: A new recommended dietary allowance of vitamin C for healthy young women. *Proc Natl Acad Sci USA* 98:9842–9846, 2001.
52. Graumlich JF, Ludden TM, Conry-Cantilena C, Cantilena Jr LR, Wang Y, Levine M: Pharmacokinetic model of ascorbic acid in healthy male volunteers during depletion and repletion. *Pharm Res* 14:1133–1139, 1997.

53. Rumsey SC, Levine M: Absorption, transport, and disposition of ascorbic acid in humans. *Nutritional Biochemistry* 9:116–130, 1998.
54. Daruwala R, Song J, Koh WS, Rumsey SC, Levine M: Cloning and functional characterization of the human sodium-dependent vitamin C transporters hSVCT1 and hSVCT2. *FEBS Lett* 460:480–484, 1999.
55. Tsukaguchi H, Tokui T, Mackenzie B, Berger UV, Chen XZ, Wang Y, Brubaker RF, Hediger MA: A family of mammalian Na⁺-dependent L-ascorbic acid transporters. *Nature* 399:70–75, 1999.
56. Jialal I, Vega GL, Grundy SM: Physiologic levels of ascorbate inhibit the oxidative modification of low density lipoprotein. *Atherosclerosis* 82:185–191, 1990.
57. Jialal I, Grundy SM: Preservation of the endogenous antioxidants in low density lipoprotein by ascorbate but not probucol during oxidative modification. *J Clin Invest* 87:597–601, 1991.
58. Jialal I, Grundy SM: Influence of antioxidant vitamins on LDL oxidation. *Ann N Y Acad Sci* 669:237–247, 1992.
59. Jialal I, Fuller CJ: Effect of vitamin E, vitamin C and beta-carotene on LDL oxidation and atherosclerosis. *Can J Cardiol* 11:97G–103G, 1995.
60. Frei B: Ascorbic acid protects lipids in human plasma and low-density lipoprotein against oxidative damage. *Am J Clin Nutr* 54:1113S–1118S, 1991.
61. Frei B, Gaziano JM: Content of antioxidants, preformed lipid hydroperoxides, and cholesterol as predictors of the susceptibility of human LDL to metal ion-dependent and -independent oxidation. *J Lipid Res* 34:2135–2145, 1993.
62. Heinecke JW: Mass spectrometric quantification of amino acid oxidation products in proteins: insights into pathways that promote LDL oxidation in the human artery wall. *Faseb J* 13:1113–1120, 1999.
63. Crawford RS, Kirk EA, Rosenfeld ME, LeBoeuf RC, Chait A: Dietary antioxidants inhibit development of fatty streak lesions in the LDL receptor-deficient mouse. *Arterioscler Thromb Vasc Biol* 18:1506–1513, 1998.
64. Heinecke JW: Is the emperor wearing clothes? Clinical trials of vitamin E and the LDL oxidation hypothesis. *Arterioscler Thromb Vasc Biol* 21:1261–1264, 2001.
65. Frei B, Stocker R, Ames BN: Antioxidant defenses and lipid peroxidation in human blood plasma. *Proc Natl Acad Sci USA* 85:9748–9752, 1988.
66. Frei B, England L, Ames BN: Ascorbate is an outstanding antioxidant in human blood plasma. *Proc Natl Acad Sci USA* 86:6377–6381, 1989.
67. Lynch SM, Morrow JD, Roberts 2nd LJ, Frei B: Formation of non-cyclooxygenase-derived prostanoids (F₂-isoprostanes) in plasma and low density lipoprotein exposed to oxidative stress in vitro. *J Clin Invest* 93:998–1004, 1994.
68. Reilly M, Delanty N, Lawson JA, FitzGerald GA: Modulation of oxidant stress in vivo in chronic cigarette smokers. *Circulation* 94:19–25, 1996.
69. Kim JG, Sabbagh F, Santanam N, Wilcox JN, Medford RM, Parthasarathy S: Generation of a polyclonal antibody against lipid peroxide-modified proteins. *Free Radic Biol Med* 23:251–259, 1997.
70. Eaton P, Hearse DJ, Shattock MJ: Lipid hydroperoxide modification of proteins during myocardial ischaemia. *Cardiovasc Res* 51:294–303, 2001.
71. Shanti A, Santanam N, Morales AJ, Parthasarathy S, Murphy AA: Autoantibodies to markers of oxidative stress are elevated in women with endometriosis. *Fertil Steril* 71:1115–1118, 1999.
72. Holvoet P, Mertens A, Verhamme P, Bogaerts K, Beyens G, Verhaeghe R, Collen D, Muls E, Van de Werf F: Circulating oxidized LDL is a useful marker for identifying patients with coronary artery disease. *Arterioscler Thromb Vasc Biol* 21:844–848, 2001.
73. Holvoet P, Collen D: Oxidation of low density lipoproteins in the pathogenesis of atherosclerosis. *Atherosclerosis* 137(Suppl):S33–S38, 1998.
