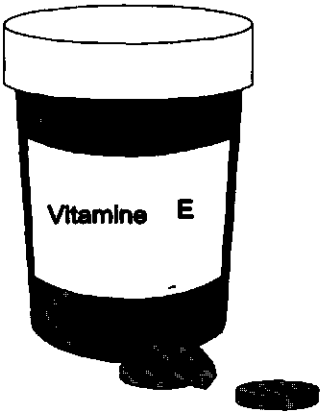
Vitamin E supplementation and atherosclerosis

Epidemiological studies in elderly and smokers

Frouwkje de Waart



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Papa voor jou!

In herinnering aan Jaap de Waart.

In de wetenschap gelijken wij op kinderen,
die aan de oever der kennis
hier en daar een steentje oprapen,
terwijl de wijde oceaan van het onbekende
zich voor onze ogen uitstrekt.

John Newton (1724-1807)

Abstract

Vitamin E supplementation and atherosclerosis. Epidemiological studies in smokers and elderly.

Ph.D.- thesis by Frouwkje de Waart, Division of Human Nutrition & Epidemiology, Wageningen University, The Netherlands, January 17, 2000

The antioxidant vitamin E may have beneficial effects on several indicators of human health. We studied the impact on atherosclerosis, immune response and total mortality in smokers and elderly people, who are at risk for increased oxidative stress. Vitamin E may exert its effect on atherosclerosis by protecting low density lipoprotein (LDL) against lipid peroxidation. Moreover, lipid peroxidation may also negatively influence the immune response. In addition to its antioxidant function, vitamin E may be beneficial through effects on cellular function, e.g., by preserving endothelium-dependent vaso-relaxation or decreasing cytokine production.

Observational studies. In a *cross-sectional* study among 158 male lifelong smokers aged 50 to 75 years, adjusted vitamin E intake and plasma levels were not associated with intima media wall thickness (IMT) of the common carotid artery. IMT ,which is a marker for atherosclerosis, was measured non-invasively by using the B-mode ultrasound technique. In a *prospective* study among 638 independently living elderly aged 65 to 85, no significant association was observed between cholesterol adjusted serum levels of vitamin E and 7.2 years total mortality. (Hazard ratio for lowest tertile vs. highest of 1.11, 95% confidence interval 0.74 to 1.65).

Intervention trials. In a randomized placebo-controlled double-blind trial among 218 lifelong male smokers, 400 IU (364 mg) vitamin E was administered daily for two years. A non significant (p=0.34) reduced progression of carotid IMT by 47% was observed compared to a significant spontaneous progression in the placebo group of 0.030 mm (p=0.006). Results were adjusted for initial IMT values and traditional CVD risk factors. Vitamin E significantly reduced the *in vitro* susceptibility of LDL to oxidation, which was not related to progression.
In this trial results were stratified by genetic predisposition. Smokers lacking the detoxifying enzyme activity of glutathion *S*-transferase μ (genotype GSTM1-*0*) were compared with those with the positive genotype (GSTM1-*1*). In the GSTM1-*0* group vitamin E reduced the proportion of smokers with increased carotid IMT by 62% (p=0.06) for the left posterior and by 73% (p=0.01) for the left anterior wall. No effects were observed for the IMT at the right side.
In a 3-month randomized double-blind placebo-controlled trial among 83 apparently healthy elderly, aged 67-87 years, 100 mg vitamin E supplementation significantly decreased the percentage of oxidized linoleic acid in LDL (10.4%) compared to the placebo group (4.6%). In this trial vitamin E supplementation did not affect the cellular and humoral immune response.

In conclusion, no supportive evidence is provided that vitamin E supplementation has beneficial effects on atherosclerosis and immune response among smokers and elderly. The observations for GSTM1-*0* genotype needs further confirmation. The protective effect of vitamin E on LDL oxidation *in vitro* lacks validation *in vivo*. Research on biomarkers of lipid oxidation and arterial damage needs priority to more adequately assess the clinical relevance and optimal intake of vitamin E.

Contents

1

Introduction

Will the 'good fairies' please prove to us that vitamin E lessens human degenerative disease?[1]

For almost eight decades now, potential health benefits of vitamin E have been studied. Vitamin E deficiency signs including neuropathic and myopathic symptoms, and disorders of the reproductive system have been well documented in studies among animals[2]. In humans vitamin E deficiency rarely occurs except in starvation, premature infants and conditions where fat absorption is limited[3]. Other than these categories with deficiencies, studies on health benefits of vitamin E in humans primarily focus on doses that go beyond correcting deficiency.

Renewed insights in the etiology of atherosclerosis[4,5] have suggested a potential key role for harmful effects of the oxidative modified low density lipoprotein (LDL)-cholesterol[6,7]. This has opened new research dimensions for vitamin E[6]. The potent antioxidant and membrane stabilizing effects of vitamin E, its presence as major antioxidant in LDL-cholesterol, and its relative non-toxicity made it an ideal candidate to study beneficial effects on the atherosclerotic process and cardiovascular disease (CVD).

The mechanism behind the potential immuno-stimulating effect of vitamin E is largely unknown but its antioxidant property[8] is likely to be involved. However, non-antioxidant functions of vitamin E have also been postulated[9,10]. The fact that functioning of specific immune defense mechanisms decreases with age[11-13] renders elderly people a specifically appropriate human target group for the confirmation of potential beneficial effects of vitamin E on immune response.

In this thesis, studies on the effect of vitamin E on atherosclerosis, immune response and total mortality are presented. Studies among smokers and elderly people are described. These groups may especially benefit from vitamin E supplementation as they are suspected to experience increased oxidative stress, which is defined as an

imbalance between reactive oxygen species (ROS) or free radicals and antioxidant defense[14].

Vitamin E

Vitamin E was discovered in 1922 by Bishop and Evans as an essential substance for normal reproduction in the rat. Vitamin E comprises of eight compounds known as tocopherols and tocotrienols differing from each other in the number and position of the methyl groups round the ring of the molecule. Tocopherols are yellow, oily liquids, freely soluble in fat solvents and remarkably stable to heat[3].

α-Tocopherol has the highest antioxidant and biological activity. It is an extremely effective chain-breaking antioxidant that protects polyunsaturated fatty acids from free radical damage. Furthermore, α-tocopherol appears to be essential for the correct functioning of cell membranes. In plasma, vitamin E is mainly transported in the lipid component of plasma lipoproteins. Vitamin E is broken down in the liver and excreted by bile. Normal plasma levels of α-tocopherol range from 11 to 37 μmol/l[15].

Vitamin E is present in small quantities in a large number of foods. The richest natural sources of vitamin E are wheat germ oil, vegetable oils, egg yolk, nuts, green plants, milk fat, and liver. The Recommended Daily Allowance (RDA) for vitamin E is 15 IU (10 mg α-tocopherol equivalents (αTE)) for men and 12 IU (8 mg αTE) for women[16].

No carcinogenic or teratogenic effects of vitamin E have been found in animal studies. Even at very high levels of intake vitamin E toxicity in animals is low. High intake of vitamin E from supplements in humans is generally considered safe[9,17-19]. Studies in individual cases showed that up to 3200 IU vitamin E per day have been taken by human subjects without significant adverse clinical effects. Interventions with megavitamin E doses ranging from 60 to 800 mg showed no negative effects on hepatic or renal function, thyroid gland, neuromuscular system and hematologic parameters in healthy subjects in periods from 4 weeks to 6 months[20-22]. One may question if this is only short-term safety and long-term accumulating effects cannot be ruled out. Moreover, specific subgroups may be vulnerable. Vitamin E may increase blood-clotting time in individuals with disease- or drug-induced vitamin K deficiency and is therefore not advised during anticoagulant therapy.

The oxidation hypothesis of atherosclerosis

An increased concentration of plasma LDL cholesterol is an established risk factor for atherosclerosis, the primary disease process underlying cardiovascular disease (CVD) cases.

The oxidation theory of atherosclerosis[7] states that oxidative modification of LDL (oxLDL) renders it more atherogenic than native LDL[6]. The oxidation of LDL by free radicals in the arterial intima is mediated by smooth muscle cells, macrophages and endothelial cells. OxLDL, but not native LDL, is recognized by scavenger receptors leading to uncontrolled uptake by macrophages in the intima and the formation of foam cells. These lipid loaden foam cells are found in early atherosclerotic lesions and at the leading edges of more advanced lesions. oxLDL inhibits the motility of tissue macrophages, it is chemotactic for circulating monocytes, and it is cytotoxic. Its cytotoxicity may be extremely important in lesion progression, specifically in the development of the extra-cellular lipid core as foam cells undergo necrosis. Furthermore, oxLDL has immunologic properties and thus induces the formation of auto-antibodies[23,24]. OxLDL has also been postulated to negatively influence endothelium functioning[25,26] such as impairment of endothelium dependent relaxation[25].

Several lines of evidence support the existence of oxLDL in vivo. OxLDL (or closely related epitopes) has been identified in animal and human lesions[23] and has been successfully extracted from these lesions[23,27]. Furthermore, auto-antibodies against epitopes of oxLDL[23,24] have been demonstrated in human plasma. The indirect measure of in vitro oxidation of LDL, which quantifies the lag phase of copper-induced oxidation of isolated LDL by monitoring conjugated dienes formation[28], has been extensively used as a marker of enhanced LDL oxidation. However, its biological relevance in vivo is debated[29,30]. Plasma auto-antibody levels against oxLDL are increasingly used as an in vivo marker for oxLDL. Several case-control studies and one cross-sectional study support a role for oxidation of LDL in atherosclerotic disease showing correlations between the above mentioned in vitro[31,32] and in vivo[30,33-35] measurements of oxLDL and atherosclerotic disease. However, studies failing to show significantly increased oxLDL in CVD patients compared to control subjects are as manifold[36-42].

The protective effect of vitamin E on CVD is believed to originate from its capacity to prevent lipid peroxidation in LDL[28,43]. Several human intervention trials confirm this, although the minimum dose required to significantly decrease the in

vitro susceptibility of LDL to oxidation is still not agreed upon and doses of 25 IU[44] as well as 400 IU[45] have been suggested as minimum requirements for effect.

Epidemiological evidence for benefits of vitamin E on cardiovascular disease

Epidemiological studies in humans including cross cultural, case-control studies and large prospective studies suggest a beneficial effect of high dietary or serum levels of vitamin E, although not conclusively (see for reviews Manson et al.[46], Stampfer and Rimm[47] and Tribble[48]). Two large prospective studies[49,50] among 87,245 nurses and 39,910 health professionals showed a risk reduction of CHD by 20 to 40% for high vitamin E intake. The reductions were most pronounced in men and women who had taken vitamin E supplements of 100 IU or more for at least two years. Another observational study[51] among 11,178 elderly showed reduced risks of 25% and 40% on respectively total- and CHD mortality for those elderly using vitamin E supplements. Non-supplementary dietary intake and plasma or adipose tissue levels of vitamin E are usually not strongly associated with CVD events. An exception is the Iowa Women's Health Study[52] among 34,486 postmenopausal women where dietary vitamin E intake (excluding supplement users) but not vitamin E supplement use was associated with a risk reduction in CHD mortality. To substantiate the apparent benefits of vitamin E in the prevention or reduction of CVD morbidity and mortality, large-scale primary and secondary intervention trials were warranted[53,53].

At this moment the available trials on vitamin E and CVD have revealed conflicting results. The Alpha-Tocopherol, Beta-Carotene (ATBC-trial) Cancer Prevention Study[54], a randomized trial among 29,133 male smokers aged 50-69, reported no reduction in cardiovascular risk after 6 years of 50 IU vitamin E treatment. There was even an increase in deaths caused by cerebral hemorrhage. Further analyses among a sub-population of smokers with a previous myocardial infarction (MI) showed that vitamin E reduced non-fatal MI but not fatal coronary heart disease[55]. A secondary randomized prevention trial[56] (CHAOS-trial) of 400 and 800 IU vitamin E among 2002 patients with angiographically proven coronary atherosclerosis showed also a reduction in nonfatal but not in fatal MI after a median follow-up of 1.4 years. However, there was a non-significant increased risk of cardiovascular death in the vitamin E group. Another large-scale secondary prevention trial[57] (GISSI-Prevenzione trial) among 11,324 MI survivors showed no reduction of 300 mg vitamin E on the combined endpoint of death, non-fatal MI, and non-fatal

stroke compared to the placebo group. However in secondary analyses a significant reduction in relative risk of 20% for all cardiovascular deaths and 35% for sudden death was found. Recently, at the European Society of Cardiology congress in Barcelona (Aug 28- Sept 1, 1999) results were presented from the HOPE trial[58]. The HOPE trial studied in a two by two factorial designed randomized trial the effect of ACE-inhibitor ramipril and vitamin E on a composite of MI, primary stroke or death from CVD among 9,541 patients with any evidence of coronary artery disease, stroke, peripheral vascular disease or diabetes. After a follow-up of 4.5 years, supplementation with 400 IU vitamin E showed no reduction in incidence of MI (16%) or stroke (7.1 %) or cardiovascular deaths (4.4%) compared to the placebo group (15.4%, 6.8% and 3.8%, respectively). The vitamin E arm of this study will be continued to assess the impact of prolonged vitamin E treatment on prevention of as well cancers as cardiovascular disease.

Several other large primary and secondary prevention trials are still ongoing which will hopefully provide a more consistent answer concerning the role of vitamin E in the prevention and treatment of CVD[59,60].

The role of vitamin E supplementation in immune response

The underlying mechanism for the immune-enhancing effect of vitamin E supplementation as found in many animal experiments is largely unknown[8]. Cells from the immune system are extra sensitive to lipid peroxidation which may negatively influence immune response by structurally changing the membranes of cells involved in immune response[9]. Furthermore, oxidative stress is increased in normal immune response as activated phagocytic cells generate hydrogen peroxides. By its antioxidant capacity vitamin E may improve immune response by counteracting these effects. Furthermore, vitamin E may exert its immune-enhancing effect by decreasing the production of immunosuppressive eicosanoids such as prostaglandin E_2, which contribute to the age-associated dysregulation of immune responsiveness[61-64]. In addition to its antioxidant function, modulation of signal transduction pathways by vitamin E may contribute to its immuno-stimulatory effect[9,10].

Epidemiological evidence for benefits of vitamin E on the immune response

Observational studies in healthy elderly human subjects found no positive associations between vitamin E intake or status and indicators of cell-mediated immune response[65-67]. Intervention trials on the effect of vitamin E on immune response are scarce[61,68,69] and

show inconsistent results. Two trials by Meydani et al.[61,68] among free-living healthy human elderly subjects reported an overall improvement in cell-mediated immune response with a daily dose of 800 mg vitamin E for 30 d, and with 60, 200 or 800 mg vitamin E after 4.5 months. In the latter study[68] beneficial effects significantly different from that of the placebo group were only found in the subjects receiving 200 mg/day. In a trial among non-institutionalized elderly people[69] testing 50 and 100 mg vitamin E for 6 months only elderly with a low baseline cellular immune response or physically less active benefited from 100 mg vitamin E supplementation by increased cellular immune response compared to the placebo group.

Rationale of this thesis

The guiding hypothesis for the studies in this thesis is that:

Vitamin E supplementation is beneficial for health in groups with a high oxidative stress.

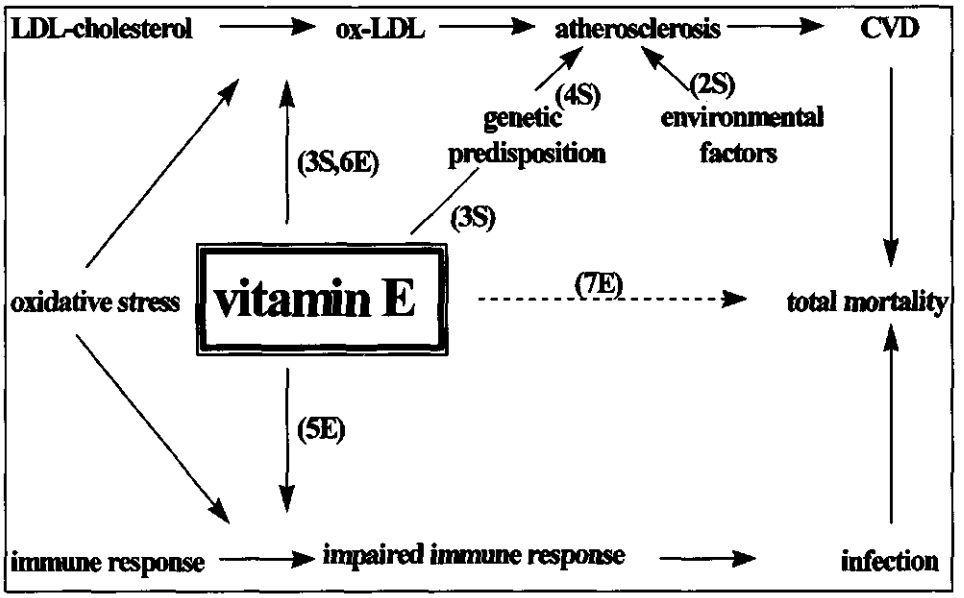
The studies focus on smokers and elderly as two at risk groups with a potential oxidant-antioxidant imbalance (oxidative stress). Risk groups with high oxidant-antioxidant imbalance are most likely to benefit from a potential effect of vitamin E on disease processes involving oxidative or free radical induced damage. Moreover, studies among these risk groups may be more efficient in terms of sample size and length of required study time.

By the constituents of the smoke they inhale, smokers put themselves at increased free radical and oxidant burden. Higher levels of plasma lipid peroxidation products in smokers than in non-smokers have been found in several studies[70-72]. Because of a more rapid metabolism of antioxidant vitamins and a generally lower intake[73-75], their antioxidant defense mechanism is lowered. Possibly as a result of this, atherosclerotic progression is more pronounced in smokers than in non-smokers[76,77].

Elderly people are the second population under study. Increased oxidative stress may accompany aging[78]. Age-associated increase in blood lipid peroxides has been reported[79,80]. The antioxidant defense may be decreased in elderly. First, the mean lower energy intake observed in elderly makes them more vulnerable for a sub-optimal vitamin status[81]. Moreover, less is known about specific needs of elderly for vitamins and they may be increased for certain vitamins[82]. Second, there are studies showing a decrease in antioxidant enzyme activity with human aging[80,83,84], although

not conclusively[78,85] and even slight increases in antioxidant enzyme activity have been reported[79,86].