74. Hsu RM, Devaraj S, Jialal I: Autoantibodies to oxidized low-density lipoprotein in patients with Type 2 diabetes mellitus. *Clin Chim Acta* 317:145–150, 2002.
75. Barja G, Lopez-Torres M, Perez-Campo R, Rojas C, Cadenas S, Prat J, Pamplona R: Dietary vitamin C decreases endogenous protein oxidative damage, malondialdehyde, and lipid peroxidation and maintains fatty acid unsaturation in the guinea pig liver. *Free Radic Biol Med* 17:105–115, 1994.
76. Kimura H, Yamada Y, Morita Y, Ikeda H, Matsuo T: Dietary ascorbic acid depresses plasma and low density lipoprotein lipid peroxidation in genetically scorbutic rats. *J Nutr* 122:1904–1909, 1992.
77. Tanaka K, Hashimoto T, Tokumaru S, Iguchi H, Kojo S: Interactions between vitamin C and vitamin E are observed in tissues of inherently scorbutic rats. *J Nutr* 127:2060–2064, 1997.
78. Cadenas S, Lertsiri S, Otsuka M, Barja G, Miyazawa T: Phospholipid hydroperoxides and lipid peroxidation in liver and plasma of ODS rats supplemented with alpha-tocopherol and ascorbic acid. *Free Radic Res* 24:485–493, 1996.
79. Kunert KJ, Tappel AL: The effect of vitamin C on in vivo lipid peroxidation in guinea pigs as measured by pentane and ethane production. *Lipids* 18:271–274, 1983.
80. Cadenas S, Rojas C, Barja G: Endotoxin increases oxidative injury to proteins in guinea pig liver: protection by dietary vitamin C. *Pharmacol Toxicol* 82:11–18, 1998.
81. Helen A, Vijayammal PL: Vitamin C supplementation on hepatic oxidative stress induced by cigarette smoke. *J Appl Toxicol* 17:289–295, 1997.
82. Dillard CJ, Kunert KJ, Tappel AL: Effects of vitamin E, ascorbic acid and mannitol on alloxan-induced lipid peroxidation in rats. *Arch Biochem Biophys* 216:204–212, 1982.
83. Kang SA, Jang YJ, Park H: In vivo dual effects of vitamin C on paraquat-induced lung damage: dependence on released metals from the damaged tissue. *Free Radic Res* 28:93–107, 1998.
84. Steinberg D, Lewis A: Conner Memorial Lecture. Oxidative modification of LDL and atherogenesis. *Circulation* 95:1062–1071, 1997.
85. Chisolm GM, Steinberg D: The oxidative modification hypothesis of atherogenesis: an overview. *Free Radic Biol Med* 28:1815–1826, 2000.
86. Mahfouz MM, Kawano H, Kummerow FA: Effect of cholesterol-rich diets with and without added vitamins E and C on the severity of atherosclerosis in rabbits. *Am J Clin Nutr* 66:1240–1249, 1997.
87. Halliwell B, Zhao K, Whiteman M: Nitric oxide and peroxynitrite. The ugly, the uglier and the not so good: a personal view of recent controversies. *Free Radic Res* 31:651–669, 1999.

88. Takahashi R, Goto S: Alteration of aminoacyl-tRNA synthetase with age: heat-labilization of the enzyme by oxidative damage. *Arch Biochem Biophys* 277:228–233, 1990.
89. Oliver CN, Ahn BW, Moerman EJ, Goldstein S, Stadtman ER: Age-related changes in oxidized proteins. *J Biol Chem* 262:5488–5491, 1987.
90. Cross CE, O'Neill CA, Reznick AZ, Hu ML, Marcocci L, Packer L, Frei B: Cigarette smoke oxidation of human plasma constituents. *Ann N Y Acad Sci* 686:72–89, 1993.
91. Reznick AZ, Cross CE, Hu ML, Suzuki YJ, Khwaja S, Safadi A, Motchnik PA, Packer L, Halliwell B: Modification of plasma proteins by cigarette smoke as measured by protein carbonyl formation. *Biochem J* 286:607–611, 1992.
92. Yan LJ, Traber MG, Kobuchi H, Matsugo S, Tritschler HJ, Packer L: Efficacy of hypochlorous acid scavengers in the prevention of protein carbonyl formation. *Arch Biochem Biophys* 327:330–334, 1996.
93. Ortwerth BJ, James HL: Lens proteins block the copper-mediated formation of reactive oxygen species during glycation reactions in vitro. *Biochem Biophys Res Commun* 259:706–710, 1999.
94. Saxena P, Saxena AK, Cui XL, Obrenovich M, Gudipaty K, Monnier VM: Transition metal-catalyzed oxidation of ascorbate in human cataract extracts: possible role of advanced glycation end products. *Invest Ophthalmol Vis Sci* 41:1473–1481, 2000.
95. Cabiscol E, Levine RL: Carbonic anhydrase III. Oxidative modification in vivo and loss of phosphatase activity during aging. *J Biol Chem* 270:14742–14747, 1995.