Figure 1.1. Simplified hypothesized mechanisms and outline of studies in this thesis (chapter number and study population; E=elderly, S=smokers)



Both the scientific and public health importance of vitamin E's protective role on health status warrants further empirical studies on the process and its (health) outcomes. The studies described in this thesis were designed as a contribution to the accumulating research evidence. From the beneficial health claims for vitamin E on degenerative disease, the described studies are restricted to the areas outlined in Figure 1.1: atherosclerosis and immune response.

Objectives and outline of the thesis

This thesis has two main objectives:

1. Among smokers, to study the effect of two-year vitamin E supplementation on atherosclerosis as operationalized by progression of common carotid intima media thickness (CCA-IMT). Moreover, the effect of vitamin E on in vitro susceptibility of LDL to oxidation was studied. A double blind placebo controlled intervention trial was conducted for this purpose.

2. Among elderly, to study the effect of vitamin E on several health characteristics. This objective was pursued through a prospective study on the association between serum vitamin E and total mortality and a 3-month intervention trial testing the effect of vitamin E on immune response and susceptibility of LDL to oxidation.

The objectives are substantiated through 3 studies, of which the key characteristics are summarized in Table 1.1

Chapters 2, 3 and 4 report studies on the effects of vitamin E on atherosclerosis among life-long smokers. In **chapter 2** we studied important environmental determinants of CCA-IMT with emphasis on smoking characteristics and plasma antioxidant vitamins. Baseline data of the intervention trial presented in chapter 3 were used. **Chapter 3** reports a placebo-controlled randomized intervention study in which we investigated the effect of two year supplementation with 400 IU vitamin E on two indicators for atherosclerosis: reduction of the progression of CCA-IMT and the in vitro susceptibility of LDL to oxidation. In **chapter 4** we investigated whether the effect of vitamin E on progression of CCA-IMT is more pronounced among smokers with the null genotype for glutathione S- transferase μ 1 (GSTM1) thus lacking the detoxification enzyme activity of GSTM1.

Chapters **5, 6 and 7** deal with vitamin E effects among elderly. Chapters 5 and 6 report the effects of 3 months vitamin E supplementation on oxidation rate of linoleic acid in LDL **(chapter 5)** and indices of cellular and humoral immune response **(chapter 6)**. In **chapter 7**, vitamin E status and health effects are put into wider perspective. Specifically, in a prospective study we investigated the association of serum carotenoids and α-tocopherol on total mortality risk.

Table 1.1 Key characteristics of the studies in this thesis.

Ch	Study type	Population	Study characteristics
2	cross-sectional (baseline data ch. 3)	158 CVD-free male smokers	* plasma and dietary intake levels of antioxidant vitamins * smoking characteristics * CCA-IMT
3	double-blind placebo controlled randomized trial	218 male life-long smokers	* 400 IU vitamin E * 2 yrs. supplementation * progression of CCA-IMT * in vitro susceptibility of LDL to oxidation (lag time)
4	see ch. 3	see ch 3	* gstm1 polymorphism * progression of CCA-IMT
5	double-blind placebo controlled randomized trial	84 independently living elderly, 65-85 yrs.	* 100 IU vitamin E * 3 months supplementation * oxidation LDL-linoleic acid * in vitro susceptibility of LDL to oxidation (lag time)
6	see ch. 5	see ch. 5	* immune response: antibodies (humoral) proliferation (cellular)
7	Prospective cohort study	638 independently living elderly above 65 yrs.	* serum carotenoids and vitamin E * 7.2 yrs. follow-up * total mortality

Ch=chapter, yrs.=years, CCA-IMT=common carotid artery intima media thickness (mm), gstm1=glutathion S-transferase μ 1

2

Smoking characteristics, antioxidant vitamins and carotid artery wall thickness among life-long smokers*

Frouwkje G. de Waart, Tineke J. Smilde,Hub Wollersheim, Anton F.H. Stalenhoef, Frans J. Kok

Abstract

We studied the associations between the common carotid-intima-media thickness (IMT), as a marker of atherosclerosis, and smoking characteristics and antioxidant vitamins among 158 male life-long CVD-free smokers. An 'increased' carotid IMT was defined as the upper 25th percentile of the carotid IMT distribution. The prevalence odds of 'increased' IMT was 2.5 times (odds ratio (OR)=2.5; 95% CI: 1.1, 5.6) higher among smokers inhaling smoke deeply into the lungs than among 'moderate and non-inhalers'. This association decreased (OR=1.6, 95% CI: 0.7, 3.8) when adjusted for other CVD risk factors. Smokers with an 'increased' carotid IMT did not differ significantly in mean antioxidant vitamin intake and status with the remaining group. However, classical CVD risk factors contributed importantly to 'increased' carotid IMT. In our study depth of inhalation was the only smoking characteristic associated with carotid IMT although attenuated after adjustment for traditional risk factors for CVD. Furthermore, in these life-long smokers, not using any vitamin supplements, no associations were found for antioxidant vitamins.

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Introduction

Smokers are extra vulnerable for free radical induced damage of the cardiovascular system. They are exposed to free radicals from cigarette smoke and in general they have lower antioxidant vitamin intake and plasma levels[73-75]. The risk of cardiovascular disease (CVD) among smokers may vary because of differences in smoking characteristics[87,88] such as number of years smoked, number of cigarettes per day, use of filters, inhalation pattern, and type of tobacco, or because of differences in antioxidant vitamin intake or status. These variations in CVD risk factors can be studied in asymptomatic smokers because they have not altered their smoking or diet habits as a consequence of CVD.

The risk of CVD in asymptomatic smokers can be estimated by intima-media wall thickness (IMT) of the common carotid artery, as measured by B-mode ultrasound. This technique is non-invasive, highly reproducible[89,90] and positively associated with generalized atherosclerosis[91], and with peripheral-[92], cerebro- and cardiovascular disease[93-95]. Moreover, several epidemiological studies show associations between CVD risk factors and carotid IMT[96-98]. These include studies reporting positive associations between smoking and IMT[76,99-101] and inverse associations between specific antioxidant vitamins and IMT[39,102-105].

To our knowledge, no previous studies have specifically addressed asymptomatic smokers' risk of increased carotid intima-media wall thickness by focusing on smoking characteristics and antioxidant vitamin intake and plasma levels. Here we report associations between carotid IMT and different smoking characteristics and antioxidant vitamin intake and status among 158 male life-long CVD-free smokers. Furthermore, the associations between carotid IMT and other CVD risk factors are presented.

Methods

Study population. This study uses baseline data from an ongoing two year double-blind intervention trial on the effects of vitamin E on progression of common carotid artery intima-media wall thickness (IMT) among lifelong male smokers. Male inhabitants from Nijmegen, a city of approximately 150,000 persons in the Netherlands, were approached using the addresses of 7,000 male inhabitants born between 1919 and 1946. The addresses were obtained from the Municipal Registration Office. An invitation letter containing a brief explanation of the study design, a participation form and a questionnaire on smoking behavior, medical history and

demographic characteristics were sent out. The recruitment period was from October 1995 to July 1996. Eligible cigarette smokers were invited to an information evening and selected based on returned questionnaires and on a medical examination. Three hundred and forty five smokers entered the selection procedure. Excluded were diabetes (n=10), users of (multi)vitamin-, vitamin E, vitamin C, ß-carotene, garlic, or fish oil supplements (n=29), users of vitamin K antagonists (marcoumar, acenocoumarol) (n=8), individuals with current illness interfering with participation (n=17), and subjects not compliant with the protocol or with second thoughts on participating or for other reasons (n=63). All together, 218 subjects ranging in age from 50 to 75 years were randomized for the trial of which 216 successfully completed the baseline measurements in three consecutive visits.

A non-response questionnaire sent to the first 1,000 participants and returned by 534 revealed that the main reasons for not participating in the study were non-smoking (n=443; 83%), current health status (n=37;7%), lack of time (n=17;3%), and several other reasons (n=37;7%). Participants and non-participants did not differ significantly in age, perceived health scores, supplement use, marital status, history and medication for CVD, or activity pattern.

Written informed consent was obtained from all participants. Their general practitioners were informed by letter about the aim and design of the study and the subject's participation. The study was approved by both the medical ethical committee of Wageningen Agricultural University and the ethics committee of Nijmegen University Hospital.

Ultrasound measurement of the common carotid artery intima-media thickness. Ultrasound examinations on both the left and right side of the intima-media thickness (IMT) of the distal 1.0 cm straight part of the common carotid artery were performed, using a high resolution B-mode ultrasound (Biosound Phase-2) with a transducer frequency of 10 MHz. Images were stored on an optical disk and analyzed with a semiautomatic software program (Eurequa; TSA company, Meudon France[106]) by one reader blinded for any information. Significant changes in density on a section perpendicular to the vessel wall from the lumen towards subadventitial structures were measured automatically: three measurements were made in a 1.0 cm long segment of both the far- and near wall. Details of the scanning and reading procedure and of the reproducibility have been described elsewhere[90]. The mean of far- and near wall at the left and right side was used in the analyses.

Smoking characteristics. The questionnaire on smoking characteristics included questions on the average number of cigarettes, cigars and/or pipes smoked per day, brand of cigarettes, type of tobacco, use of filter, depth of inhalation, age at which participants started smoking and number of times and duration of attempts to quit-smoking. Nicotine and tar content of manufactured cigarettes was noted from the package brought to one of the visits. Duration of smoking was calculated as the age at the start of this study minus the age at which participants started smoking minus number of non-smoking years in between this period. Pack-years were calculated by multiplying duration of smoking by the number of cigarettes smoked per day divided by 20. Depth of inhalation[107] was elicited by asking "When you smoke cigarettes, how deeply do you usually inhale the smoke?" The 5 answer categories ranged from 'into the mouth/just puff' to 'deeply into the lungs'. In the analyses 'inhaling deeply into the lungs' will be compared with the other 4 categories combined. For three subjects no information was available on depth of inhalation. Plasma cotinine was measured by packed column gas-liquid chromatography[108] as biomarker of short-term exposure to cigarette smoke.

Antioxidant vitamins. Food frequency questionnaires[109] were checked by a nutritionist and any inconsistencies or incomplete answers were solved by telephone. Coding of nutrients was done by use of an adapted version of the 1993 computerized Dutch nutrient base[110]. Vitamin E intake was calculated as mg α-tocopherol equivalents.

Fasting blood samples were obtained for vitamin and lipid profile analyses. All samples were stored at -80° C until analyzed. For the vitamin C analyses 0.5 ml heparinized plasma was stabilized with 4.5 ml 5% metaphosphoric acid within one hour after blood collection. Vitamin C was measured fluoremetrically[111]. Vitamin C levels under the detection limit were assigned two-thirds of the value of the limit of detection. The plasma concentrations of α-tocopherol and ß-carotene were analyzed by reverse phase high-performance liquid chromatography (HPLC)[112].

CVD risk factors and other measurements. Medical history, current medication, questions on claudication[113], and family history of CVD was obtained by a research nurse.

Total plasma cholesterol and triglycerides were analyzed by enzymatic methods using commercially available reagents (CHOD-PAP, no 237574, Boehringer

Mannheim GmbH, FRG and Sera-PAK, Tournai, Belgium cat. no. 6639 respectively). HDL cholesterol was determined with the polyethylene glycol method[114]. LDL cholesterol was calculated by the Friedewald equation[115]. Blood pressure was measured in duplicate on the right arm with a sphygmomanometer with subjects in supine position after a 10-minute rest. Height and weight were measured. Body mass index was calculated as weight divided by squared height (kg/m^2).

Data analyses. An additional 58 subjects were excluded from this cross-section analyses, namely, those reporting CVD history (previous myocardial infarction, stroke or bypass operation); using CVD medication including lipid lowering drugs, aspirin, ß-blockers, Ca-antagonists, ACE-inhibitors, nitrates, and antihypertensive drugs; or having positive scores on a standard angina and intermittent claudication questionnaire[113]. This left 158 individuals for the analysis.

The smokers in the upper 25th percentile of carotid IMT were defined as the "increased" carotid IMT group. Age-adjusted mean values of smoking characteristics, antioxidant vitamins and CVD risk factors were compared between the "increased" carotid IMT group and the remaining group by ANCOVA (table 2.1 and 2.2). Means were compared by the Mann-Whitney *U*-test when not-normally distributed.

Age-adjusted correlation coefficients were calculated by age-adjusted standardized regression coefficients (ß). Unadjusted correlations are given by Spearman correlation coefficients (r).

The dietary intakes of vitamin E and ß-carotene were correlated with energy intake. These vitamins were adjusted for energy intake by calculating their residuals from linear regression models with energy intake as the dependent variable[116]. Plasma α-tocopherol and ß-carotene levels were highly correlated with plasma cholesterol. These plasma vitamins were adjusted for plasma cholesterol by calculating their residuals from linear regression models with plasma cholesterol as the dependent variable[116]. For ease of interpretation, descriptives are expressed as unadjusted data.

The contribution of independent variables to carotid IMT was assessed through three increasingly comprehensive models (table 2.3). The first model included age and those smoking characteristics that in exploratory stepwise regression analysis significantly ($p<0.05$) contributed to carotid IMT (only depth of inhalation met that criterion). The second model also included plasma antioxidants vitamins. The third model extended the first model with a selection of relevant risk indicators for CVD including body mass index, systolic- and diastolic blood pressure, plasma

concentrations of LDL, HDL, triglycerides and glucose and alcohol consumption. Again, these CVD risk factors were selected through exploratory stepwise regression analysis, revealing plasma LDL and HDL and systolic blood pressure as significant contributors to carotid IMT.

The strengths of associations of independent variables with carotid IMT in the above-mentioned three models were expressed both as regression coefficients (b) from multiple regression analysis and as prevalence odds ratios (OR) from multiple logistic regression analysis. The prevalence odds ratios of having an "increased" carotid IMT (dependent) were estimated for dichotomized independent variables using their median as the cut-off point.

The statistical package SAS (version 6.09, 1989) was used for all the analyses.

Table 2.1 Unadjusted mean (SD) of general characteristics and cardiovascular disease risk factors between lifelong male smokers in the upper quartile[a] of common carotid intima-media thickness (CCA-IMT) with the remaining group

	Total group n=158		lower 75%[a] n=118		upper 25%[a] n=40		
	mean	SD	mean	SD	mean	SD	95% CI[b]
CCA-IMT (mm)	0.93	0.15	0.86	0.09	1.13	0.09	0.2- 0.3
age (yr.)	59	6	58	6	63	6	2.4- 6.7
height (m)	1.76	6.3	1.76	0.06	1.75	0.06	-0.03- 0.01
weight (kg)	79.6	11.2	78.7	11.2	82.0	10.9	0.2- 8.7
body mass index ($kg/m^{2)}$	25.8	3.4	25.4	3.5	26.9	3.0	0.4- 3.0
Blood pressure (mmHg)							
systolic	138	15	135	16	144	13	1.1- 12.4
diastolic	83	7	82	8	84	7	-1.1- 4.6
Plasma lipid & glucose mmol/l							
total cholesterol	6.0	1.0	5.9	1.0	6.3	1.1	0.05- 0.8
LDL-cholesterol	4.1	1.0	4.0	0.9	4.5	1.0	0.2- 0.9
HDL-cholesterol	1.17	0.36	1.20	0.37	1.09	0.30	-0.3- -0.03
triglycerides	1.68	0.98	1.67	1.06	1.69	0.71	-0.2- 0.6
glucose	5.6	1.0	5.6	1.0	5.8	0.9	-0.2- 0.5
Intake							
energy (MJ/d)	10.4	2.5	10.6	2.5	9.8	2.3	-1.6- 0.3
alcohol (g/d[c])	14.9	0-65	16.2	0-64	10.5	0-82	

[a]Smokers in the lower 75% of CCA-IMT (ranging from 0.62 mm to 1.02 mm), and in the upper 25% of CCA-IMT (ranging from 1.03 mm to 1.43 mm). [b]95% confidence intervals for differences in age-adjusted means between subjects in the upper 25% and lower 75% of CCA-IMT. [c]For alcohol intake the median and 90% range is given.

Results

The lifelong male smokers in this study had a mean (SD) common carotid artery intima-media thickness (IMT) of 0.93 (0.15) mm. Carotid IMT and age were highly correlated, as shown by a Spearman correlation coefficient of r=0.48 (p=0.0001). Furthermore, age was significantly associated with most of the other cardiovascular risk factors. Consequently, all associations between carotid IMT and CVD risk indicators were adjusted for age. The CVD risk indicators plasma total- and LDL cholesterol, body mass index, and systolic- and diastolic blood pressure were significantly ($p \leq 0.01$) associated with carotid IMT. These age-adjusted correlations were all between 0.18 and 0.27. Plasma HDL-cholesterol levels were inversely (p=0.006) associated with carotid IMT.

Smokers in the upper 25% of carotid IMT (range from 1.03 to 1.43 mm) were defined as the 'increased' carotid IMT-group. Compared with the remaining smokers, they had significantly higher age-adjusted mean plasma levels of total- and LDL-cholesterol, lower plasma HDL-cholesterol levels, higher systolic blood pressure and higher mean body mass index (table 2.1).

The mean (SD) number of smoking years was around 42 (8) years, ranging from 16 to 62 years. Inhaling smoke deeply into the lungs was reported by 51% in the 'increased' carotid IMT group and by 35% in the remaining group ($p<0.05$). None of the other age-adjusted smoking characteristics were significantly different between the 'increased' carotid IMT group and the rest (table 2.2).

A closer examination of the inhalation pattern showed a positive trend (p=0.01) in carotid IMT with 3 categories of inhalation after adjustment for age (figure 2.1a). Deep inhalers smoked more cigarettes per day (p=0.02) but still a positive trend (p=0.04) was found for plasma cotinine with inhalation (three categories) after adjustment for cigarette consumption and age.

Figure 2.1b shows that depth of inhalation was inversely associated with plasma vitamin C levels (p value for trend is 0.03).

Intake of vitamin E and ß-carotene were slightly lower in the 'increased' carotid IMT group. However, comparison of mean vitamin intake values, adjusted for age and energy intake, between the 'increased' IMT group and the remaining group were not statistically significant. Moreover, plasma vitamin levels were not associated with carotid IMT. Because fruit and vegetables are an important source of vitamin C and ß-carotene and vegetable oils an important source of vitamin E, their associations with carotid IMT were also studied. Similar to vitamins, no associations were found

Table 2.2 Unadjusted mean (SD) or prevalence (%) of smoking characteristics and antioxidant vitamins between lifelong male smokers in the upper quartile[a] of common carotid intima-media thickness (CCA-IMT) with the remaining group

	Total group n=158		lower 75%[a] n=118		upper 25%[a] n=40		
	mean or %	SD	mean or %	SD	mean or %	SD	95 % CI[b]
cigarettes/d	18.4	9.8	18.7	10.5	17.4	7.2	-3.9- 3.6
plasma cotinine g/ml	256.6	113.1	256.0	118.1	258.3	98.1	-25.7- 60.0
Depth of inhalation %							
deeply into the lungs (yes)	39		35		51		*
Type of tobacco %							
manufactured cigarettes	33		35		28		
hand-rolled cigarettes only	51		49		55		
mix	16		16		18		
Manufactured cigarettes only (n 71)							
light cigarettes (%)	23		22		24		
filter cigarettes (%)	65		67		59		
tar (mg)	12.5	3.8	12.4	3.9	12.9	3.5	-1.4- 3.0
nicotine (mg)	0.98	0.26	0.97	0.28	1.00	0.22	-0.1- 0.2
History							
age started smoking (yr.)	17	4	17	4	16	3	-1.7- 1.0
pack-years	38.2	21.7	37.7	23.3	39.8	16.6	-8.4- 8.2
Vitamin intake mg/d[c]							
vitamin E[d]	15.2	5.2	15.3	5.2	14.7	5.1	-1.3- 1.7
ß-carotene	1.66	0.70	1.70	0.73	1.54	0.61	-0.2- 0.2
vitamin C	85.7	36-197	85.7	32-197	87.6	42-189	
vegetables g/d	163.0	62-310	159.9	61-315	166.8	67-303	
fruit g/d	78.4	4-251	78.4	4-251	78.6	3-269	
vegetable oils g/d	3.1	0-15	3.0	0-15	4.2	0-21	
plasma vitamins µmol/l							
α-tocopherol	29.7	7.1	29.6	7.2	30.0	6.6	-3.2- 0.8
ß-carotene	0.29	0.17	0.28	0.17	0.30	0.18	-0.05- 0.08
ascorbic acid	33.4	19.2	34.0	19.4	31.7	18.9	-9.2- 5.7

* $p < 0.05$ (age-adjusted) [a]Smokers in the lower 75% of CCA-IMT (ranging from 0.62 mm to 1.02 mm), and in the upper 25% of CCA-IMT (ranging from 1.03 mm to 1.43 mm). [b]95% confidence intervals for differences in age-adjusted means between subjects in the upper 25% and lower 75 % of CCA-IMT. Plasma α-tocopherol and ß-carotene are also adjusted for plasma cholesterol and vitamin E and ß-carotene intake for energy intake as described in the method section. [c]For intake of vitamin C, vegetables, fruit, and vegetable oils the median and 90% range is given [d]Vitamin E is mg of α-tocopherol equivalent

Table 2.3 Multivariate models showing regression coefficients ± standard errors of carotid IMT (dependent) and prevalence odds ratios (OR) of having an 'increased' IMT(dependent) with smoking characteristics, antioxidant vitamin status and other risk factors for cardiovascular disease (CVD) (independent variables) in a group of 158 male lifelong smokers free of CVD

	Linear regression[a]						Logistic regression[b]					
Independent variables	Model I		Model II		Model III		Model I		Model II		Model III	
Smoking												
deep inhalation (yes->no)	0.053	±0.022**	0.048	± 0.023*	0.036	±0.021	2.5	(1.1-5.6)	2.4	(1.1-5.6)	1.6	(0.7-3.8)
Plasma antioxidant vitamins[c]												
α-tocopherol			0.0	±0.002					0.8	(0.4-1.8)		
β-carotene			-0.001	±0.07					1.3	(0.6-3.0)		
ascorbic acid			-0.0002	±0.0006					1.0	(0.4-2.2)		
CVD risk indicators												
age	0.01	± 0.002***	0.01	±0.002***	0.009	±0.002***	4.8	(2.1-11.2)	4.3	(1.8-10.2)	3.3	(1.3-8.3)
systolic blood pressure (mmHg)					0.002	±0.001***					2.9	(1.1-7.3)
LDL cholesterol (mmol/l)					0.03	±0.01**					3.3	(1.4-8.0)
HDL cholesterol (mmol/l)					-0.06	±0.03*					0.6	(0.2-1.4)

* $p < 0.05$; **$p<0.01$, *** $p< 0.001$; regression coefficients significantly different from 0 [a]Regression coefficients ± standard error (SE). Continuous independent variables except for 'deep inhalation' into the lungs which is coded 1 for deep inhalers and 0 for non-deep and non inhalers [b]Prevalence odds ratios and 95% confidence intervals. Independent variables are dichotomized with the median as cut-off point. [c]plasma α-tocopherol and plasma ß-carotene are adjusted for plasma cholesterol by taking into the model the residuals of these variables from linear regression with plasma cholesterol as the dependent variable.

for these foods either. The intake of vegetables and fruit was inversely related to plasma cotinine levels (r=-0.17 and r=-0.23 respectively, both $p<0.05$). Fruit intake was also inversely associated with cigarettes smoked (r=-0.36, $p<0.001$). The recommended vegetable intake of 200 gram per day was met only by 28% of the subjects.

Figure 2.1 Age-adjusted mean and SE of common carotid artery intima media thickness (CCA-IMT) and plasma vitamin C levels over categories of inhalation of cigarette smoke.

Fig. 2.1a

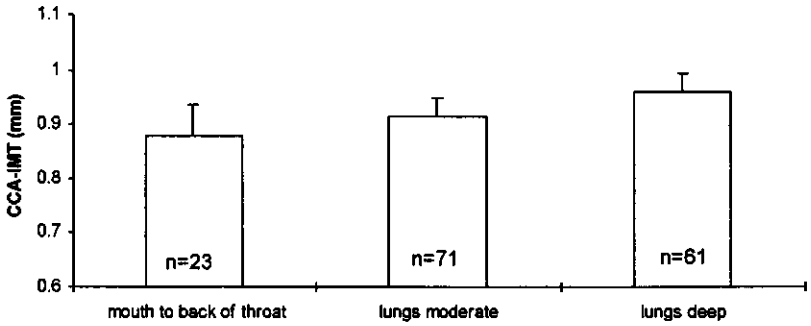

Fig. 2.1b

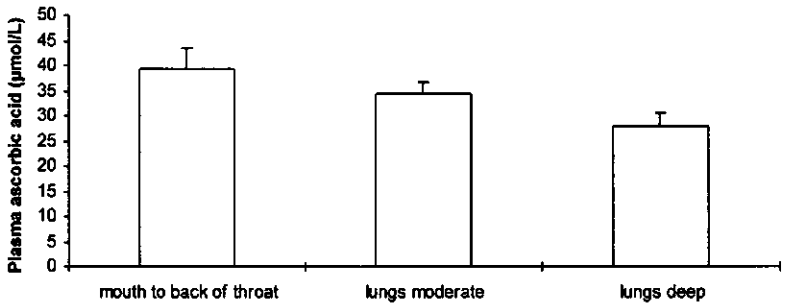

Table 2.3 shows three multivariate regression models. Of the smoking characteristics, only depth of inhalation was included in the models as it proved to be the only significant predictor of carotid IMT in exploratory stepwise regression. Model I shows that smokers inhaling deeply into the lungs had an age-adjusted carotid IMT that was 0.053 mm (0.022), $p< 0.01$, thicker than non-or moderate inhalers. Furthermore, the prevalence odds of 'increased' IMT was 2.5 times higher among deep inhalers, after adjustment for age, than in the remaining group (odds ratio (OR);

95% confidence interval (CI) OR=2.5 (1.1-5.6)). Including plasma antioxidant vitamins in model II does not significantly change any of the regression coefficients or prevalence odds ratios from model I. Including other CVD risk factors (model III), also selected by exploratory stepwise regression, explained 34% of the variance in carotid IMT. The regression coefficient for deep inhalation decreased from 0.053 mm to 0.036 mm, still borderline significant (p=0.08). Age, systolic blood pressure, LDL- and HDL-cholesterol levels were independent predictors of carotid IMT. Prevalence odds ratios revealed a similar pattern with age (OR=3.3; CI=1.3-8.3), systolic blood pressure (OR=2.9;CI=1.1-7.3) and LDL-cholesterol levels (OR=3.3;CI=1.4-8.0) as independent predictors of increased carotid IMT. Smokers with combined high levels of LDL and low antioxidant status with the median taken as cut-off point revealed age-adjusted prevalence odd ratios of having an increased IMT for α-tocopherol (OR=2.44; CI=1.1-5.5), ß-carotene (OR=1.66, CI=0.7-3.7) and vitamin C (OR=2.41; CI=1.1-5.5) not exceeding the age-adjusted risk for only high LDL levels (OR=3.57; CI=1.6-8.1). Therefore, in this group of smokers the combination of high LDL levels and low antioxidant status did not subsequently increases the risk of increased IMT.

Discussion

Depth of inhalation was the only smoking characteristic significantly associated with carotid intima-media thickness (IMT) within our study population of male lifelong smokers. 'Deep inhalers' had a mean (standard error) age-adjusted carotid IMT that was 0.053 mm (0.022) thicker (6%) than that of 'moderate and non-inhalers' ($p<0.05$). This association decreased and became borderline significant when adjusted for other CVD risk factors. Mean plasma levels and intake of antioxidant vitamins were not associated with carotid IMT. However, plasma vitamin C levels were significantly lower in smokers inhaling deeply. The CVD risk factors age, plasma LDL- and HDL cholesterol , and systolic blood pressure significantly contributed to carotid IMT.

A problem in studying associations between smoking characteristics, diet and CVD is that people may adjust these habits in light of emerging health problems. Moreover, recall bias caused by smokers being deceptive about their smoking habits when smoking-related disease is present may lead to misclassification. Therefore, we studied a pre-clinical population of 158 smokers selected from available data on 216 smokers. Because we selected an asymptomatic population, our results cannot be generalized to lifelong smokers. Also, self-selection may have occurred because the smokers under study were participants in a long-term intervention trial. They may differ

from individuals not willing to commit themselves to such a trial. In our study this effect is likely to be small. The results of a non-response study comparing 534 non-responders with 73 responders revealed no significant differences in age, history or presence of CVD, physical activity, demographic characteristics and subjective health score. Although the non-responders differed in being mainly non-smokers.

A reliable pre-clinical indicator of atherosclerosis is required to assess the association between smoking characteristics and antioxidant vitamins in an asymptomatic population such as ours. The use of carotid IMT measurements by B-mode ultrasound as such an indicator is justified by reported associations between carotid IMT and generalized atherosclerosis[91] and cardio- and cerebrovascular events at various sites[92-95]. Furthermore, the carotid IMT measurement is highly reproducible[89,90] and it is a preferable method for studying asymptomatic populations as it is non-invasive, harmless, and has a low burden for the individual measured. The clinical relevance of our results of 0.053 mm (0.036 mm after adjustment for CVD risk factors) difference in IMT for smokers inhaling deeply may be judged by comparison with these other studies. This is complicated by differences in study design. In a 8.8 years follow-up of the cholesterol lowering atherosclerosis study[117] for every 0.03 mm/year progression in IMT a relative risk of 3.1 (95% CI [2.1-4.5]) on any coronary event was reported. In a cross sectional study, Bots et al.[95] reported a relative risk of 1.34 for stroke and 1.25 for MI for every additional difference of 0.16 mm of CCA-IMT after adjustment for major cardiovascular risk factors.

The usefulness of smokers' self-reported inhalation pattern as measure of smoke exposure was supported in our study by the significant trend ($p<0.05$) between inhalation pattern and plasma cotinine after allowing for the effects of cigarette consumption. Stepney[118] did not find an association of self-reported inhalation and long-term smoke exposure as measured by carbonmonoxide in exhaled air. However, only moderate and deep inhalers were compared as the group of non-inhalers and slightly inhalers were too small to include in the analyses. Higenbottam et. al[88] found that the ten-year mortality rates for coronary heart disease in male cigarette smoking British civil servants were higher among self-reported inhalers than non-inhalers.

Studies[76,100,101] relating smoking to carotid IMT in the general population showed that pack-years of smoking was dose-dependently associated with carotid IMT. It is not clear why we did not find a significant association between pack-years (number of cigarettes times years smoked divided by 20) and carotid IMT. However, in our study, the age-adjusted mean number of pack-years was slightly higher in the group with 'increased' carotid IMT. Recent consumption of cigarettes, used to calculate the

number of pack-years, may not be a good indicator of lifelong consumption. Non-differential misclassification of pack-years of smoking, therefore, may have attenuated the association with carotid IMT towards the null value.

We did not find any significant associations between antioxidant vitamins and carotid IMT within our population of smokers. Antioxidant vitamins may be important in smokers in counteracting the effects of lipid peroxidation[119]. Smokers have high oxidative stress and usually both a lower intake and plasma level of antioxidant vitamins, which may render their LDL cholesterol more susceptible to lipid peroxidation[120]. Oxidation of LDL cholesterol is thought to play an important role in early atherosclerosis by depositing cholesterol in macrophages, leading to the formation of fatty streaks[121]. Increased oxidation of LDL-cholesterol in smokers is supported by reported higher levels of lipid peroxides in the plasma of smokers[70,71,122]. Moreover, the susceptibility of LDL to oxidation decreased in smokers after they had quit[123].

Several reasons may account for the fact that we did not find an association, if any, between carotid IMT and unsupplemented antioxidant vitamin plasma levels within the group of smokers in our study. First, our measure of antioxidant vitamin intake and plasma levels may not accurately represent lifetime intake, and thereby have decreased possible associations. Erythrocyte vitamin E was significantly inversely associated with carotid IMT in the EVA study[102] and the authors stated that erythrocyte vitamin E may reflect longer-term dietary intake and bioavailability than do plasma concentrations. Second, our study only included smokers not using any antioxidant supplements. Physiological concentrations of antioxidant vitamins may be insufficient to decrease carotid IMT. Azen et al.[103] showed less annual progression of carotid IMT in high vitamin E supplement users (>= 100 IU) as compared with low vitamin E supplement users (<100 IU). However, no such differences were found between high- and low vitamin C supplement users. Kritchevsky et al.[105] reported that the inverse associations between carotid IMT and vitamin C and vitamin E intake were lower when supplement users were omitted from the analyses in the group of older women in the Atherosclerosis Risk in Community (ARIC) study. Further, in the ARIC study no differences were found for plasma values of α-tocopherol and ß-carotene and increased carotid IMT[39].

The associations we found between carotid IMT and classical CVD risk factors such as increased total plasma cholesterol, increased plasma LDL cholesterol, decreased HDL cholesterol, higher age, and increased blood pressure support the overall validity of our cross-sectional analyses. Furthermore, those associations are consistent with

findings of other epidemiological studies[96-98]. Several studies have shown a higher progression rate of atherosclerosis in smokers than in nonsmokers[76,77]. From a public health point of view decreasing CVD risk of smokers would best be served by quitting smoking. But among smokers some will be at higher CVD risk than other smokers.

In conclusion, our results seem to indicate that (self-reported) depth of smoke inhalation contributes to atherosclerosis as measured by carotid IMT. However, traditional risk factors for CVD are more important contributors. In future epidemiological studies among smokers the addition of a short questionnaire on smoking characteristics, including depth of smoke inhalation, may be beneficial for a closer identification of smokers who are more sensitive than others to the atherogenic effect of smoking.

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3

Effect of vitamin E supplementation on 2-year progression of carotid intima media thickness in lifelong male smokers*

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Abstract

The beneficial role of a high dose of vitamin E in cardiovascular disease prevention as supported by observational data awaits confirmation from intervention trials. Therefore, we studied the effect of a daily dose of 400 IU vitamin E on the 2-year change of the common carotid intima media thickness (CCA-IMT). The study was designed as a randomized placebo-controlled double-blind intervention trial among 218 male lifelong smokers from the general population, 50-75 yr. with a mean (SD) number of 43 (8) smoking years. CCA-IMT was measured as a marker for generalized atherosclerosis by B-mode ultrasound. The combination of posterior and anterior wall measurements at the right and left side was used in the data analyses. The baseline mean CCA-IMT was 0.95 mm (0.16) for the group (n=189) including persons with both baseline and 2-year CCA-IMT measurements. In the placebo group (n=93) the mean 2-year change ('progression') in CCA-IMT was 0.026, 95% CI:0.005; 0.047, p=0.02 after adjustment for baseline CCA-IMT and intervention period. A non-significant reduced progression of 35% was seen in the vitamin E group (n=96) compared to placebo. After adjustment for traditional CVD risk factors vitamin E reduced CCA-IMT progression by 47% (p=0.34) compared to the progression in the placebo group (0.030 mm, 95% CI 0.009; 0.050, p=0.006). Although a tendency of reduced progression was observed in the vitamin E supplemented group of smokers our data do not support a clear beneficial effect of vitamin E on atherosclerosis.

* *Submitted*

Introduction

The accumulation of oxidized LDL-cholesterol in foam cells in the intima of the vessel wall and migration and proliferation of smooth muscle cells are important factors in the onset and progression of atherosclerosis[6].Vitamin E may have a protective effect against atherosclerosis by preventing low density lipoprotein (LDL) from oxidative modification.

Progression or regression of atherosclerosis in a general population can suitably be studied by following the change of the intima media thickness of the common carotid artery over time by B-mode ultrasound. This technique is non-invasive, highly reproducible[89] and associated with generalized atherosclerosis[91] and cardiovascular disease (CVD)[95,117].

Evidence for a beneficial effect of vitamin E on CVD is limited to two large trials. A randomized trial reported no reduction in coronary risk for male smokers[54] for 50 IU vitamin E. However, in a secondary analysis a reduction in non-fatal myocardial infarction (MI) but not on fatal coronary heart disease among smokers with previous MI was observed[55]. A secondary prevention trial of 400 and 800 IU/day showed also a reduction in nonfatal but not in fatal MI[56].

Several studies have shown a higher progression rate of atherosclerosis in smokers than in nonsmokers[76,77]. Smokers have a high exposure to free radicals and usually a lower intake and/or status of antioxidant vitamins[71,73].

Therefore, we studied the effect of a dose of 400 IU of vitamin E on two-year progression of common carotid artery intima media thickness (CCA-IMT), as a marker for atherosclerosis among a group of 218 male lifelong cigarette smokers from the general population.