96. Yan LJ, Sohal RS: Mitochondrial adenine nucleotide translocase is modified oxidatively during aging. *Proc Natl Acad Sci USA* 95:12896–12901, 1998.
97. Pennathur S, Wagner JD, Leeuwenburgh C, Litwak KN, Heinicke JW: A hydroxyl radical-like species oxidizes cynomolgus monkey artery wall proteins in early diabetic vascular disease. *J Clin Invest* 107:853–860, 2001.
98. Frederikse PH, Garland D, Zigler Jr JS, Piatigorsky J: Oxidative stress increases production of beta-amyloid precursor protein and beta-amyloid (A β) in mammalian lenses, and A β has toxic effects on lens epithelial cells. *J Biol Chem* 271:10169–10174, 1996.
99. Fu S, Dean R, Southan M, Truscott R: The hydroxyl radical in lens nuclear cataractogenesis. *J Biol Chem* 273:28603–28609, 1998.
100. Taylor A, Smith DE, Palmer VJ, Shepard D, Padhye N, Theriault C, Morrow F: Relationships between acetone, cataracts, and ascorbate in hairless guinea pigs. *Ophthalmic Research* 25:30–35, 1993.
101. Taylor A: Nutritional influences on risk for cataract. *Int Ophthalmol Clin* 40:17–49, 2000.
102. Drouin R, Rodriguez H, Gao SW, Gebreyes Z, O'Connor TR, Holmquist GP, Akman SA: Cupric ion/ascorbate/hydrogen peroxide-induced DNA damage: DNA-bound copper ion primarily induces base modifications. *Free Radic Biol Med* 21:261–273, 1996.
103. Fischer-Nielsen A, Poulsen HE, Loft S: 8-Hydroxydeoxyguanosine in vitro: effects of glutathione, ascorbate, and 5-aminosalicylic acid. *Free Radic Biol Med* 13:121–126, 1992.
104. Hu ML, Shih MK: Ascorbic acid inhibits lipid peroxidation but enhances DNA damage in rat liver nuclei incubated with iron ions. *Free Radic Res* 26:585–592, 1997.
105. Singh NP: Sodium ascorbate induces DNA single-strand breaks in human cells in vitro. *Mutat Res* 375:195–203, 1997.
106. Ames BN: DNA damage from micronutrient deficiencies is likely to be a major cause of cancer. *Mutat Res* 475:7–20, 2001.
107. Cadenas S, Barja G, Poulsen HE, Loft S: Oxidative DNA damage estimated by oxo8dG in the liver of guinea-pigs supplemented with graded dietary doses of ascorbic acid and alpha-tocopherol. *Carcinogenesis* 18:2373–2377, 1997.
108. Reddy VN, Giblin FJ, Lin LR, Chakrapani B: The effect of aqueous humor ascorbate on ultraviolet-B-induced DNA damage in lens epithelium. *Invest Ophthalmol Vis Sci* 39:344–350, 1998.
109. Weimann A, Belling D, Poulsen HE: Quantification of 8-oxoguanine and guanine as the mucelobase, nucleoside and deoxynucleoside forms in human urine by high-performance liquid chromatography-electrospray tandem mass spectrometry. *Nucleic Acids Res* 30:E7, 2002.
110. Tanaka K, Hayatsu T, Negishi T, Hayatsu H: Inhibition of N-nitrosation of secondary amines in vitro by tea extracts and catechins. *Mutat Res* 412:91–98, 1998.
111. Kessler H, Husemann B, Wagner W: Potential protective effect of vitamin C on carcinogenesis caused by nitrosamine in drinking water: an experimental study on Wistar rats. *Eur J Surg Oncol* 18:275–281, 1992.
112. Hagiwara A, Murai T, Yoshino H, Goshima H, Mori S, Takashima A, Shirai T, Fukushima S: Hepatocarcinogenic activity of N-butyl-N-(4-hydroxybutyl)nitrosamine in rats is not modified by sodium L-ascorbate. *Teratog Carcinog Mutagen* 19:33–42, 1999.
113. Chen T, Na Y, Wanibuchi H, Yamamoto S, Lee CC, Fukushima S: Loss of heterozygosity in (LewisX^{F344})F1 rat urinary bladder tumors induced with N-butyl-N-(4-hydroxybutyl)nitrosamine followed by dimethylarsinic acid or sodium L-ascorbate. *Jpn J Cancer Res* 90:818–823, 1999.
114. Haffner SM: Clinical relevance of the oxidative stress concept. *Metabolism* 49:30–34, 2000.
115. Baynes JW, Thorpe SR: Glycooxidation and lipoxidation in atherogenesis. *Free Radic Biol Med* 28:1708–1716, 2000.
116. Maxwell SR: Coronary artery disease-free radical damage, antioxidant protection and the role of homocysteine. *Basic Res Cardiol* 95(Suppl 1):I65–I71, 2000.