Methods

Population and design. The study was designed as a two-year randomized double-blind placebo-controlled trial. Male inhabitants born between 1919 and 1946 from Nijmegen, a city of approximately 150,000 persons in the Netherlands, were approached using 7,000 addresses obtained from the Municipal Registration Office. The recruitment period was from October 1995 to July 1996. Eligible cigarette smokers were invited to an information evening and selected from returned questionnaires and on a medical examination. Three hundred and forty five smokers entered the selection procedure. Excluded were diabetes patients (n=10), users of (multi)vitamin-, vitamin E, vitamin C, ß-carotene, garlic, or fish oil supplements

(n=29), users of vitamin K antagonists (phenprocoumon, acenocoumarol) (n=8), individuals with current illness interfering with participation (n=17), and subjects not compliant to the protocol or unwillingness to participate (n=63). All together, 218 subjects ranging in age from 50 to 75 years were randomly assigned to intervention (n=109) and placebo group (n=109). The subjects received either capsules containing a daily dose of 400 IU (268 mg) vitamin E as dl-α-tocopherol or placebo capsules (provided by F Hoffman La Roche Ltd, Basel).

In 191 subjects, CCA-IMT measurements were obtained both at baseline and after a mean follow-up time of 1.8 years. Main reasons for the 27 subjects lost to follow-up regarding CCA-IMT measurements were either health related (n=4 vitamin E group, n=5 placebo group) of which 2 related to CVD (one person in the placebo group died of aneurysm of the aorta abdominalis), or unwilling to comply to the study protocol, not motivated to continue the study or having adverse feelings related to the intake of the capsules (n=7 vitamin E and n=11 placebo group). An additional 2 subjects were excluded from the analyses both from the vitamin E group as in the presence of plaques, IMT could not be properly measured at baseline or at 2-year. This resulted in 189 subjects included in the data analysis.

Written informed consent was obtained from all participants. The study was approved by the ethics committees of Wageningen University and Nijmegen University Hospital.

Ultrasound measurements. Ultrasound examinations were performed at baseline and after 2 years on both the left and right side of the distal 1.0 cm straight part of the common carotid artery (CCA), using a high resolution B-mode ultrasound (Biosound Phase-2) with a transducer frequency of 10 MHz. Images were stored on an optical disk and analyzed with a semiautomatic software program (Eurequa; TSA company, Meudon France[106]) by one reader blinded to treatment assignment or any other study information. Three measurements were made in a 1.0 cm long segment of both the posterior- and anterior wall. When a plaque was present a smaller segment was taken with a minimum of 0.2 cm. For the scanning at 2-year a worksheet with data of the baseline measurement on head position and scanning angle and an on line video image of the baseline measurement was used by the ultrasonographer to assure high reproducibility. Details of the scanning and reading procedure and of the reproducibility have been described elsewhere[90]. The mean of left and right posterior wall and left and right anterior wall and the mean of the combination of posterior and

anterior wall at right and left side were used in the data analyses. Progression is defined as the difference of common carotid artery intima media thickness (CCA-IMT) measurements at baseline and after two-years.

Low density lipoprotein oxidation and antioxidant vitamins. Fasting EDTA blood samples for oxidation of LDL were collected and directly supplemented with saccharose. Fasting heparinized samples were obtained for vitamin and lipid analyses. All samples were stored at -80°C until analysis.

LDL was isolated by density gradient ultracentrifugation (40,000 rpm for 18 h at 4°C) using a SW40 rotor (Beckman, Palo Alto, California, USA). The oxidation experiments were performed as described by Esterbauer et al.[28] and as modified by Princen et al.[124]. Briefly, the oxidation of LDL (60 μg apolipoprotein/ml) was initiated by the addition of 18 μM $CuSO_4$. The change of the 234-diene absorption was followed spectrophotometrically(Lambda 12, Perkin Elmer, GmBH, FRG). The lag time (min) was determined as the intercept of the tangents drawn to the segments of the absorbance curves of the lag and propagation phases of conjugated dienes. All samples of the same person were analyzed in the same run.

For the vitamin C analyses 0.5 ml plasma was stabilized with 4.5 ml 5% metaphosphoric acid within one hour after blood collection. Vitamin C was measured fluoremetrically. The plasma concentrations of α-tocopherol and ß-carotene and vitamin levels in LDL were analyzed by reverse phase high-performance liquid chromatography (HPLC).

Smoking and other CVD risk factors. Pack-years of smoking were calculated by multiplying duration of smoking (smoking years) by the number of cigarettes smoked per day divided by 20. Plasma cotinine was measured at baseline by packed column gas-liquid chromatography[108].

Medical history, current medication, and family history of CVD was obtained at baseline by a research nurse. Baseline medication for CVD included lipid lowering drugs, digitalis, daily aspirin use, ß-blockers, Ca-antagonists, ACE-inhibitors, and nitrates. Blood pressure measurements, changes in smoking habits and other lifestyle factors and medical consumption or current illness were checked every half year. Body mass index (BMI) was calculated as weight divided by squared height (kg/m^2).

Total plasma cholesterol and triglycerides were analyzed enzymatically (Boehringer Mannheim GmbH, FRG and Sera-PAK, Tournai, Belgium). HDL

cholesterol was determined with the polyethylene glycol method[114]. LDL cholesterol was calculated by the Friedewald equation[115].

Data analyses. Differences within the study groups and between vitamin E and placebo group were tested by student t-test. Means and mean individual changes were compared by the Mann-Whitney U-test and Wilcoxon-signed rank test, respectively, when not normally distributed. Correlations are given by Spearman or Pearson correlation coefficients (r) where appropriate. ANCOVA was used to compare adjusted mean changes in CCA-IMT within and between vitamin E and placebo group. Multiple regression was used to study the treatment effect adjusted for classical risk indicators for CVD. The intervention effect is given as the regression coefficient for treatment group with 95% confidence intervals (CI). The statistical package SAS (version 6.09,1989) was used for all the analyses. A p-value of 0.05 or a 95% confidence interval not including 0 when testing mean differences between and within groups was considered statistically significant.

Results

The lifelong male smokers in this study had a mean (SD) age of 60 (6) years and smoked an average of 43 (8) years ranging from 16 to 62 years. Table 3.1 gives the baseline characteristics by study group. No significant differences were observed in smoking characteristics, body mass index, blood pressure and health characteristics between the vitamin E and placebo group. In the placebo group 14% of the subjects had experienced CVD and in the vitamin E group 9%.

Baseline plasma lipid values did not differ between vitamin E and placebo group (table 3.2). Except for a small but significant difference in change in plasma total cholesterol, caused by changes in LDL cholesterol, no differences in changes in plasma lipid values, blood pressure or weight were observed between both study groups during the intervention period. As expected plasma and LDL-vitamin E increased significantly ($p < 0.05$) in the vitamin E supplemented group. More than 80% of the capsules was taken by 83% of the subjects according to pill counts.

The lagtime during the in vitro oxidation of LDL was 18.1 minutes (95% CI:15.5 to 20.7) longer in the vitamin E than in the placebo group. This increase in lagtime correlated highly with increase in LDL-vitamin E in the intervention group as assessed by Spearman correlation r=0.7 (p=0.0001).

Table 3.1. Baseline characteristics by study group for male lifelong smokers.

	Placebo group n=93		Vitamin E group n=96	
	mean or %	SD	mean or %	SD
age (y)	59.9	6.0	60.2	6.1
body mass index (kg/m^2)	26.1	3.3	25.9	3.2
systolic blood pressure	141	17	141	16
diastolic blood pressure	84	8	84	8
ankle brachial pressure index	1.08	0.07	1.08	0.06
CCA-IMT (mm)				
posterior wall	0.97	0.22	0.93	0.15
anterior wall	0.95	0.19	0.96	0.18
combination 4 sites	0.96	0.18	0.94	0.14
cigarettes (number/d)	18	9	17	9
smoking years	43	7	43	8
pack years	38	19	37	21
plasma cotinine µg/ml	248	125	252	122
cardiovascular disease (CVD) history*	14		9	
hypertension (%)†	22		19	
medication for CVD (%)‡	12		11	

* Self-reported CVD history included MI, stroke or bypass surgery.

† Hypertension: subjects using antihypertensive medication or having systolic and/or diastolic blood pressure ≥ 160 mmHg and ≥ 95 mmHg, respectively.

‡ See method section.

The smokers in this study had a mean unadjusted CCA-IMT (4 sites) of 0.95 (0.16), and 0.95 (0.19) and 0.96 (0.18) for the posterior and anterior wall, respectively (table 1). Correlations for baseline CCA-IMT with CVD risk indicators, i.e., age, systolic and diastolic blood pressure, BMI, plasma total- and LDL cholesterol were all between 0.17 and 0.48 (all $p<0.05$).

In the placebo group the 2-year unadjusted change of CCA-IMT (4 sites) was 0.022 (0.12) mm (95% CI:-0.002; 0.05,p=0.07). The baseline values for posterior wall and for CCA-IMT (4 sites) were somewhat lower in the vitamin E group compared to the placebo group while 2-year change was highly dependent on baseline values. Pearson correlations of change with baseline values were r=-0.37, -0.33, -0.46 (all p=0.0001) for posterior and anterior wall and CCA-IMT (4 sites), respectively. In table 3.3 changes in CCA-IMT adjusted for baseline values and difference in intervention period (model I) are presented. A significant progression of CCA-IMT of 0.026 mm, 95% CI:0.005;0.047 was seen in the placebo group compared to 0.017, 95% CI:-0.004;0.038 in the vitamin E group. Reduction in progression in the vitamin

Table 3.2. Mean (SD) baseline and two-year change of lipids, vitamins, and LDL oxidation by study group for male lifelong smokers

	Placebo group, N=93*				Vitamin E group, N=96*			
	baseline		2-yr change		baseline		2-yr change	
	mean	SD	mean	SD	mean	SD	mean	SD
Plasma lipids (mmol/L)								
total cholesterol	6.1	1.0	-0.3†	0.9	6.0	1.0	0.1‡	0.8
triglycerides	1.71	1.02	-0.09	0.94	1.80	1.07	0.07	0.78
HDL-cholesterol	1.16	0.29	0.02	0.20	1.19	0.42	-0.05	0.2
LDL-cholesterol	4.2	1.0	-0.3†	0.7	4.0	1.0	0.2†‡	0.7
Plasma vitamins µmol/L								
α-tocopherol	30.2	7.5	2.3†	5.9	30.9	6.8	41.6†‡	20.7
ß-carotene	0.31	0.19	-0.02	0.10	0.28	0.14	0.02	0.13
ascorbic acid	33.9	19.1	-1.8	49.4	36.1	18.5	-4.9	53.9
LDL-cholesterol								
α-tocopherol (nmol/mg apoB)	12.9	2.3	0.4	2.4	13.4	2.5	10.5†‡	5.5
lagtime (min)	87.9	8.8	-2.2†	6.2	89.6	11.1	15.9†‡	10.5

*Baseline or 2-yr blood sampling missing (n=8 placebo, n=13 vitamin E group)

† $p < 0.05$ different from 0, ‡ $p<0.05$ for testing differences in 2-year changes between study groups

Table 3.3 Two-year CCA-IMT (mm) progression by study group and intervention effects for male lifelong smokers.

	Placebo group, n=93			Vitamin E group, n=96			Intervention effect: difference vitamin E-placebo		
Change CCA-IMT	mean	95% CI		mean	95% CI		mean	95% CI	
Model 1									
posterior wall	0.020	-0.006;	0.045	0.013	-0.013;	0.038	-0.007	-0.043;	0.029
anterior wall	0.032	0.004;	0.059	0.021	-0.006;	0.048	-0.011	-0.049	0.028
combination 4 sites	0.026	0.005;	0.047	0.017	-0.004;	0.038	-0.009	-0.039;	0.020
Model 2									
posterior wall	0.026	0.001;	0.051	0.010	-0.014;	0.034	-0.016	-0.051;	0.019
anterior wall	0.029	0.003;	0.056	0.023	-0.003;	0.049	-0.007	-0.045;	0.031
combination 4 sites	0.030	0.009;	0.050	0.015	-0.005;	0.036	-0.014	-0.043;	0.015

Model 1: adjusted for baseline CCA-IMT and intervention period

Model 2: model 1 + adjusted for, age and baseline values of plasma LDL- and HDL-cholesterol, systolic blood pressure, BMI, presence of CVD, baseline antihypertensive or CVD medication and pack years of smoking.

E group was -0.009 mm 95% CI:-0.039;0.020 (35%) as compared to the placebo group. Further adjustments (model II) for classical risk indicators for CVD revealed a non significant difference of -0.014 mm 95% CI:-0.043;0.015 (47%) in CCA-IMT (4 sites) between vitamin E and placebo group. For the posterior wall this was 62% and for the anterior wall 24%.

At baseline the Spearman correlations between lagtime and CCA-IMT (4 sites), posterior wall and anterior wall were -0.13 (p=0.09), -0.13 (0.09) and -0.08 (p=0.31), respectively. There were no significant associations between changes of CCA-IMT (4 sites), posterior and anterior wall and changes in lagtime in the vitamin E group.

Discussion

Our vitamin E intervention trial among lifelong smokers showed a non-significant 47% reduction of 0.014 mm in progression of CCA-IMT for the vitamin E compared to the placebo group.

Lifelong smokers are showing faster progression rates of CCA-IMT than nonsmokers[76,77]. The high baseline CCA-IMT values of 0.95 mm we observed are comparable to those of other high CVD risk groups, e.g. patients with familial hypercholesterolemia[125]. Furthermore, the high free radical exposure and lower antioxidant status[73] make lifelong smokers particularly suitable to study effects of antioxidant supplementation. The associations we found between baseline CCA-IMT and CVD risk factors are consistent with findings from other epidemiological studies[96].

Our study design was based on the a priori expectation that a daily dosis of 400 IU vitamin E would generate a relevant and statistically significant reduction in progression of CCA-IMT. Despite a 47% reduction in 2-year progression no statistical significance was reached, probably because of limited power. It turned out that, the spontaneous 2-year progression in the placebo group and the absolute difference in progression between vitamin E and placebo group were smaller than was expected based on the limited information available at the start of our trial[77,126]. In a 2-year population-based study[77] faster progression (0.21 mm progression in maximum CCA-IMT) for male smokers than non smokers was reported. Ongoing lipid lowering trials at that moment suggested progression rates of 0.008 to 0.10 mm/year[126]. Based on this information, we anticipated a spontaneous 2-year progression of CCA-IMT of at least 0.10 mm in our high risk population of older, lifelong smoking males. After adjustment for CVD risk factors we found in the placebo group a spontaneous progression of only 0.030 mm, seriously reducing the power of our statistical test.

This smaller than expected progression is not specific to our methodology as it is in line with reports from recent lipid lowering trials and the ARIC study[76,127]. Also, the fact that high baseline values, like ours, have higher levels of measurement error[128] has further contributed to the lower than expected power of our study.

Poor compliance will not have attenuated our study results since mean plasma vitamin E levels increased more than twofold and an individual twofold increase was seen in 75% of the subjects, in line with dose response studies[45]. Moreover, the dose of 400 IU vitamin E is justified, according to the CVD endpoint trial[56] on vitamin E (400 IU) that showed reduction in nonfatal MI (but not fatal MI nor total mortality) in coronary patients.

Increased oxidation of LDL in smokers is supported by higher levels of plasma lipid peroxides[71,73] and a decrease in susceptibility to oxidation of LDL after quitting[123]. Our observation of an increase in lagtime after vitamin E supplementation is in line with other studies[45,124,129].

The increasing number of studies reporting positive associations between carotid IMT and generalized atherosclerosis[91] and cardio- and cerebrovascular events at various sites[95,117] justified the use of carotid IMT measurements by B-mode ultrasound as intermediate endpoint for atherosclerosis in our study. Also, this allows for direct comparison to other studies to qualitatively assess the clinical relevance of our intervention effect of 0.014 mm reduction in progression of CCA-IMT. The 8.8 years follow-up of the cholesterol lowering atherosclerosis study[117] reported for every 0.03 mm/year progression in IMT a relative risk of 3.1 (95% CI [2.1-4.5]) on any coronary event. Also, in a cross sectional study, Bots et al.[95] reported a relative risk of 1.34 for stroke and 1.25 for MI for every additional difference of 0.16 mm of CCA-IMT after adjustment for major cardiovascular risk factors. Together, these findings suggest that our results may be of clinical relevance, but the lack of statistical significance of our intervention effect clearly implies that such conclusion would require further substantiation from additional intervention studies.

Thus, as one of the first intervention trials, our study has not clearly shown a protective effect of vitamin E supplementation on atherosclerosis for the high risk population of older male lifelong smokers. Studies with more statistical power are needed to provide clear evidence for the potential beneficial action of vitamin E on the progression of atherosclerosis. Several large-scale vitamin E intervention trials are underway[59,60] and will allow for generalization across different populations. So far, recommendations regarding vitamin E supplementation for the prevention of atherosclerosis are premature in light of the available evidence.

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4

Glutathione *S*-transferase M1 genotype in smokers mediates effect of vitamin E on atherosclerosis*

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Abstract

We hypothesize that smokers with the null genotype for GSTM1 (GSTM1-*0*), who thus lack the detoxification enzyme glutathione S-transferase μ1, a) show increased progression of atherosclerosis and b) may benefit more from vitamin E supplementation in respect of reduced progression of atherosclerosis than smokers with the positive genotype (GSTM1-*1*). In a randomized placebo-controlled trial we studied the effect of vitamin E (400 IU) on atherosclerosis, as measured by 2-year change of left and right posterior and anterior common carotid intima media thickness (CCA-IMT) by B-mode ultrasound, among 189 male lifelong smokers. The frequency of GSTM1-*0* was 0.50. In the placebo group, smokers with GSTM1-*0* genotype had a higher mean 2-year progression of CCA-IMT of 0.045 mm (95% CI 0.016; 0.074, p=0.002) compared to 0.012 mm (95% CI -0.020;0.043, p=0.47) for those with GSTM1-*1* genotype after adjustment for baseline IMT, intervention time and major CVD risk factors. This was more pronounced for the posterior wall of CCA-IMT shown by a difference of 0.052 mm (95% CI 0.001;0.103, p=0.046) between GSTM1-*0* and GSTM1-*1* groups. For the smokers with GSTM1-*0* genotype, vitamin E supplementation reduced the proportion of smokers with increased CCA-IMT progression at respectively the left posterior and anterior wall by 73% (95% CI 26%; 90% p=0.01) and 62% (95% CI –4%; 86%, p=0.06), as compared to placebo. In conclusion, our data suggest that smokers lacking the detoxifying enzyme GST μ1 show stronger progression of atherosclerosis and may benefit more from vitamin E supplementation.