117. Lipinski B: Pathophysiology of oxidative stress in diabetes mellitus. *J Diabetes Complications* 15:203–210, 2001.
118. Anderson JW, Gowri MS, Turner J, Nichols L, Diwadkar VA, Chow CK, Oeltgen PR: Antioxidant supplementation effects on low-density lipoprotein oxidation for individuals with type 2 diabetes mellitus. *J Am Coll Nutr* 18:451–461, 1999.
119. Ho E, Bray TM: Antioxidants, NF κ B activation, and diabetogenesis. *Proc Soc Exp Biol Med* 222:205–213, 1999.
120. Cameron NE, Cotter MA: Metabolic and vascular factors in the pathogenesis of diabetic neuropathy. *Diabetes* 46(Suppl 2):S31–S37, 1997.
121. Miyata T, Kurokawa K, van Ypersele de Strihou C: Relevance of oxidative and carbonyl stress to long-term uremic complications. *Kidney Int* 58(Suppl 76):S120–S125, 2000.
122. Descamps-Latscha B, Witko-Sarsat V: Importance of oxidatively modified proteins in chronic renal failure. *Kidney Int* 59(Suppl 78):S108–S113, 2001.
123. Nguyen-Khoa T, Massy ZA, De Bandt JP, Kebede M, Salama L, Lambrey G, Witko-Sarsat V, Druke TB, Lacour B, Thevenin M: Oxidative stress and haemodialysis: role of inflammation and

- duration of dialysis treatment. *Nephrol Dial Transplant* 16:335–340, 2001.
124. Blake DR, Winyard PG, Marok R: The contribution of hypoxia-reperfusion injury to inflammatory synovitis: the influence of reactive oxygen intermediates on the transcriptional control of inflammation. *Ann N Y Acad Sci* 723:308–317, 1994.
 125. Schulz HU, Niederau C, Klonowski-Stumpe H, Halangk W, Luthen R, Lippert H: Oxidative stress in acute pancreatitis. *Hepato-gastroenterology* 46:2736–2750, 1999.
 126. Heller R, Unbehaun A, Schellenberg B, Mayer B, Werner-Felmayer G, Werner ER: L-Ascorbic Acid Potentiates Endothelial Nitric Oxide Synthesis via a Chemical Stabilization of Tetrahydrobiopterin. *J Biol Chem* 276:40–47, 2001.
 127. Huang A, Vita JA, Venema RC, Keaney JF: Ascorbic acid enhances endothelial nitric-oxide synthase activity by increasing intracellular tetrahydrobiopterin. *J Biol Chem* 275:17399–17406, 2000.
 128. Brown AA, Hu FB: Dietary modulation of endothelial function: implications for cardiovascular disease. *Am J Clin Nutr* 73:673–686, 2001.
 129. Drexler H, Hornig B: Endothelial dysfunction in human disease. *J Mol Cell Cardiol* 31:51–60, 1999.
 130. Levine GN, Frei B, Koulouris SN, Gerhard MD, Keaney Jr JF, Vita JA: Ascorbic acid reverses endothelial vasomotor dysfunction in patients with coronary artery disease. *Circulation* 93:1107–1113, 1996.
 131. Motoyama T, Kawano H, Kugiyama K, Hirashima O, Ohgushi M, Yoshimura M, Ogawa H, Yasue H: Endothelium-dependent vasodilation in the brachial artery is impaired in smokers: effect of vitamin C. *Am J Physiol* 273:H1644–H1650, 1997.
 132. Beckman JA, Goldfine AB, Gordon MB, Creager MA: Ascorbate restores endothelium-dependent vasodilation impaired by acute hyperglycemia in humans. *Circulation* 103:1618–1623, 2001.
 133. Vita JA, Frei B, Holbrook M, Gokce N, Leaf C, Keaney Jr JF: L-2-Oxothiazolidine-4-carboxylic acid reverses endothelial dysfunction in patients with coronary artery disease. *J Clin Invest* 101:1408–1414, 1998.
 134. Jackson TS, Xu A, Vita JA, Keaney Jr JF: Ascorbate prevents the interaction of superoxide and nitric oxide only at very high physiological concentrations. *Circ Res* 83:916–922, 1998.
 135. Duffy SJ, Gokce N, Holbrook M, Hunter LM, Biegelsen ES, Huang A, Keaney JF, Vita JA: Effect of ascorbic acid treatment on conduit vessel endothelial dysfunction in patients with hypertension. *Am J Physiol Heart Circ Physiol* 280:H528–H534, 2001.
 136. Padayatty SJ, Levine M: Vitamin C and coronary microcirculation. *Circulation* 103:E117, 2001.
 137. Ness AR, Chee D, Elliott P: Vitamin C and blood pressure—an overview. *J Hum Hypertens* 11:343–350, 1997.
 138. Bates CJ, Walmsley CM, Prentice A, Finch S: Does vitamin C reduce blood pressure? Results of a large study of people aged 65 or older. *J Hypertens* 16:925–932, 1998.