* *Submitted*

Introduction

Differences in genotype for enzyme systems involved in the biotransformation from cigarette smoke metabolites may differentiate the risk for cardiovascular disease among smokers[130]. The glutathione S-transferase μ 1 (GSTM1) polymorphism is particularly relevant as fifty percent of the general Caucasian population lacks GSTM1 enzyme activity due to two deficient GSTM1 null alleles (GSTM1-*0*)[131]. The enzyme GSTM1 is involved in detoxification of potential atherogenic substances such as lipid peroxides, reactive oxygen species and polycyclic aromatic hydrocarbons from cigarette tar.

By preventing lipid oxidation of LDL, the antioxidant vitamin E is believed to protect against the development of atherosclerosis[129]. As smokers are particularly exposed to free radicals and generally have a lower antioxidant vitamin status[73] they may especially benefit from vitamin E supplementation.

We hypothesize that smokers with the GSTM1-*0* genotype have increased progression of atherosclerosis and benefit more from vitamin E supplementation. This was studied among male lifelong smokers in a double-blind placebo-controlled intervention trial on the effect of 400 IU vitamin E supplementation on the 2-year change in the common carotid intima media thickness (CCA-IMT) measured by B-mode ultrasound as a valid and highly reproducible marker for atherosclerosis[89,90,132].

Methods

For a double-blind placebo controlled 2-year intervention trial male inhabitants born between 1919 and 1946 from Nijmegen, a city in the Netherlands, were approached using addresses obtained from the Municipal Registration Office. The recruitment period was from October 1995 to July 1996. Eligible cigarette smokers were selected from returned questionnaires and on a medical examination. Three hundred and forty five smokers entered the selection procedure. Excluded were diabetes patients (n=10), users of (multi)vitamin-, vitamin E, vitamin C, ß-carotene, garlic, or fish oil supplements (n=29), users of vitamin K antagonists (phenprocoumon, acenocoumarol) (n=8), individuals with current illness interfering with participation (n=17), and subjects not compliant to the protocol or unwillingness to participate (n=63). All together, 218 subjects were randomized and received either 400 IU (364 mg) vitamin E as dl-α-tocopherol or placebo capsules (provided by F Hoffman La Roche Ltd, Basel). In 189 subjects, CCA-IMT measurements were obtained both at baseline and after a mean follow-up time of 1.8 years. Main reasons for the 29 subjects lost to

follow-up regarding CCA-IMT measurements were either health related (n=4 vitamin E group, n=5 placebo group) of which 2 related to CVD (one person in the placebo group died of aneurysm of the aorta abdominalis), or unwilling to comply to the study protocol, not motivated to continue the study or having adverse feelings related to the intake of the capsules (n=7 vitamin E and n=11 placebo group), or for two subjects (vitamin E group) in the presence of plaques IMT could not be properly measured at baseline or at 2-year.

Written informed consent was obtained from all participants. The study was approved by the ethics committees of Wageningen University and Nijmegen University Hospital.

At baseline and at two year ultrasound examination were performed on both the left and right side of the distal 1.0 cm straight part of the common carotid artery, using a high resolution B-mode ultrasound (Biosound phase-2) with a transducer frequency of 10 MHz[90]. Images were stored on an optical disk and analyzed with a semiautomatic software program (Eurequa; TSA company, Meudon France[106]) by one reader blinded to treatment assignment or any other study information. For the scanning at 2-year a worksheet with data of the baseline measurement on head position and scanning angle and an on line video image of the baseline measurement was used by the ultrasonographer to assure high reproducibility. Details of the scanning and reading procedure and of the reproducibility have been described elsewhere[90].

GSTM1 genotypes were detected from buffycoat by PCR-based assays as originally described by Brockmöller et al.[133]. Written informed consent for isolation of DNA was given separately by all participants prior to GSTM1 genotype detection.

All statistical analyses were performed by SAS-PC. Mean differences and 95% confidence intervals in 2-year change in CCA-IMT were calculated by ANCOVA by computing least square means for the interaction effects of intervention and of GSTM1 polymorphism (PROC GLM). Adjustments were made for differences in baseline IMT and intervention time. In a second model further adjustments were made for major CVD risk factors. Increased progression of CCA-IMT was defined as CCA-IMT values above the median in the placebo group. Logistic regression with increased proportion of IMT was used as an additional measure to calculate the vitamin E intervention effect by genotype for the left and right posterior and anterior wall separately.

Table 4.1 Mean (SD) baseline characteristics by GSTM1 genotype for 189 male smokers

Characteristics	GSTM1-*1* (n=94)	GSTM1-*0* (n=95)	P-value
Vitamin E intervention group (%)	54 (n=51)	47 (n=45)	0.34
Posterior wall (PW) CCA-IMT* (mm)	0.92 (0.17)	0.97 (0.21)	0.09
Anterior wall (AW) CCA-IMT (mm)	0.95 (0.18)	0.96 (0.19)	0.78
Combination PW, AW CCA-IMT (mm)	0.94 (0.15)	0.97 (0.17)	0.19
Age (y)	60 (6)	60 (6)	0.75
Body mass index (kg/m^2)	25.8 (3.1)	26.1 (3.4)	0.59
Cigarettes (number/day)	18 (9)	17 (9)	0.33
Pack years of cigarette smoking	38.7 (21.4)	35.4 (18.4)	0.44
Cardiovascular disease(CVD) history (%)†	9	15	0.18
Hypertension (%)‡	20	20	0.97
Medication for CVD (%)	6	20	0.006

*CCA-IMT=common carotid artery intima media thickness.

†Self-reported CVD history included myocardial infarction, stroke or bypass surgery

‡Hypertension: using antihypertensive medication or having systolic and/or diastolic blood pressure >= 160 mmHg and >= 95 mmHg, respectively.

Results

Baseline values for CCA-IMT (combination of right and left posterior (PW) and anterior (AW) common carotid intima media thickness) and for PW and AW separately were non-significantly higher in the group with the GSTM1 null- compared to the positive genotype (table 1). In the placebo group, the increase in CCA-IMT was 0.036 (p=0.01) in individuals with the GSTM1-*0* genotype and 0.014 mm (p=0.38) in those with the GSTM1-*1* genotype (Table 2). After adjustment for major CVD risk factors (model 2) the GSTM1-*0* typed smokers in the placebo group showed 0.033 mm (95% CI -0.010; 0.076, p=0.13) more increase in CCA-IMT than the GSTM1-*1* genotype group. This difference was most pronounced in the PW (difference of 0.052 mm, 95% CI 0.001; 0.103, p=0.046). No significant intervention effect (difference between vitamin E and placebo group) was found for progression CCA-IMT and progression of PW and AW by GSTM1 genotype. Although a larger change in CCA-IMT was seen in the GSTM1-*0* group (CCA-IMT change= -0.022 mm) than in the GSTM1-*1* group (CCA-IMT change= -0.003 mm)(model 2).

For the PW and AW at the left and right site separately a variable less sensitive towards distribution of individual values was created by defining subjects with values

Table 4.2. Two year change in common carotid intima media thickness (mm) by GSTM1 polymorphism for the placebo and vitamin E group and intervention effects (vitamin E - placebo) for 189 male lifelong smokers.

	GSTM1-*1*	GSTM1-*0*	
	mean (95% CI)	mean (95% CI)	P-value*
Model 1	***2-yr change posterior and anterior wall combined (mm)***		
Placebo	0.014 (-0.017; 0.045)	0.036 (0.008; 0.065)	0.29
Vitamin E	0.015 (-0.014; 0.044)	0.019 (-0.011; 0.049)	0.85
Intervention effect	0.001 (-0.042; 0.044)	-0.017 (-0.059; 0.024)	0.54
Model 2	***2-yr change posterior and anterior wall combined (mm)***		
Placebo	0.012 (-0.020; 0.043)	0.045 (0.016; 0.074)	0.13
Vitamin E	0.008 (-0.020; 0.037)	0.023 (-0.007; 0.054)	0.49
Intervention effect	-0.003 (-0.045; 0.039)	-0.022 (-0.063; 0.020)	0.54
	2-yr change posterior wall (mm)		
Placebo	-0.002 (-0.038; 0.035)	0.050 (0.016; 0.084)	0.046
Vitamin E	-0.005 (-0.039; 0.028)	0.027 (-0.008; 0.063)	0.20
Intervention effect	-0.004 (-0.053; 0.046)	-0.023 (-0.072; 0.026)	0.58
	2-yr change anterior wall (mm)		
Placebo	0.024 (-0.017; 0.065)	0.034 (-0.003; 0.071)	0.73
Vitamin E	0.021 (-0.016; 0.057)	0.025 (-0.014; 0.064)	0.88
Intervention effect	-0.003 (-0.058; 0.052)	-0.009 (-0.063; 0.045)	0.89

* P-value for testing differences between genotype groups

Model 1: adjusted for baseline CCA-IMT and intervention period;

Model 2: model 1 + adjusted for age and baseline values of plasma LDL-, HDL-cholesterol, systolic blood pressure, BMI, presence of CVD, baseline antihypertensive or CVD medication and pack years of smoking

above the median of 2-year IMT change (calculated from the placebo group) as having increased progression of IMT (Table 3). In the GSTM1-*0* genotype group for the left PW and left AW the proportion of smokers with increased progression was reduced by 60% (95% CI 3%; 83%,p= 0.04) and 52% (95% CI -19%; 80%,p=0.11), respectively, by supplementation with vitamin E as compared to placebo. This reduction was more pronounced after adjustment for major CVD risk factors (reduction in left PW of 73%,p=0.01 and 62%, p=0.06 in left AW). An unexpected non significant higher proportion of men with increased progression in the right PW and AW by vitamin E supplementation was observed in the GSTM1-*0* subjects.

Table 4.3 Prevalence odds ratios (OR) for the intervention effect (vitamin E minus placebo) of having an increased 2-yr CCA-IMT by GSTM1 polymorphism for 189 male lifelong smokers.*

	GSTM1-*1*	GSTM1-*0*	
	OR (95% CI)	OR (95% CI)	P-value†
Model 1			
Posterior wall right	1.13 (0.46; 2.80)	1.97 (0.79; 4.95)	0.39
Posterior wall left	1.19 (0.48; 2.93)	0.40 (0.17; 0.97)	0.09
Anterior wall right	1.23 (0.49; 3.09)	1.59 (0.58; 4.35)	0.75
Anterior wall left	1.03 (0.36; 2.97)	0.48 (0.20; 1.19)	0.19
Model 2			
Posterior wall right	1.05 (0.40; 2.81)	1.91 (0.69; 5.27)	0.33
Posterior wall left	1.03 (0.36; 2.98)	0.27 (0.10; 0.74)	0.07
Anterior wall right	1.06 (0.38; 2.94)	1.70 (0.53; 5.44)	0.85
Anterior wall left	1.36 (0.40; 4.59)	0.38 (0.14;1.04)	0.08

*An increased 2-yr CCA-IMT is defined as the upper median levels of change in the placebo group. †P-value for testing differences between genotype groups

Model 1: adjusted for baseline CCA-IMT and intervention period

Model 2: model 1 + adjusted for, age and baseline values of plasma LDL-, HDL-cholesterol, systolic blood pressure, BMI, presence of CVD, baseline antihypertensive or CVD medication and pack years of smoking.

Discussion

In this study we showed that the 2-year progression of common carotid intima media thickness (CCA-IMT) was clearly more increased for smokers with the GSTM1-*0* genotype, thus lacking the detoxification enzyme glutathione S-transferase μ, compared to smokers with the GSTM1-*1* genotype. Vitamin E supplementation in smokers with the GSTM1-*0* type was beneficial for specific sites of CCA-IMT shown by a 60 to 73% reduced proportion of smokers having increased progression of CCA-IMT.

For cancer at various sites studies are available on GSTM1 genotype showing increased risk for individuals with the GSTM1-*0* type, especially in interaction with other factors as e.g. smoking[131]. It is hypothesized that the detoxification role of GSTM1 in smokers is not only important in the development of specific cancers but also in the pathogenesis of atherosclerosis. However, studies on GSTM1 genotype and atherosclerosis are lacking. Lack of glutathione transferase activity towards trans-stilbene oxide determined by phenotype was found more frequently among smokers with intermittent claudication than among healthy smokers of the same age[134]. However, in a study among 159 heavy smokers no higher levels of polycyclic

aromatic hydrocarbon-DNA adducts were found in the presence of GSTM1-*0* genotype compared to GSTM1-*1* genotype[135].

To give an overall picture we presented the combination of 4 sites of CCA-IMT as well as the posterior and anterior wall and the individual left and right site CCA-IMTs separately. Differences in strength and significance of individual associations may reflect real differences or may be related to measurement variability. The anterior wall of the common carotid artery may systematically underestimate the true IMT as the image does not anatomically reflect the intima media complex exactly[136]. However, it appears to reflect the presence of atherosclerosis elsewhere in the arterial system as strongly as the posterior wall[132]. Furthermore, the general association between different sites[137] favors a combination of sites in estimating progression as this reduces the variability considerably, leading to increased precision[132]. But, differences in IMT values between left and right site associated with carotid atherosclerosis have been reported [138] and differences in focal nature of the atherosclerotic process would favor presentation of individual sites, thereby increasing specificity but reducing precision.

The use of carotid IMT by B-mode ultrasound in our study as an indicator of atherosclerosis is justified by reported associations between carotid IMT and generalized atherosclerosis[91]. At the start of our trial we anticipated a spontaneous 2-year progression of CCA-IMT of at least 0.10 mm in our high risk smoking population[77]. The smaller than expected progression is not specific to our study as it is in line with findings from other studies in smokers[76].

Evidence for a beneficial effect of vitamin E on CVD is limited and results are inconsistent. A randomized trial reported no reduction in coronary risk for male smokers[54] for 50 IU vitamin E. However, in a secondary analysis a reduction in non-fatal myocardial infarction (MI) but not on fatal coronary heart disease among smokers with previous MI was observed[55]. A secondary prevention trial of 400 and 800 IU/day showed also a reduction in nonfatal but not in fatal MI[56]. A recently published trial[57] among MI survivors showed no reduction after 3.5 year of 300 mg vitamin E supplementation on the combined endpoint of death, nonfatal MI, and nonfatal stroke. However, a significant reduction in cardiovascular deaths was found.

As smokers with the GSTM1-*0* genotype will have a lower enzymatic defense system against reactive metabolites of cigarette smoke this group was hypothesized to especially benefit from the antioxidant vitamin E. Our study is the first to report beneficial effects of vitamin E on progression of IMT in relation to GSTM1 polymorphism in smokers. If further studies confirm our data, GSTM1 genotype may

importantly improve the predictive value of smoking for CVD. Also the commonly present GSTM1 polymorphism could be considered as a useful tool to refine in an easy and cheap way the risk group smokers to improve statistical power in future epidemiological studies on the etiology of atherosclerosis.

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5

Vitamin E supplementation in elderly lowers the oxidation rate of linoleic acid in LDL*

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Abstract

Oxidation of LDL-linoleic acid (LDL-LA), major substrate for lipid peroxidation, may be counteracted by the antioxidant vitamin E. In a 3-month randomized double-blind placebo-controlled trial in 83 apparently healthy Dutch elderly, aged 67-85 years, the direct protective effect of 100 IU vitamin E on the rate of oxidized LDL-LA was studied. The oxidation of LDL-LA was measured by its disappearance after a 5-h in vitro Cu-oxidation of LDL isolated from 1 ml plasma. In the vitamin E group, the decrease in oxidized LDL-LA of 10.4% ($p<0.05$) was significantly different ($p<0.05$) from the smaller 4.6% ($p<0.01$) decrease in the control group. Moreover, within the vitamin E group the decrease was even more marked over tertiles of α-tocopherol to LDL-LA ratio with a significant difference in decrease ($p<0.05$) from the lowest compared to the highest tertile of, respectively, 18.4% [-24%;-2%] (median and range) and 2.0% [-16%;34%]. In conclusion, supplementation with 100 IU vitamin E in elderly is beneficial in lowering the rate of oxidation of LDL-LA. The protective effect of vitamin E might best be monitored by using the ratio of α-tocopherol to LDL-LA as this reflects the degree of α-tocopherol available to protect the amount of LDL-LA present.

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Introduction

An inverse association between both intake and status of vitamin E (α-tocopherol) and coronary heart disease (CHD) morbidity and mortality is supported by an increasing number of epidemiological studies[49-52,56,139-143], although not conclusively[54,144-146]. A randomized controlled trial[56] showed a reduced risk of non-fatal myocardial infarction in patients with coronary atherosclerosis upon 400 IU or 800 IU α-tocopherol daily, while another[54] showed no effect on cardiovascular mortality upon 50 mg α-tocopherol daily. A follow-up study among 11,178 elderly showed significant reduced risks on total and CHD mortality for those elderly using vitamin E supplements[51]. The protective effect of the antioxidant vitamin E on CHD is thought to originate from its capacity to prevent lipid peroxidation in low density lipoproteins (LDL)[28,43]. Human intervention trials[44,45,124,147,148], are in line with this hypothesis, although the dose required to decrease the susceptibility of LDL to oxidation is still not agreed upon[44,45]. In addition to vitamin E, interindividual differences in linoleic acid, the major substrate for lipid peroxidation[149], may contribute to the interindividual variation in LDL peroxidation[150]. The rate of LDL oxidation depends on the poly unsaturated fatty acid (PUFA) concentration of LDL[151].

We extend on previous trials by investigating whether 3 months of supplementation with 100 IU vitamin E has a direct protective effect on the rate of LDL-linoleic acid (LDL-LA) oxidation, and on the absolute amount of oxidized LDL-LA and formation of conjugated dienes. Furthermore, as the amount of α-tocopherol required to prevent oxidation might be directly related to the amount of LDL-LA we also studied the role of the ratio of α-tocopherol to LA in the LDL particle. We conducted our study in elderly (> 65 years) as few data on oxidation of LDL are available for this age group[152,153]. Moreover, oxidized LDL seems not only to play a role in the formation of fatty streaks but also in more advanced lesions[6,154].

Methods

Population and design. The study was designed as a 3-month randomized double-blind placebo-controlled trial and conducted in 1994. In two consecutive steps, a total of 83 apparently healthy elderly, aged 67-85 years, were selected from a database of 1012 elderly from the city of Arnhem, the Netherlands[155]. The first selection step included the following criteria; still living in Arnhem, not institutionalized, no use of prescribed drugs for cardiovascular disease, high blood pressure or diabetes mellitus; no reported cancer or chronic diseases of the bowel or stomach, kidney, or liver. In the second step,

the 355 remaining persons were invited by mail and received a questionnaire on health status, smoking habits, and use of supplements and medication. Seventy-five subjects did not respond, 129 refused and 151 were willing to participate. From this group 68 were not eligible because of the following criteria; use of vitamin supplements (A, A/D, C, E or multivitamin) (n=25), stopped smoking less than 5 years before (n=5), current treatment for CHD, heart attack or stroke (n=31), use of medication for hypertension or high cholesterol (n=6), diabetes (n=2). The 83 eligible subjects were randomly assigned to intervention (n=42) and placebo group (n=41). To ensure group comparability, stratification was conducted before randomization on the following variables: smoking habits (current versus non-smokers), five-year age groups and gender. The subjects received either 100 IU vitamin E (twice daily 50 IU) as dl-α-tocopheryl acetate or placebo (lactose) capsules.