 139. Duffy SJ, Gokce N, Holbrook M, Huang A, Frei B, Keaney Jr JF, Vita JA: Treatment of hypertension with ascorbic acid. *Lancet* 354:2048–2049, 1999.
 140. Fotherby MD, Williams JC, Forster LA, Craner P, Ferns GA: Effect of vitamin C on ambulatory blood pressure and plasma lipids in older persons. *J Hypertens* 18:411–415, 2000.
 141. Bassenge E, Fink N, Skatchkov M, Fink B: Dietary supplement with vitamin C prevents nitrate tolerance. *J Clin Invest* 102:67–71, 1998.
 142. Hallberg L, Brune M, Rossander-Hulthen L: Is there a physiological role of vitamin C in iron absorption? *Ann N Y Acad Sci* 498:324–332, 1987.
 143. Hallberg L: Wheat fiber, phytates and iron absorption. *Scand J Gastroenterol Suppl* 129:73–79, 1987.
 144. Hallberg L, Brune M, Rossander L: The role of vitamin C in iron absorption. *Int J Vitam Nutr Res Suppl* 30:103–108, 1989.
 145. Davidsson L, Walczyk T, Morris A, Hurrell RF: Influence of ascorbic acid on iron absorption from an iron-fortified, chocolate-flavored milk drink in Jamaican children. *Am J Clin Nutr* 67:873–877, 1998.
 146. Hunt JR, Mullen LM, Lykken GI, Gallagher SK, Nielsen FH: Ascorbic acid: effect on ongoing iron absorption and status in iron-depleted young women. *Am J Clin Nutr* 51:649–655, 1990.
 147. Stack T, Aggett PJ, Aitken E, Lloyd DJ: Routine L-ascorbic acid supplementation does not alter iron, copper, and zinc balance in low-birth-weight infants fed a cows'-milk formula. *J Pediatr Gastroenterol Nutr* 10:351–356, 1990.
 148. Harju E, Lindberg H: Ascorbic acid does not augment the restoration effect of iron treatment for empty iron stores in patients after gastrointestinal surgery. *Am Surg* 52:463–466, 1986.
 149. Rhode BM, Shustik C, Christou NV, MacLean LD: Iron absorption and therapy after gastric bypass. *Obes Surg* 9:17–21, 1999.
 150. Hunt JR, Gallagher SK, Johnson LK: Effect of ascorbic acid on apparent iron absorption by women with low iron stores. *Am J Clin Nutr* 59:1381–1385, 1994.
 151. Rathbone BJ, Johnson AW, Wyatt JI, Kelleher J, Heatley RV, Losowsky MS: Ascorbic acid: a factor concentrated in human gastric juice. *Clin Sci* 76:237–241, 1989.
 152. Sobala GM, Schorah CJ, Sanderson M, Dixon MF, Tompkins DS, Godwin P, Axon AT: Ascorbic acid in the human stomach. *Gastroenterology* 97:357–363, 1989.
 153. Sobala GM, Schorah CJ, Shires S, Lynch DA, Gallacher B, Dixon MF, Axon AT: Effect of eradication of *Helicobacter pylori* on gastric juice ascorbic acid concentrations. *Gut* 34:1038–1041, 1993.
 154. Sobala GM, Schorah CJ, Pignatelli B, Crabtree JE, Martin IG, Scott N, Quirke P: High gastric juice ascorbic acid concentrations in members of a gastric cancer family. *Carcinogenesis* 14:291–292, 1993.
 155. Helsler MA, Hotchkiss JH, Roe DA: Influence of fruit and vegetable juices on the endogenous formation of N-nitrosoproline and N-nitrosothiazolidine-4-carboxylic acid in humans on controlled diets. *Carcinogenesis* 13:2277–2280, 1992.
 156. Byers T, Guerrero N: Epidemiologic evidence for vitamin C and vitamin E in cancer prevention. *American Journal of Clinical Nutrition* 62:1385S–1392S, 1995.
 157. Ayaori M, Hisada T, Suzukawa M, Yoshida H, Nishiwaki M, Ito T, Nakajima K, Higashi K, Yonemura A, Ohsuzu F, Ishikawa T, Nakamura H: Plasma levels and redox status of ascorbic acid and levels of lipid peroxidation products in active and passive smokers. *Environ Health Perspect* 108:105–108, 2000.
 158. Schectman G: Estimating ascorbic acid requirements for cigarette smokers. *Ann N Y Acad Sci* 686:335–345, discussion 345–346, 1993.
 159. Stankova L, Riddle M, Larned J, Burry K, Menashe D, Hart J,

- Bigley R: Plasma ascorbate concentrations and blood cell dehydroascorbate transport in patients with diabetes mellitus. *Metabolism* 33:347–353, 1984.
160. Will JC, Byers T: Does diabetes mellitus increase the requirement for vitamin C? *Nutr Rev* 54:193–202, 1996.
161. Sinclair AJ, Taylor PB, Lunec J, Girling AJ, Barnett AH: Low plasma ascorbate levels in patients with type 2 diabetes mellitus consuming adequate dietary vitamin C. *Diabet Med* 11:893–898, 1994.
162. Hume R, Weyers E, Rowan T, Reid DS, Hillis WS: Leucocyte ascorbic acid levels after acute myocardial infarction. *Br Heart J* 34:238–243, 1972.
163. Riemersma RA, Carruthers KF, Elton RA, Fox KA: Vitamin C and the risk of acute myocardial infarction. *Am J Clin Nutr* 71:1181–1186, 2000.
164. Bonham MJ, Abu-Zidan FM, Simovic MO, Sluis KB, Wilkinson A, Winterbourn CC, Windsor JA: Early ascorbic acid depletion is related to the severity of acute pancreatitis. *Br J Surg* 86:1296–1301, 1999.
165. Scott P, Bruce C, Schofield D, Shiel N, Braganza JM, McCloy RF: Vitamin C status in patients with acute pancreatitis. *Br J Surg* 80:750–754, 1993.
166. Dallongeville J, Marecaux N, Fruchart JC, Amouyel P: Cigarette smoking is associated with unhealthy patterns of nutrient intake: a meta-analysis. *J Nutr* 128:1450–1457, 1998.
167. Padayatty SJ, Levine M: Vitamin C and myocardial infarction: the heart of the matter. *Am J Clin Nutr* 71:1027–1028, 2000.
168. Enstrom JE, Kanim LE, Klein MA: Vitamin C intake and mortality among a sample of the United States population. *Epidemiology* 3:194–202, 1992.
169. Gey KF: Cardiovascular disease and vitamins. Concurrent correction of ‘suboptimal’ plasma antioxidant levels may, as important part of ‘optimal’ nutrition, help to prevent early stages of cardiovascular disease and cancer, respectively. *Bibl Nutr Dieta* 52:75–91, 1995.
170. Kritchevsky SB, Shimakawa T, Tell GS, Dennis B, Carpenter M, Eckfeldt JH, Peacher-Ryan H, Heiss G: Dietary antioxidants and carotid artery wall thickness. The ARIC Study. *Atherosclerosis Risk in Communities Study. Circulation* 92:2142–2150, 1995.
171. Josphipura KJ, Ascherio A, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, Hennekens CH, Spiegelman D, Willett WC: Fruit and vegetable intake in relation to risk of ischemic stroke. *JAMA* 282:1233–1239, 1999.
172. Ness AR, Powles JW: Fruit and vegetables, and cardiovascular disease: a review. *Int J Epidemiol* 26:1–13, 1997.
173. Daviglus ML, Orenca AJ, Dyer AR, Liu K, Morris DK, Persky V, Chavez N, Goldberg J, Drum M, Shekelle RB, Stamler J: Dietary vitamin C, beta-carotene and 30-year risk of stroke: results from the Western Electric Study. *Neuroepidemiology* 16: 69–77, 1997.
174. Gey KF, Moser UK, Jordan P, Stahelin HB, Eichholzer M, Ludin E: Increased risk of cardiovascular disease at suboptimal plasma concentrations of essential antioxidants: an epidemiological update with special attention to carotene and vitamin C. *Am J Clin Nutr* 57:787S–797S, 1993.
175. Josphipura KJ, Hu FB, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, Colditz G, Ascherio A, Rosner B, Spiegelman D, Willett WC: The effect of fruit and vegetable intake on risk for coronary heart disease. *Ann Intern Med* 134:1106–1114, 2001.
176. Ascherio A, Rimm EB, Hernan MA, Giovannucci E, Kawachi I, Stampfer MJ, Willett WC: Relation of consumption of vitamin E, vitamin C, and carotenoids to risk for stroke among men in the United States. *Ann Intern Med* 130:963–970, 1999.
177. Kushi LH, Folsom AR, Prineas RJ, Mink PJ, Wu Y, Bostick RM: Dietary antioxidant vitamins and death from coronary heart disease in postmenopausal women. *N Engl J Med* 334:1156–1162, 1996.
178. Ascherio A, Rimm EB, Hernan MA, Giovannucci E, Kawachi I, Stampfer MJ, Willett WC: Relation of consumption of vitamin E, vitamin C, and carotenoids to risk for stroke among men in the United States. *Ann Intern Med* 130:963–970, 1999.
179. Hodis HN, Mack WJ, LaBree L, Cashin-Hemphill L, Sevanian A, Johnson R, Azen SP: Serial coronary angiographic evidence that antioxidant vitamin intake reduces progression of coronary artery atherosclerosis. *JAMA* 273:1849–1854, 1995.
180. Losonczy KG, Harris TB, Havlik RJ: Vitamin E and vitamin C supplement use and risk of all-cause and coronary heart disease mortality in older persons: the Established Populations for Epidemiologic Studies of the Elderly. *Am J Clin Nutr* 64:190–196, 1996.