Data collection. In the beginning and at the end of the 3-month intervention period subjects were picked up at home and brought to the examination room in the department. Fasting EDTA and heparinized blood samples were taken for determination of plasma cholesterol and antioxidant vitamin levels and measurements of LDL oxidation. Body weight, height and blood pressure were determined at both occasions as well. At baseline the subjects filled in a questionnaire on socio-demographic characteristics, physical activity and perceived health score. They were asked to monitor during the intervention period occurring illness and/or medicine use in a diary. To improve and monitor compliance the subjects were visited at home once during the trial. At the end of the study compliance was assessed by pill counts.

For the first 34 subjects who entered the study no heparinized tubes, necessary for the measurements of oxidation, fatty acid- and antioxidant profile of LDL, were available. Thus, the data on the oxidation rate of linoleic acid are restricted to a subsample of n=49 subjects. The study was approved by the Medical Ethical Committee of the Agricultural University and all subjects gave a written informed consent.

Laboratory measurements.

Plasma vitamins. Plasma was prepared within 3 h after collection under nitrogen and stored at -80°C until analysis, to minimize analytical variation. Laboratory baseline and post-intervention measurements on an individual were performed in the same run. Following extraction, the concentrations of α-tocopherol, ß + γ tocopherol, ß-carotene, a-carotene and lutein were measured in plasma, by reverse-phase high-performance

liquid chromatography (HPLC) (adapted from Hess et al.[112]). The column was a prepackaged 25 cm x 4.6 mm Vydac 201TP54, C_{18} 300 Å (Hesperia, CA, USA). Detection after separation was carried out using two UV detectors, one for determination of retinol and carotenoids (UV2000) (set at 0-10 min: 325 nm; 10-32 min: 450 nm; 32-40 min: 470 nm) and one detector (UV1000) for determination of the tocopherols (set at 0-9 min: 325 nm; 9-40 min:292 nm). Total cholesterol and triglycerides (Ames, Bayer Diagnostics, Belgium) and HDL cholesterol were analyzed in duplo enzymatically (CHOD-PAP method, Boehringer Mannheim GmbH, Germany). LDL cholesterol was calculated using the Friedewald equation[115].

LDL oxidation. LDL oxidation was performed as described by Esterbauer et al.[28] by incubation of LDL isolated from 1 ml plasma with a freshly prepared 2.5 mmol/L $CuCl_2$ solution in phosphate buffer saline (PBS), pH 7.4 at 20° for 5 h, with a major modification in the isolation step[156].

In short, heparinized plasma (EDTA interferes with the Cu-oxidation) was adjusted to a density of 1.21 g/ml by addition of solid KBr and overlaid with 150 mMol/L NaCl. The samples were centrifuged in a TL-100 ultracentrifuge (Beckman) for 25 min. at 10° C and 440,000 x g. The LDL was removed by slicing the polycarbonate tube, adjusted to a density of 1.21 and centrifuged a second time for 90 min. at 10° C and 440,000 x g in order to remove attached proteins. The LDL was again removed by slicing the polycarbonate tube and used directly for the LDL oxidation experiments since any dialysis steps would remove also antioxidants and carotenoids. ApoB was determined by RID (Immuno AG, Vienna). Formation of conjugated dienes was measured every 15 min for 5 h by continuously monitoring the increase of the 234 nm absorbance following the above described Cu-oxidation of LDL. The length of the lag phase (min) was determined as the intercept of the tangents drawn to the segments of the absorbance curves corresponding to the lag and propagation phases of conjugated dienes formation as described by Frei and Gaziano[157].

LDL vitamins and fatty acids. α-tocopherol and ß-carotene concentrations in the LDL fractions were analyzed simultaneously by reversed phase HPLC. The lipids of the LDL were extracted by the method of Bligh and Dyer[158]. The fatty acids were transesterified and the methyl esters analyzed by gas chromatography on a capillary OV-1 column[159]. The data were normalized by comparing the areas of the fatty acid peaks with the area

of the internal standard peak (heptadecanoic acid) after correction for the various response factors.

Rate of linoleic acid oxidized was calculated as the disappearance of linoleic acid upon LDL oxidation divided by the LDL-LA concentration prior to oxidation times 100. Rate of arachidonic acid oxidized (LDL-AA) was calculated similarly. The absolute amount of LDL-LA oxidized, LDL-AA oxidized, amount of conjugated dienes formed, and vitamin concentrations in LDL are expressed per mg apoB. The molar extinction coefficient for dienes (29,500 $M^{-1}cm^{-1}$) was used to calculate the maximum amount of conjugated dienes formed. The ratio of α-tocopherol to linoleic acid in LDL was calculated as (LDL-α-tocopherol/linoleic acid)*100.

Data analysis. Baseline results are expressed as mean ± standard deviations (SD) or percentage ± SD. Changes in plasma- and LDL vitamins and lipid profiles after 3-month intervention are expressed as median and range of individual percentage change. Changes in oxidation measures (LDL-LA and LDL-AA oxidized, amounts of conjugated dienes formed and lag times) are expressed as median and range of absolute change.

One subject in the vitamin E group taking only 15% of the distributed capsules was excluded. Therefore, for plasma vitamin and plasma lipid profile analyses 41 subjects were included in both study groups. For LDL oxidation analyses, this resulted in data on 24 in both study group. Mean baseline values ± SD and mean individual changes during intervention were compared between the study groups by using the Mann-Whitney U test. Mean individual changes over the intervention period for the control- and vitamin E group were tested using the Wilcoxon-signed rank test for paired comparisons within groups. Presented correlations are expressed as Spearman correlation coefficients. Analysis were performed using the statistical package SAS.

Results

Table 5.1 presents the mean baseline characteristics of the placebo- and vitamin E supplemented group. Pre-stratification on sex, smoking behavior and 5-year age groups was successful, as shown by equal distribution of these variables in both study groups. No differences were observed for the other characteristics either. During intervention similar changes in both study groups occurred in body mass index (control group -0.9%, $p<0.05$; vitamin E group -1.3%, $p<0.001$) and diastolic blood pressure (control group 6.0%, $p<0.01$; vitamin E group 3.3%, $p=0.2$).

Table 5.1. Baseline characteristics of the control- and vitamin E study groups

	Control group (n 41)	Vitamin E group (n 41)
Age (yr.)	74.8±5.3	74.2±5.3
Male (%)	61	61
Smokers (%)	24	24
Body mass index (kg/m2)	25.3±3.5	26.2±3.6
Blood pressure (mmHg)		
systolic	153.5±19.6	156.8±23.5
diastolic	77.3±10.3	80.4±9.5
Perceived health score[a]	8.2±1.3	8.3±1.3
Physical active (%)[b]	62.5	70.0
Intervention period (days)	84±6	84±4
Capsules taken (%)	93.9±8.6	92.3±8.3

Values are mean ±SD, or percentages ±SD when indicated. [a] rating on a scale ranging from 1 (worst) to 10 (best). [b] based on subjective comparison of physical activity to people of the same age and health status

Intervention with vitamin E induced a significant 43.0 % (absolute median change of 14.0 mmol/L, p=0.0001) increase in plasma α-tocopherol (Table 5.2) and a 40.0% (absolute median change of 4.9 mmol/mg apoB, p=0.0001) in LDL-α -tocopherol (Table 5.3). Note however, that there was substantial individual variation in the increase in α-tocopherol ranging from -31% to +176% increase in plasma, and -12% to +381% increase in LDL. The percentage increase in plasma- and LDL α-tocopherol in the vitamin E group was associated with a similar percentage decrease in plasma- and LDL-ß+γ tocopherol (both r=-0.5, p<0.01). Baseline characteristics as shown in Table 5.1 and plasma vitamin values as shown in Table 5.2 yielded similar figures for the subpopulation of 48 with oxidation measurement data.

The plasma lipid profile did not importantly change during the intervention and did not differ between the control and vitamin E group (Table 5.2). The small change in plasma HDL cholesterol in the vitamin E group, reached statistical significance however.

About 44% of the fatty acids (FA) in LDL are PUFAs, of which 74 % was linoleic acid (C18:2), the main PUFA in LDL, and 14% arachidonic acid (C20:4), the second

most important PUFA in LDL and very prone to oxidation. FA profile in LDL as % of total FA did not differ between the study groups or change during intervention (data not shown).

Table 5.2. Plasma antioxidant vitamins and lipid profile at baseline and after intervention

	Control group (*n* 41)				Vitamin E group (*n* 41)			
	baseline		change 3 months		baseline		change 3 months	
	mean	SD	median	Range	mean	SD	median	range
Plasma antioxidant vitamins (μmol/l)								
α-tocopherol	34.3	6.9	0.6	-8-7	33.5	8.9	14.0**‡	-7-49
β+γ tocopherol	4.4	6.0	-0.1	-2-4	5.2	7.6	-1.2*‡	-30-1
β-carotene	0.42	0.2	0.01	-0.6-0.1	0.34	0.2	0.04†	-0.3-0.3
α-carotene	0.05	0.04	0.00	-0.1-0.1	0.05	0.03	0.00	-0.1-0.2
lutein	0.2	0.08	-0.01	-0.1-0.1	0.2	0.09	0.01	-0.2-0.2
Plasma lipid profile (mmol/l)								
total cholesterol	5.5	0.9	0.1	-2-1	5.5	1.3	0.03	-1.2-1.8
triglycerides	1.3	0.5	0.01	-2-1	1.4	0.5	0.01	-1.4-1.0
LDL cholesterol	3.2	0.8	0.05	-2-1	3.2	1.1	0.06	-1.1-1.4
HDL cholesterol	1.7	0.4	0.03*	-0.2-0.4	1.7	0.5	0.03†	-1.0-0.5

Changes significantly different from 0: * $p < 0.05$, ** $p < 0.001$. Significantly different from control group; † $p < 0.05$, ‡ $p < 0.001$

Figure 5.1A shows LDL oxidation over time expressed as the median amount of conjugated dienes per mg apoB formed. In Fig. 5.1B the decrease from baseline values in conjugated dienes formed during the intervention is illustrated. From the time point of 3 h to 4.25 h the observed decrease is significantly higher in the vitamin E group ($p<0.05$). No difference at baseline was observed in lag time (phase preceding the propagation phase of lipid peroxidation) between vitamin E (mean lag time (SD); 156 min (25)) and control group (166 min (22)). The median change in the lag time during intervention in the vitamin E group (median lag time [25%-75% range]; 28 min [-2;37] was significantly ($p< 0.05$) higher as compared to the control group (-2 min. [-19;8]).

The observed similar median decrease in control- and vitamin E group during intervention in absolute amount of oxidized LDL-LA (respectively 231.3 and 238.8 nmol/mg apo B) and amount of conjugated dienes formed (respectively 9.5 and 10.4) (Table 4)), can be explained by an observed decrease in LDL-LA concentration during intervention in both study groups (respectively 8.7% (156 nmol/mg apoB) and 13.8% (223 nmol/mg apoB) (Table 5.3). The correlation in the control group between changes in LDL-LA concentration during intervention and changes in oxidized LDL-LA is $r=0.9$ ($p<0.001$). Therefore, to account for differences in LDL-LA concentrations, the amount

Table 5.3.Antioxidants and fatty acid profile in LDL at baseline and after interventions.

	Control group (*n* 24)				Vitamin E group (*n* 24)			
	baseline		change 3 months		baseline		change 3 months	
LDL antioxidant vitamins (nmol/mg apoB)	mean	SD	median	range	mean	SD	median	range
α-tocopherol	13.9	3.6	-1.0	-6-7	12.4	4.3	4.9**‡	-1-20
β+γ tocopherol	1.6	0.8	-0.1	-2-6	1.3	0.6	0.7**†	-2-1
β-carotene	0.3	0.2	0.0	-0.3-0.1	0.3	0.1	0.03†	-0.3-0.2
lycopene	0.08	0.07	0.1	-0.1-0.1	0.1	0.0	0.0	-0.1-0.2
LDL fatty acids (nmol/mg apoB)								
total fatty acid	5207	552	-480**	-1565-1209	5542	2078	-546*	-7578-1541
polyunsaturated FA	2348	333	-240**	-790-574	2349	692	-309*	-2727-780
linoleic acid (18:2)	1747	300	-156*	-657-445	1759	503	-223*	-1929-699
arachidonic acid (20:4)	301	77	-23*	-139-113	327	124	-20	-450-152

Table 5.4 Oxidation of linoleic acid, arachidonic acid and formation of conjugated dienes after 5 h in vitro Cu-oxidation of LDL baseline and after 3 months.

	Control group (*n* 24)				Vitamin E group (*n* 24)			
	baseline		change 3 months		baseline		change 3 months	
Oxidation linoleic acid in LDL	mean	SD	median	range	mean	SD	median	range
absolute (nmol/mg apoB)	1127	227	-231**	-454-131	1152	374	-239**	-1664-374
relative (%)	64.3	6.2	-4.6**	-14-9	66.5	9.0	-10.4*†	-44-34
Oxidation arachidonic acid in LDL								
absolute (nmol/mg apoB)	262	67.4	-21.8**	-138-77	288	115	-32.8*	-449-98
relative (%)	87.1	3.7	-2.8**	-7-6	88.1	4.8	-6.4*	-22-17
Conjugated dienes (nmol/mg apoB)	81.9	13.4	-9.5**	-34-12	90.2	27.6	-10.4*	-117-20

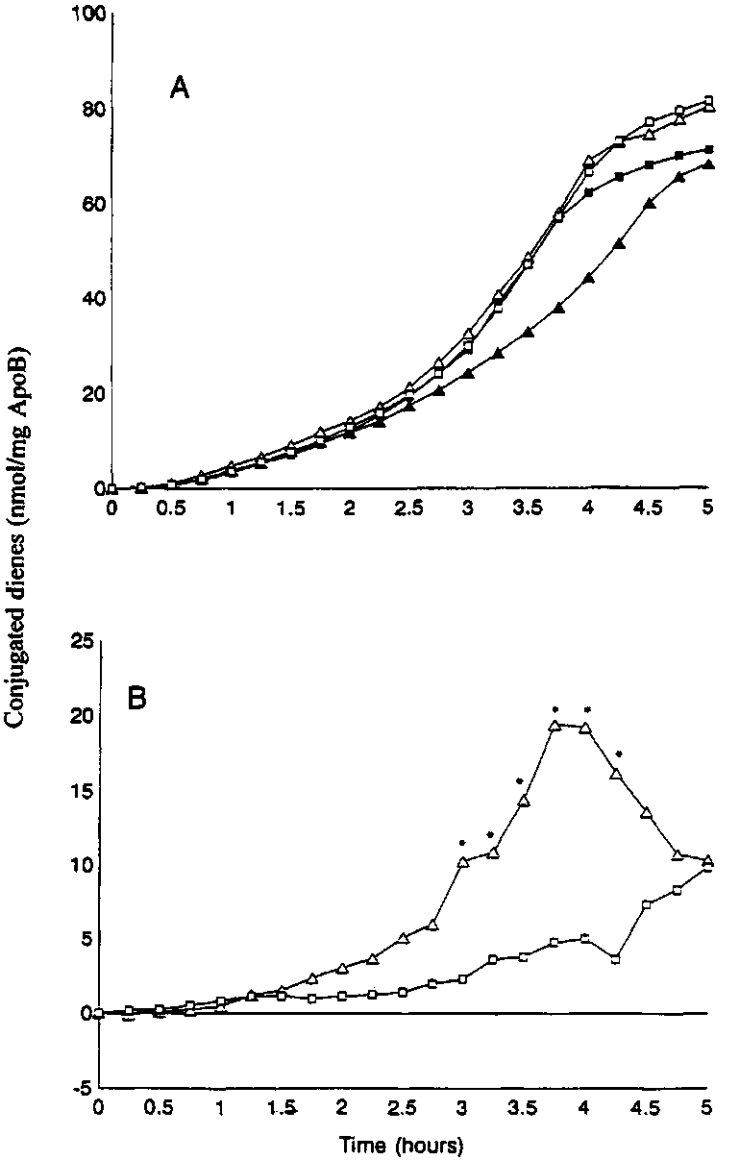
Changes significantly different from 0: * $p < 0.05$, ** $p < 0.001$. Significantly different from control group; † $p < 0.05$

*Table 5.5 Oxidation of linoleic acid (LA), arachidonic acid (AA) and formation of conjugated dienes stratified for baseline (α-tocopherol/LA)*100 ratio in LDL <> for the vitamin E group*

	I <-0.24; 0.66> *n* 8				II <0.67; 0.80> *n* 8				III <0.84; 1.20> *n* 8			
	baseline		change 3 months		baseline		change 3 months		baseline		change 3 months	
	mean	SD	median	range	mean	SD	median	range	mean	SD	median	range
Ratio α-tocopherol/LA LDL	0.49	0.15	0.55*	0.3-1	0.72	0.05‡	0.40*	0.2-1.0	0.96	0.11‡	0.10*†	-0.03-0.5
Oxidation LA in LDL												
absolute (nmol/mg apoB)	1334	517	-382*	-1664-79	1033	164	-264*	-687-108	1111	362	-123	-474-374
relative (%)	71.0	8.1	-18.4*	-24--2	65.6	3.7	-8.5*	-44--0.1	63.4	12.4	-2.0†	-16-34
Oxidation AA acid in LDL												
absolute (nmol/mg apoB)	347	151	-104	-449-25	247	68.5	-29.0	-118-98	277	107	-23.3†	-65-51
relative (%)	90.5	4.2	-11.5*	-22--0.9	88.2	2.8	-6.0*	-13-0.7	85.9	6.3	-2.0†	-9-18
Conjugated dienes (nmol/mg apoB)	92.4	34.8	-12.9	-117-3	81.2	18.9	-8.0	-38-20	94.9	27.7	-7.5*	-27--0.2

Changes significantly different from 0: * $p < 0.05$, ** $p < 0.001$. Significantly different from group I; † $p < 0.05$

*Figure 5.1. Time-course curves showing measurement of conjugated diene formation (nmol/mg apoB) during copper-mediated low density lipoprotein (LDL) oxidation. LDL was isolated from 1 ml plasma and oxidized with 2.5 μmol/L Cu^{2+} and absorbance at 234 nm was measured for 5 h at 15-min intervals. In (A), the oxidation curves are given for the control group (□) and vitamin E group (Δ) at baseline as well as after 3-month intervention; control group (■) and vitamin E group (▲). In (B), the decrease from baseline values of formation of conjugated dienes during intervention are given for control (□) and vitamin E group (Δ). Median values per time point are given; * denotes a significant difference between control and vitamin E group ($p < 0.05$).*



of oxidized LDL-LA was also expressed as percentage of concentrations of linoleic acid in LDL prior to oxidation. In the vitamin E group, the median decrease in percentage of oxidized LDL-LA of 10.4% ($p<0.05$) was significantly different ($p<0.05$) from the smaller 4.6% ($p<0.01$) decrease in the control group (Table 5.4). Similar results were seen for the oxidation of arachidonic acid (LDL-AA) although the twofold decrease in the percentage LDL-AA oxidized seen in the vitamin E group as compared to the decrease in the control group did not reach statistical significance.

To account for changes in LDL-LA concentrations as well as changes in LDL-α -tocopherol upon supplementation, the α-tocopherol/LA ratio in LDL was studied. Changes in the α-tocopherol/LDL-LA ratio in the vitamin E group during the intervention were inversely correlated with changes in the absolute amount of LDL-LA oxidized ($r=-0.5$; $p< 0.05$), the percentage LDL-LA oxidized ($r=-0.6$; $p<0.01$), the amount of conjugated dienes formed ($r=-0.3$; $p=0.1$) and the percentage of LDL-AA oxidized ($r=-0.6$; $p<0.01$). Subjects with the largest increase in the α-tocopherol/LDL-LA had the lowest baseline values for the ratio ($r=-0.6$ ($p<0.001$). From tertiles of baseline α-tocopherol/LDL-LA ratios (Table 5.5) it can be seen that subjects in the lowest tertile (I) benefited most from the supplementation in terms of absolute and relative amount of oxidized LDL-LA with median changes in values of -382 nmol/mg apoB LDL-LA and -18.4% LDL-LA respectively, in tertile I as compared to -123 nmol/mg apoB LDL-LA and -2.0% in tertile III.

For the oxidation measurements the vitamin E- and control group both contained four smokers. Excluding them from the analysis did not reveal different results.

Discussion

In this double-blind placebo controlled intervention study in an elderly population, supplementation with 100 IU vitamin E resulted in a significant two-fold decrease in percentage of oxidized LDL-LA between intervention- and control group. Moreover, within the vitamin E group this protective effect was even more marked with increase in α-tocopherol to linoleic acid ratio in LDL.

Our study population was selected as "healthy" using a questionnaire on disease and medicine use, and can therefore not be compared with the elderly population in general. Underlying subclinical diseases may well have been present but are not expected to have influenced the comparison between both study groups because of randomization. Schmuck et al.[152] showed no differences in indices of oxidation of LDL between healthy elderly as compared to a younger age group. Stulnig et al.[153] recently

showed marginally increased amounts of oxidized LDL, estimated by antibodies against LDL modified by 4-hydroxynonenal, in serum from healthy elderly as compared to a young control group. But in contrast they[153] observed an increased lag time and decreased maximal rate of LDL-oxidation in vitro for their elderly study group.

Plasma cholesterol levels in our study of 5.5 mmol/L, which are relatively low for an elderly population, have also been found in healthy elderly by Schmuck and colleagues[152].

The relative high baseline plasma α-tocopherol values of 33 mmol/L we found are not unusual for a Dutch elderly population as seen in the SENECA study (a study on nutrition and the elderly in 11 different countries in Europe)[160], and comparable with the values found in healthy elderly by Stulnig et al.[153]. This does not mean, however, that healthy elderly might not benefit from vitamin E supplementation. Also healthy elderly will have more accelerated atherosclerosis than younger individuals and it is hypothesized that oxidized LDL may not only play a role in the formation of fatty streaks but also in more complicated lesions through its cytotoxicity and formation of new foam cells at the leading edges of more advanced atherosclerotic lesions[6,154,161]. Although not consistently significant Verlangieri et al.[162] found in non-human primates that α-tocopherol does not prevent atherosclerosis but appears to lessen the severity and reduce the rate of the disease. Hodis et al.[142] demonstrated less coronary artery lesion progression in subjects with previous coronary artery graft surgery with supplementary vitamin E intake of at least 100 IU per day. Vitamin E studies on CHD end-points in elderly populations are scarce[51], but worth to consider since studies in middle-aged subjects suggest beneficial effects[49,50,52,56,141,142]. It is hypothesized that vitamin E favors a protective effect on atherosclerosis by protecting LDL against oxidation[43,54]. However, an animal experiment[163] in hyperlipidemic Watanabe rabbits showed that protecting of LDL against oxidation by vitamin E does not necessarily lead to less aortic atherosclerosis. In future research attention should be paid to find out if a protective effect of vitamin E on atherosclerosis can be partly explained by oxidation susceptibility of LDL.

Instead of looking at oxidation products formed we investigated the disappearance of LDL-LA as main substrate for LDL lipidperoxidation upon vitamin E supplementation. It showed that pre-oxidative LDL-LA concentrations are not necessarily stable across the intervention period as seen in a small but significant decrease in both study groups in our study. This was due to an overall decrease in fatty acids (FA) in LDL over the intervention period, therefore, the contribution of the individual FA to total fatty acids remained constant. The decrease in FA might be due to

a seasonal effect in dietary habits where fat intake being higher in winter (start study in February) than in summer (end of study in June), as supported by a study in young Dutch women[164]. The pre-oxidation LDL-LA concentrations largely determine the absolute amount of LDL-LA oxidized ($r=0.9$, $p<0.001$ in our study). Changes in the concentration of LDL-LA are therefore influencing the amount of LDL-LA oxidized (our study $r=0.8$, $p=0.0001$) and may mask the protective effect of vitamin E. Therefore, to account for pre-oxidative LDL-LA concentrations the ratio of oxidized LDL-LA to pre-oxidative LDL-LA values has to be used. The interindividual variation in elevation of plasma and LDL α-tocopherol in the vitamin E group in response to the same doses of vitamin E has been observed by others[45]. Dimitrov et al.[165] observed that fat-intake affected plasma elevation of α-tocopherol in their supplemented group and suggested this as one of the contributing factors to intra- and interindividual variation. To relate the degree of protection afforded by α-tocopherol to amount of LA present in LDL we investigated the ratio of α-tocopherol to LA in LDL. Our results suggest that this ratio may contribute to the individual variation in increase in LDL-α -tocopherol upon supplementation as subjects with the lowest (most unfavorable) ratio benefited more from the vitamin E supplementation. Although regression towards the mean may be part of the explanation, this could not account for the observed decreasing effects on oxidation of LDL with improvement of the α-tocopherol to LA ratio in LDL. The importance of this ratio is also illustrated by a finding of Croft et al.[166] who found a positive correlation between LDL-α -tocopherol and extent of FA oxidation and concluded a possible pro-oxidant effect of vitamin E while α-tocopherol expressed per mg LA showed no correlations with indices of LDL oxidation. When LA is not taken into account a positive correlation of α-tocopherol with lipid peroxidation products (in our study measured as absolute amount of oxidized LDL-LA) might lead to a possibly false suggestion that α-tocopherol has a pro-oxidative effect.

Lowering the amount of LDL-LA through the diet is not necessarily beneficial in decreasing oxidation susceptibility of LDL. After all, food products high in LA are usually also high in vitamin E and lower total plasma and LDL-cholesterol thereby decreasing the total amount of LDL particles present for possible oxidation. Intervention trials have shown that mono unsaturated fatty acids (MUFAs) as oleic acid are less susceptible to oxidation than linoleic acid[150,167,168] and just as effective in lowering total plasma, and LDL-cholesterol levels. In a group of Watanabe hyperlipidemic rabbits[163], diets high in oleic acid and vitamin E increased the lag time

(onset to LDL oxidation) with 140%, LA and vitamin E increased lag time by 59% while LA alone decreased lag times for LDL-oxidation with 30%.

The dose of 100 IU vitamin E in our study is relatively low to obtain an effect according to a dose-response study by Jialal and colleagues[45] suggesting that doses of 400 IU per day are required to show a protective effect against LDL oxidation. The results observed in our study are more in line with another dose-response study by Princen et al.[44] who found already oxidative protection after ingestion of only 25 IU vitamin E for two weeks. Increases of 40.0% (median) in LDL-α -tocopherol and 43.0% (median) plasma α-tocopherol in our study are in the same order of magnitude compared to the aforementioned studies[44,45]. High baseline plasma vitamin E levels as found in our study have been postulated by others[145] to be no longer beneficial in reducing CHD risk. Recently, however Stephens et al.[56] found in a double-blind placebo controlled study a relative risk of 0.23 of non-fatal MI upon 400 or 800 IU vitamin E daily in patients with angiographically proven atherosclerosis who had baseline plasma α-tocopherol values of 32.4 mmol/L. This shows that subjects with high baseline values comparable with those in our study might still benefit from vitamin E supplementation.

In conclusion this study in elderly shows that supplementation with 100 IU vitamin E in elderly is beneficial in increasing LDL- and plasma α-tocopherol concentrations and lowering the oxidation rate of LDL-LA. The protective effect of vitamin E supplementation is monitored more accurately by taking the ratio of LDL-α -tocopherol to LDL-LA since it reflects the degree in which the LA present in LDL is protected by α-tocopherol against oxidation.

6

Effect of 3 months vitamin E supplementation on indices of the cellular and humoral immune response in elderly subjects*

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Abstract

It has been suggested that decreased immune responsiveness in the elderly may be counteracted by the antioxidant vitamin E. In a 3-month double-blind placebo-controlled intervention trial among elderly subjects aged 65 years and over we studied the effects of a daily dose of 100 mg dl-α-tocopheryl acetate on the cellular immune responsiveness (*n* 52) measured by the *in vitro* response of peripheral blood mononuclear cells (PBMC) to the mitogens concanavalin A (ConA) and phytohemagglutinin (PHA). Also effects on the humoral immune responsiveness (*n* 74) were investigated by measuring immunoglobulin (Ig)G, IgG4 and IgA antibody concentrations against various common antigens. In the vitamin E group plasma α-tocopherol increased by 51% (P=0.0001) during intervention whereas no significant changes were observed in the control group. Initial proliferative PBMC responses differed between the vitamin E group and the control group whereas all other baseline characteristics were comparable. No significant changes were observed in cellular immune responsiveness when adjusted for initial values in either the control group or the vitamin E group and, after the trial period, responses in the two groups were not significantly different. Similarly, in the vitamin E group no significant changes were found in levels of IgG and IgA raised against *Penicillium* or IgG4 raised against egg, milk, or wheat proteins. In the control group small but significant increases in IgG anti-*Penicillium* (P <0.05) and decreases in IgG4 against milk proteins (P <0.05) were observed. Thus, the results of this study performed with the relatively low dose of 100 mg dl-α-tocopheryl acetate do not support the claims of a beneficial effect of vitamin E intake on the overall immune responsiveness of elderly subjects.

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Introduction

A decreased functioning of specific immune defense mechanisms with aging[11-13,169,170] is presumed to contribute to increased morbidity and mortality in the elderly[170-174]. In addition to chronic diseases that accompany aging that may lead to a decrease of immune responsiveness in the elderly, the aging process itself is thought to play a major role too[175] highlighting the potential relevance of improving the immune response in 'healthy' elderly people. Increased lipid peroxidation and production of eicosanoids such as prostaglandin E_2 (PGE_2) may be important determinants in age-associated dysregulation of immune responsiveness. Vitamin E may improve immune responsiveness by counteracting these effects[61-63,176-179].

Effects of vitamin E supplementation on the cellular and humoral immune response in experimental animals have been reported extensively[178,180-186]. Observational studies in healthy elderly human subjects found no positive associations between vitamin E intake or status and indicators of cell-mediated immune response[65-67]. However, these observational studies were cross-sectional and not designed to provide information on the effect of (long term) specific vitamin supplementation. In an intervention study among free-living healthy human elderly subjects with a daily dose of 800 mg dl-α-tocopheryl acetate for 30 d, Meydani and colleagues[61] observed an overall improvement in cell-mediated immune response as shown by increased frequency and size of positive delayed type hypersensitivity skintest responses, interleukin-2 production and *in vitro* proliferative response of peripheral blood mononuclear cells (PBMC) to the mitogen concanavalin A (ConA). Also a reduced PGE_2 synthesis by PBMC was seen, which argues in favor of reduction of PGE_2 production as a possible working mechanism. In the same study no significant differences were found in humoral immune response measured as total serum concentrations of immunoglobulin (Ig)M, IgG and IgA, nor in the T-cell independent proliferative response of B cells to the mitogen *Staphylococcus aureus* Cowan. In another intervention study among institutionalized healthy elderly women, supplementation with 200 mg vitamin E for 1 year had no effect on total IgM, IgG and IgA antibody concentrations[187].

The optimal dose for potential beneficial effects of supplemental vitamin E on immune variables in the elderly has not yet been determined. Studies investigating low or relatively (compared with other trials) low doses of supplemental vitamin E can contribute to the debate on whether it is warranted to advise elderly people to increase their intake of vitamin E-rich foods, or to explore the possibilities of enrichment of eligible food products or to advise vitamin E supplement use. But, at this point there is

only limited evidence that the positive effects of vitamin E on immune responses, as found in animal experiments, generalize to human elderly subjects. Therefore, we have investigated the effects of a 3 month period of 100 α-tocopheryl acetate supplementation on cell-mediated immune responsiveness, expressed as *in vitro* response of PBMC to the mitogens ConA and phytohemagglutinin (PHA), and on humoral immune responsiveness, assessed by measuring IgG, IgG4 and IgA antibody concentrations against various common antigens. To avoid inter-assay variation, inherent to the mitogen proliferation assays, isolated PBMC were stored in liquid N_2 and mitogen proliferative response of pre-and post-intervention PBMC of each individual were measured simultaneously in the same run.

Methods

Population and design. The study was designed as a 3-month randomized double-blind placebo-controlled trial to investigate the association between vitamin E and health characteristics in elderly subjects, such as immune response, lung function and as the main question the effect on oxidation of LDL- cholesterol, and was conducted in 1994. In two consecutive steps a total of eighty-three apparently healthy elderly subjects, aged 67-85 years, were selected from a database of a population of 1012 elderly in the city of Arnhem, the Netherlands[155]. The first selection step included the following criteria: still living in Arnhem, not institutionalized, no use of prescribed drugs for cardiovascular disease (CVD), high blood pressure or diabetes mellitus; no reported cancer or chronic diseases of the bowel or stomach, kidney, or liver. In the second step, the 355 remaining persons were invited by mail and received a questionnaire on health status, smoking habits, and use of supplements and medication. Seventy-five subjects did not respond, 129 refused and 151 were willing to participate. From this latter group sixty-eight were not eligible because of the following criteria: use of vitamin supplements (A, A+D, C, E or multivitamin; (*n* 25), stopped smoking less than 5 years before (*n* 5), current treatment for CVD, heart attack or stroke (*n* 31), use of medication for hypertension or high cholesterol (*n* 6), or diabetes (*n* 2). The eighty-three eligible subjects were randomly assigned to intervention (*n* 42) and placebo groups (*n* 41). To ensure group comparability, subjects were prestratified before randomization on the following variables: smoking habits (current *v.* non-smokers), 5-year age groups and sex. The subjects received either vitamin E (100 mg dl-α-tocopheryl acetate; Hoffmann-La Roche, Mijdrecht, The Netherlands; 50 mg twice daily) or placebo (lactose) capsules. Of the eighty-three subjects entering the study one subject in the vitamin E group who took

only 15% of the distributed capsules was excluded from the data analysis. Eight subjects had missing values on baseline or post-intervention humoral immune response measurements. A random group of thirty subjects had missing values for baseline and/or post-intervention values for the mitogenic proliferation responses due to shortage of plasma or isolated blood mononuclear cells to perform all the tests. Data analysis was performed on complete data sets. Therefore, baseline- and humoral immune characteristics are presented for seventy-four subjects (thirty-six in the placebo group and thirty-eight in the vitamin E group). For cell-mediated immune response, data on fifty-two subjects (twenty-five placebo and twenty-seven vitamin E group) are presented.

Data collection. At the beginning and end of the 3-month intervention period subjects were picked up at home and brought to the examination room at the University. Fasting blood samples were taken into EDTA for determination of antioxidant vitamin levels and for measurement of indices of the cellular and humoral immune response. Body weight, height and blood pressure were determined at both occasions as well. At baseline the subjects filled in a questionnaire on socio-demographic characteristics, physical activity and perceived health score. A semi-quantitative food frequency questionnaire was administered and used to estimate antioxidant vitamin intake[109]. Subjects were asked to monitor intercurrent illness and/or medicine use in a diary. To improve and monitor compliance subjects were visited at home once during the trial. At the end of the study compliance was assessed by pill counts.

The study was approved by the Medical Ethical Committee of the Agricultural University and all subjects gave a written informed consent to participate.

Laboratory measurements. Plasma was prepared under N_2 within 3 h after venepuncture and stored at -80°Cuntil analysis, to minimize analytical variation. Laboratory baseline and post-intervention measurements for each individual were performed in the same run. Concentrations of α-tocopherol, ß + γ tocopherol, and ß-carotene were measured in plasma, by reverse-phase HPLC (adapted from Hess *et al.*[112]). The HPLC analyses were performed using a system from Thermo Seperation Products (Freemont, CA, USA). The column was a prepacked 0.25 m x 4.6 mm Vydac 201TP54, C_{18} 300 Å (Hesperia, CA, USA). The mobile phase used for the Vydac column was methanol-tetrahydrofuran-water in the following proportions: 0 min, 89: 2: 9; 10 min, 98: 2: 0; 20 min 97: 3: 0; 30 min 90: 10: 0; 40 min, 90: 10: 0 by volume, and the flow rate was 1 ml/min. Detection after separation was carried out using two u.v. detectors,

one for determination of carotenoids (u.v. 2000) and one (u.v.1000) for determination of the tocopherols.