181. Mark SD, Wang W, Fraumeni Jr JF, Li JY, Taylor PR, Wang GQ, Guo W, Dawsey SM, Li B, Blot WJ: Lowered risks of hypertension and cerebrovascular disease after vitamin/mineral supplementation: the Linxian Nutrition Intervention Trial. *Am J Epidemiol* 143:658–664, 1996.
182. Salonen JT, Nyyssonen K, Salonen R, Lakka HM, Kaikkonen J, Porkkala-Sarataho E, Voutilainen S, Lakka TA, Rissanen T, Leskinen L, Tuomainen TP, Valkonen VP, Ristonmaa U, Poulsen HE: Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study: a randomized trial of the effect of vitamins E and C on 3-year progression of carotid atherosclerosis. *J Intern Med* 248:377–386, 2000.
183. Ness AR, Powles JW, Khaw KT: Vitamin C and cardiovascular disease: a systematic review. *J Cardiovasc Risk* 3:513–521, 1996.
184. Lonn EM, Yusuf S: Is there a role for antioxidant vitamins in the prevention of cardiovascular diseases? An update on epidemiological and clinical trials data. *Can J Cardiol* 13:957–965, 1997.
185. Jha P, Flather M, Lonn E, Farkouh M, Yusuf S: The antioxidant vitamins and cardiovascular disease. A critical review of epidemiologic and clinical trial data. *Ann Intern Med* 123:860–872, 1995.
186. Taylor A, Jacques PF, Chylack Jr LT, Hankinson SE, Khu PM, Rogers G, Friend J, Tung W, Wolfe JK, Padhye N, Willett WC: Long-term intake of vitamins and carotenoids and odds of early age-related cortical and posterior subcapsular lens opacities. *Am J Clin Nutr* 75:540–549, 2002.
187. Chylack Jr LT, Brown NP, Bron A, Hurst M, Kopcke W, Thien U, Schalch W: The Roche European American Cataract Trial (REACT): a randomized clinical trial to investigate the efficacy of an oral antioxidant micronutrient mixture to slow progression of age-related cataract. *Ophthalmic Epidemiol* 9:49–80, 2002.
188. Age-Related Eye Disease Study Research Group: A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E and beta carotene for age-related cataract and vision loss: AREDS report no. 9. *Arch Ophthalmol* 119: 1439–1452, 2001.

189. Gale CR, Hall NF, Phillips DI, Martyn CN: Plasma antioxidant vitamins and carotenoids and age-related cataract. *Ophthalmology* 108:1992–1998, 2001.
190. Block G: Epidemiologic evidence regarding vitamin C and cancer. *Am J Clin Nutr* 54:1310S–1314S, 1991.
191. McCullough ML, Robertson AS, Jacobs EJ, Chao A, Calle EE, Thun MJ: A prospective study of diet and stomach cancer mortality in United States men and women. *Cancer Epidemiol Biomarkers Prev* 10:1201–1205, 2001.
192. Zhang S, Hunter DJ, Forman MR, Rosner BA, Speizer FE, Colditz GA, Manson JE, Hankinson SE, Willett WC: Dietary carotenoids and vitamins A, C, and E and risk of breast cancer. *J Natl Cancer Inst* 91:547–556, 1999.
193. Hunter DJ, Manson JE, Colditz GA, Stampfer MJ, Rosner B, Hennekens CH, Speizer FE, Willett WC: A prospective study of the intake of vitamins C, E, and A and the risk of breast cancer. *N Engl J Med* 329:234–240, 1993.
194. Hunter DJ, Colditz GA, Stampfer MJ, Rosner B, Willett WC, Speizer FE: Diet and risk of basal cell carcinoma of the skin in a prospective cohort of women. *Ann Epidemiol* 2:231–239, 1992.
195. Zhang SM, Hunter DJ, Rosner BA, Giovannucci EL, Colditz GA, Speizer FE, Willett WC: Intakes of fruits, vegetables, and related nutrients and the risk of non-Hodgkin's lymphoma among women. *Cancer Epidemiol Biomarkers Prev* 9:477–485, 2000.
196. Jacobs EJ, Connell CJ, Patel AV, Chao A, Rodriguez C, Seymour J, McCullough ML, Calle EE, Thun MJ: Vitamin C and vitamin E supplement use and colorectal cancer mortality in a large American Cancer Society cohort. *Cancer Epidemiol Biomarkers Prev* 10:17–23, 2001.
197. Greenberg ER, Baron JA, Tosteson TD, Freeman Jr DH, Beck GJ, Bond JH, Colacchio TA, Collier JA, Frankl HD, Haile RW, et al.: A clinical trial of antioxidant vitamins to prevent colorectal adenoma. *Polyp Prevention Study Group*. *N Engl J Med* 331:141–147, 1994.
198. Blot WJ, Li JY, Taylor PR, Guo W, Dawsey SM, Li B: The Linxian trials: mortality rates by vitamin-mineral intervention group. *Am J Clin Nutr* 62:1424S–1426S, 1995.
199. Block G, Norkus E, Hudes M, Mandel S, Helzlsouer K: Which plasma antioxidants are most related to fruit and vegetable consumption? *Am J Epidemiol* 154:1113–1118, 2001.
200. Food and Nutrition Board: "Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids: A Report of the Panel on Dietary Antioxidants and Related Compounds, Subcommittees on Upper Reference Levels of Nutrients and of Interpretation and Use of Dietary Reference Intakes, and the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, Food and Nutrition Board, Institute of Medicine." Washington, DC: National Academy Press, pp 95–184, 2000.
201. Pratico D: F(2)-isoprostanes: sensitive and specific non-invasive indices of lipid peroxidation in vivo. *Atherosclerosis* 147:1–10, 1999.
202. Morrow JD, Frei B, Longmire AW, Gaziano JM, Lynch SM, Shyr Y, Strauss WE, Oates JA, Roberts 2nd LJ: Increase in circulating products of lipid peroxidation (F2-isoprostanes) in smokers. Smoking as a cause of oxidative damage. *N Engl J Med* 332:1198–1203, 1995.
203. Steinberg FM, Chait A: Antioxidant vitamin supplementation and lipid peroxidation in smokers. *Am J Clin Nutr* 68:319–327, 1998.
204. Porkkala-Sarataho E, Salonen JT, Nyyssonen K, Kaikkonen J, Salonen R, Ristonmaa U, Diczfalussy U, Brigelius-Flohe R, Loft S, Poulsen HE: Long-term effects of vitamin E, vitamin C, and combined supplementation on urinary 7-hydro-8-oxo-2'-deoxyguanosine, serum cholesterol oxidation products, and oxidation resistance of lipids in nondepleted men. *Arterioscler Thromb Vasc Biol* 20:2087–2093, 2000.
205. Dietrich M, Block G, Hudes M, Morrow JD, Norkus EP, Traber MG, Cross CE, Packer L: Antioxidant supplementation decreases lipid peroxidation biomarker F(2)-isoprostanes in plasma of smokers. *Cancer Epidemiol Biomarkers Prev* 11:7–13, 2002.
206. Chevion M, Berenshtein E, Stadtman ER: Human studies related to protein oxidation: protein carbonyl content as a marker of damage. *Free Radic Res* 33(Suppl):S99–S108, 2000.
207. Carty JL, Bevan R, Waller H, Mistry N, Cooke M, Lunec J, Griffiths HR: The effects of vitamin C supplementation on protein oxidation in healthy volunteers. *Biochem Biophys Res Commun* 273:729–735, 2000.
208. Daube H, Scherer G, Riedel K, Ruppert T, Tricker AR, Rosenbaum P, Adlkofer F: DNA adducts in human placenta in relation to tobacco smoke exposure and plasma antioxidant status. *J Cancer Res Clin Oncol* 123:141–151, 1997.
209. Huang HY, Helzlsouer KJ, Appel LJ: The effects of vitamin C and vitamin E on oxidative DNA damage: results from a randomized controlled trial. *Cancer Epidemiol Biomarkers Prev* 9:647–652, 2000.
210. Jacobson JS, Begg MD, Wang LW, Wang Q, Agarwal M, Norkus E, Singh VN, Young TL, Yang D, Santella RM: Effects of a 6-month vitamin intervention on DNA damage in heavy smokers. *Cancer Epidemiol Biomarkers Prev* 9:1303–1311, 2000.
211. Lee BM, Lee SK, Kim HS: Inhibition of oxidative DNA damage, 8-OHdG, and carbonyl contents in smokers treated with antioxidants (vitamin E, vitamin C, beta-carotene and red ginseng). *Cancer Lett* 132:219–227, 1998.
212. Podmore ID, Griffiths HR, Herbert KE, Mistry N, Mistry P, Lunec J: Vitamin C exhibits prooxidant properties. *Nature* 392:559, 1998.
213. van den Berg R, van Vliet T, Broekmans WM, Cnubben NH, Vaes WH, Roza L, Haenen GR, Bast A, van den Berg H: A vegetable/fruit concentrate with high antioxidant capacity has no effect on biomarkers of antioxidant status in male smokers. *J Nutr* 131:1714–1722, 2001.
214. van't Veer PV, Kok FJ: Human studies to substantiate health effects of antioxidants. What is needed? *Free Radic Res* 33:S109–S115, 2000.
215. Boaz M, Green M, Fainauru M, Smetana S: Oxidative stress and cardiovascular disease in hemodialysis. *Clin Nephrol* 55:93–100, 2001.
216. Weinstein T, Chagnac A, Korzets A, Boaz M, Ori Y, Herman M, Malachi T, Gafer U: Haemolysis in haemodialysis patients: evidence for impaired defence mechanisms against oxidative stress. *Nephrol Dial Transplant* 15:883–887, 2000.

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