Humoral immune response: antigen-specific immunoglobulins G and A. The following specific antibodies were measured by enzyme immunoassay essentially as described previously[188,189]: IgG and IgA against a mixture of extracts of four different strains of *Penicillium* (Allergolisk Laboratorium Kopenhagen, Benelux, Houten, The Netherlands; art.no. 25-00) and IgG4 against egg (Laboratorium Diephuis, Groningen, The Netherlands; art.no. 58-04), milk (art.no. 58-01) and wheat[188] proteins. High-capacity microtitre plates (Greiner no. 655061; Greiner, Nuertingen, Germany) were coated overnight at 4° with the antigen preparations in phosphate-buffered saline (PBS). After blocking of free binding sites with gelatin-containing PBS-Tween (PBTG), duplicate 0.1 ml portions of sera diluted 1:100 (IgG4) and 1:500 (IgA, IgG) in PBTG were incubated in wells coated with the various antigens and in non-coated control wells. Binding of IgG, IgG4 or IgA was measured with peroxidase-labelled mouse monoclonal anti-human IgG (Central Laboratory of the Red Cross Blood transfusion Service, Amsterdam, the Netherlands (CLB) no. M1304), anti-human IgG4 (CLB no. M1331), or anti-IgA (CLB, no. M1354) respectively, followed by 30 min incubation with *o*-phenylenediamine (OPD). The reaction was read at 405 nm, terminated by addition of HCl, and then read at 492 nm. Absorbance values at 492 nm (A_{492}) > 3.0 were obtained by extrapolation from the A_{405} values according to the method of Doekes *et al.*[190].

Each plate included a positive control (pooled plasma) and no-plasma controls in each type of coated and non-coated well. Antibody concentrations were expressed as A_{492}* values corrected for values observed in control wells, as follows:

$$A_{492}^{*}(i,a_1) = [A_{492}(i,a_1) - A_{492}(0,a_1)] - [A_{492}(i,0) - A_{492}(0,0)]$$

in which $A_{492}(i,a_1)$ is the value obtained with plasma i on antigen a_1, $A_{492}(0,a_1)$ the value obtained with buffer on antigen a_1, and $A_{492}(i,0)$ and $A_{492}(0,0)$ are the analogous values obtained in non-coated wells.

Pre- and post-intervention plasma samples of each subject were tested in the same microtitre plate, to avoid inter-assay variation.

Cellular immune response: isolation and mitogenic stimulation of peripheral blood mononuclear cells. Within 6 h after venepuncture, PBMC were isolated from

EDTA-blood by Ficoll-Hypaque (Pharmacia BiotechAB, Uppsala Sweden) density gradient centrifugation (Boyum, 1968). PBMC were harvested and washed twice with sterile PBS and resuspended in 1 ml culture medium (RPMI 1640;Sigma-Aldrich Co. Ltd., Irvine Strathclyde) with 100 ml/l dimethylsulfoxide (DMSO) (hybri-max, Sigma-Aldrich Co. Ltd) in polypropylene vials. The cells were frozen overnight at -80° and stored in liquid N_2 (-196°) until analysis.

For mitogenic stimulation, vials containing pre- and post-intervention PBMC were removed from the liquid N_2 simultaneously and thawed quickly at 37° in a 5% CO_2-incubator. DMSO was removed by washing with RPMI 1640. This washing procedure was repeated twice to ensure all DMSO had been removed, after which the pellets were resuspended in 750 ml RPMI-1640 and the concentration of viable cells was determined with the Trypan Blue exclusion method.

Portions of 0.5 x 10^6 cells were cultured in twenty-four-well plates (Costar, Cambridge, MA, USA) in 0.50 ml RPMI-1640 containing 2 mM L-glutamine (Sigma), 50 mM 2-mercaptoethanol (Sigma), 30 mg/ml gentamycin (Sigma), and 100 ml/l normal human serum, in the presence of PHA (Sigma) or ConA (Sigma), both at 1 and 5 mg/ml. Normal human serum was a pool of sera from five healthy department members; these sera had all been tested individually in pilot experiments, and were free of detectable mitogenic activity. They were heat inactivated by incubation at 56° for exactly 30 min followed by 15 min centrifugation at 1500 rev./min before storage. On each culture-plate control wells were included with pre- and post-intervention PBMC of the same subject in the same medium without mitogens.

After incubation for 90 h at 37°, 5% CO_2 and 95-98% relative humidity, cells were washed twice in cold serum-free saline, and resuspended in the original volume (0.5 ml) of RPMI-1640, with glutamine, gentamycin and mercaptoethanol, but without human serum. In these suspensions metabolic cell activity was assessed by the MTT method[191] with adaptations taken from Denizot & Lang[192]. Thus, of each 0.5 ml suspension three 0.12 ml portions were applied to a ninety-six-well flat-bottom plate, mixed with 10 ml (10 mg/L) solution of 3,4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT;Sigma) stock solution. After 4 h incubation 120 ml isopropanol was added to each well to stop MTT conversion and to dissolve the produced formazan, which was quantified spectrophotometrically at 570 nm.

Mitogenic stimulation was expressed as the difference in A_{570} between the mean of duplicate cells cultured with mitogens and the control cell cultures.

Data analysis. Baseline results are expressed as mean and standard deviations (SD) or median and 25-75 % range. Mean baseline values and mean individual changes during intervention were compared between the study groups by using the unpaired *t* test in case of normal distribution and the Mann-Whitney test. Similarly, mean individual changes over the intervention period for the control- and vitamin E groups were tested with the paired *t* test or with the Wilcoxon-signed rank test for paired comparisons within groups.

Multiple linear regression was used to adjust differences in change in proliferative response values between the placebo group and vitamin E group for initial values[193]. Presented correlations are expressed as Spearman correlation coefficients. All analyses were performed using the statistical package SAS[194].

Table 6.1.Characteristics of elderly subjects participating in a 3-month intervention trial with vitamin E. (Mean values with their standard deviations or frequency (%))

	Control Group (*n* 36)		Vitamin E Group (*n* 38)	
	Mean	SD	Mean	SD
Age (years)	74.5	5.2	74.2	5.2
Male (%)	67		61	
Smokers (%)	25%		24%	
Intervention period (days)	83.7	6.8	84.2	4.5
Pills taken (%)	93.9	9.0	93.0	6.9
Health score‡	8.2	1.3	8.2	1.3
Height	1.70	0.09	1.70	0.09
Body mass index (kg/m^2)	25.1	3.2	25.9	3.2
No reported illness or medicine use †(%)	44%		55%	
Reported illness (days)†‡	5.5	8.7	3.5	3.4

* rating on a scale from 1 (worst) to 10 (best),

† based on self-reported occurrence of illness and use of medicine, recorded in a health diary during the 3-month intervention period.

‡ only for the group who reported illness or medicine use in their diary: placebo group, *n* 20, and vitamin E group, *n* 17.

Results

Table 6.1 presents the mean baseline characteristics of the placebo and vitamin E-supplemented groups. Pre-stratification on sex, smoking behavior and 5-year age groups

was successful, as shown by equal distribution of these variables in both study groups. Subjects participated for, on average, 3 months in the study (84 d; Table 6.1) with a variation from 61 to 94 d. Differences in supplementation period, mainly due to measuring during the holiday period (May-June), were not associated with immune response nor with particular population characteristics. The number of subjects not reporting any intercurrent illness or medicine use during the trial was slightly higher in the vitamin E group (*n* 21) than in the control group (*n* 16).

Baseline plasma antioxidant vitamin concentrations and estimated dietary antioxidant vitamin intake did not differ significantly between intervention and control groups (Table 6.2). In the vitamin E group plasma α-tocopherol increased during intervention by 51% (mean 16.7 (SD 12.7) mmol/L, P=0.0001). In the vitamin E group the increase in α-tocopherol was correlated with an observed decrease of plasma ß+γ tocopherol (Spearman correlation r -0.5, P=0.006). At baseline only one subject had a plasma α-tocopherol level below the cut-off value of 11.6 mmol/L for risk of vitamin E deficiency in human subjects and none below the 2.2 mmol α-tocopherol/mmol cholesterol value. Taking the optimal levels to prevent chronic disease as postulated by Gey [195], 32% fell below the 30 mmol plasma α-tocopherol/l limit and 18% below the 5.2 mmol α-tocopherol/mmol cholesterol value. In this study similar values for the reported immune variables were observed between subjects above and below the above mentioned optimal α-tocopherol levels mentioned earlier.

Humoral Immune Response; immunoglobulin G and A antibody concentrations.
At baseline, median concentrations of IgA and IgG raised against *Penicillium* and IgG4 raised against three different food antigens were comparable in vitamin E and placebo groups (Table 6.3). In the vitamin E-supplemented group no significant changes in antibody concentrations were observed after 3 months. In the control group a small but significant increase was seen for the IgG concentration against *Penicillium* (median change A_{492} 0.1; P=0.01) and a small decrease in IgG4 against milk protein (median change A_{492} -0.01; P=0.04). During the intervention period, similar changes were observed in the intervention- and placebo groups. Baseline and 3-month antibody concentrations were highly correlated (r 0.9 for all antibody concentrations in both groups). Baseline concentrations of IgG4 against the three different food antigens showed strong mutual correlations ($0.6 < r < 0.7$; P=0.0001).

Baseline antibody concentrations for the total study population were not significantly correlated with age, BMI, antioxidant plasma concentrations or antioxidant

Table 6.2. Plasma antioxidant vitamin concentrations and antioxidant vitamin intakes of elderly subjects during a 3-month intervention trial with vitamin E (Mean values and standard deviations or median values and their 25- 75% range)

	Control group (*n* 36)				Vitamin E group (*n* 38)			
	Baseline		Change		Baseline		Change	
	Mean or median	SD or 25-75%	Mean or median	SD or 25-75%	Mean or median	SD or 25-75%	Mean or median	SD or 25-75%
Plasma antioxidant vitamins (µmol/l)								
α-tocopherol	34.4	7.1	-0.8	3.7	33.0	8.1	16.7**	12.7
ß + γ tocopherol	2.7	2.1-4.3	-0.06	-0.4-0.4	3.2	2.1-4.2	-1.2**	-1.9--0.6
b-carotene	0.3	0.3-0.5	0.01	-0.06-0.03	0.3	0.2-0.4	0.03	-0.02-0.09
Antioxidant vitamins intake (mg/d)								
Energy (MJ/d)	7.6	2.1			7.6	1.7		
Vitamin E	9.2	7.2-15.8			11.0	8.2-13.4		
b-carotene	1.3	0.9-1.5			1.4	1.0-1.6		
Vitamin C	100.1	73.7-143.2			84.0	73.2-130.9		

* Mean or median values were significantly different from those for the control group, $P < 0.01$

Table 6.3. Concentrations of antibodies against common antigens (absorbance at 492 nm) in elderly subjects and changes to these concentrations during a 3-month intervention trial with vitamin E.

	Control group (*n* 36)				Vitamin E group (*n* 38)			
	Baseline		Change		Baseline		Change	
	Median	25-75%	Median	25-75%	Median	25-75%	Median	25-75%
IgA, *Penicillium*	1.2	0.5-2.0	0.01	-0.1-0.1	1.0	0.6-2.2	-0.01	-0.1-0.1
IgG, *Penicillium*	2.8	1.5-3.6	0.1*	-0.0-0.3	3.0	2.4-4.0	-0.04	-0.2-0.2
IgG4, egg protein	3.3	2.1-5.5	-0.1	-0.3-0.3	3.9	0.6-5.8	0.02	-0.1-0.2
IgG4, milkprotein	1.1	0.1-3.1	-0.01*	-0.2-0.0	1.0	0.0-4.2	0.0	-0.1-0.1
IgG4, wheat protein	0.2	0.0-2.6	0.0	-0.1-0.0	0.5	0.1-2.4	0.0	-0.1-0.1

Ig, Immunoglobulin. * Changes significantly different from zero, $P < 0.05$

Table 6.4. Mitogenic stimulation of peripheral blood mononuclear cells from elderly subjects and changes in these characteristics during a 3-month intervention trial with vitamin E‡. (Mean values and standard deviations)

	Control group (*n* 25)8				Vitamin E group (*n* 27)				Treatment effect§ Change (vitamin E-control group)			
	baseline		change		baseline		change		unadjusted		adjusted for initial values	
Stimulant	mean	SD	mean	SD	mean	SD	mean	SD	mean	SE	mean	SE
PHA, 5 μg/ml	0.41	0.23	0.15	0.21	0.67*	0.32	0.03*	0.20	-0.12†	0.06	-0.04	0.06
ConA, 5 μg/ml	0.26	0.09	0.13	0.17	0.40*	0.20	0.06	0.17	-0.07	0.05	-0.05	0.05

PHA, phytohaemagglutinin; ConA, concanavalin A * Mean values were significantly different form control group, $P < 0.05$. † Mean changes were significantly different from zero, $P < 0.05$. ‡ Proliferation was assessed by the MTT method and expressed as the absorbance value (at 570 nm) of the formazan produced.

§ Treatment effect is expressed as difference in 3-month changes in mitogenic stimulations between vitamin E and placebo-supplemented groups and expressed as the β_1 and standard error from the regression equation: $X_2 = \beta_0 + \beta_1 T + \beta_2 X_1$, where X_2 is change in proliferative mitogenic response, X_1 is baseline proliferative value and T is treatment group (1=intervention group, 0=placebo group).

vitamin intake. Higher baseline values of IgG4 against food antigens were seen for females than for males (IgG4 against milk-protein: median A_{492} 2.6 (25-75 % interval 0.3-4.3) for females and 0.5 (interval 0.03 -2.2) for males, P = 0.03). These sex differences persisted when comparing non-smoking males (n 30) and females (n 26). No sex differences for IgG and IgA raised against *Penicillium* were observed. The difference between smokers and non-smokers was evaluated among men because only one female smoker was present. Only for IgG against *Penicillium* was a significantly (P= 0.0004) lower baseline value for smokers (median 2.1 (interval 1.2 - 2.7)) seen as compared with non-smokers (median 3.5 (interval 2.7-4.3)).

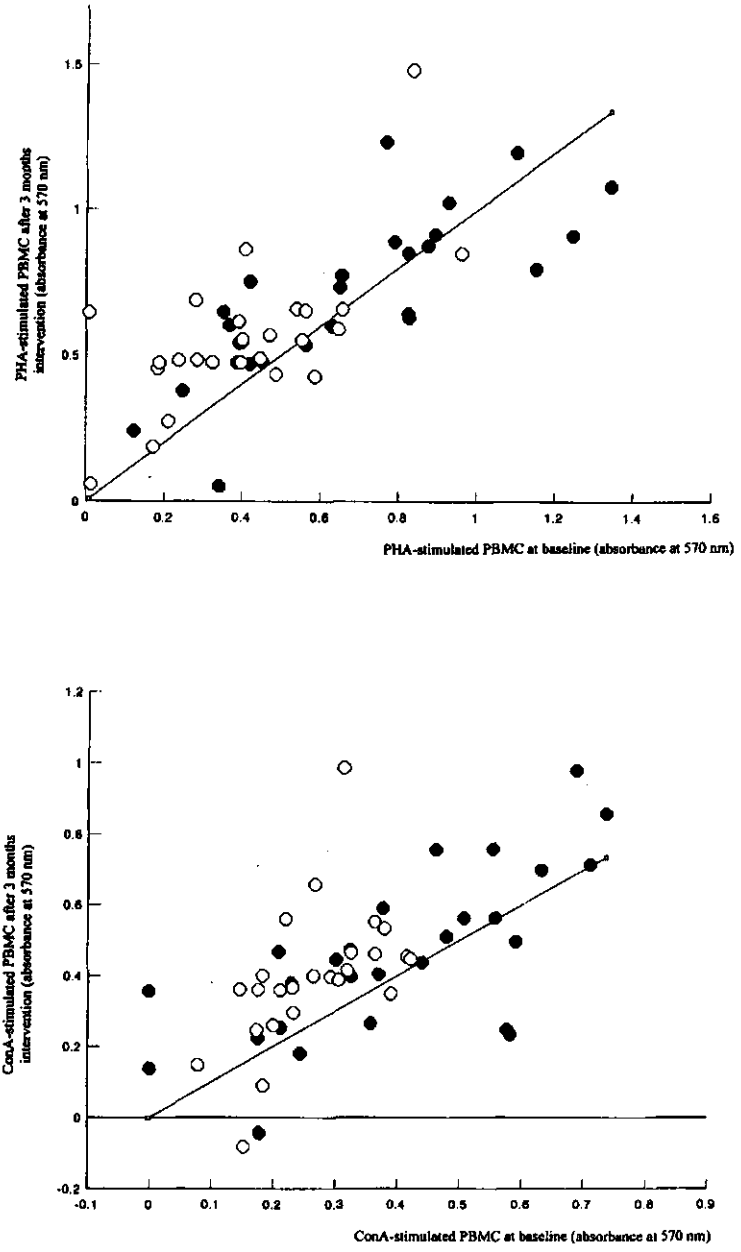
Cellular immune response; mitogen stimulation of peripheral blood mononuclear cells with phytohemagglutinin *and concanavalin A.*

Data for cellular immune responses were available for a subpopulation of fifty-two subjects from the total study population. Baseline characteristics of this subpopulation were similar to those given for the total study population in Tables 6.1 and 6.2.

At baseline lymphocyte proliferation to both mitogens ConA and PHA (5 mg/ml) was significantly lower (P =0.04) in the control group (Table 6.4). Furthermore, changes in PHA proliferative responses during intervention were negatively correlated with initial values (r -0.5, P< 0.02 for both study groups). To obtain an unbiased estimate, differences in change in proliferative response between the vitamin E group and the placebo group were adjusted for initial proliferative response values. As shown in Table 6.4 the adjusted treatment effect indicates that vitamin E had no effect on mitogenic stimulation of PBMC with PHA and ConA. To explore a potential effect of baseline α-tocopherol plasma values on immune response we analyzed separately the subjects in the lower two tertiles of baseline α-tocopherol values. This left thirty-two subjects (n 16 for both control and vitamin E groups) for analysis, with mean α-tocopherol baseline values of 28.9 (SD 4.9) mmol/L and 27.5 (SD 5.6) mmol/L and increases during intervention of 0.4 (SD 3.4) and 11.2 (SD 9.6) μmol/l in the control and vitamin E groups respectively. The results on mitogenic response for this sub-sample did not differ from those obtained from the total sample.

A_{570} values for unstimulated PBMC were similar for control and vitamin E group showing means of 0.10 (SD 0.05) and 0.11 (SD 0.08) respectively. Correlations between baseline and post-intervention measurements for PHA-stimulated PBMC were r 0.5 (P=0.006) and r 0.8 (P=0.0001) for control and vitamin E groups respectively. For ConA, correlations of r 0.6 (P=0.0008) and r 0.7 (P=0.0001) were observed (Fig. 6.1).

Figure 6.1. Stimulation of peripheral blood mononuclear cells (PBMC) from elderly subjects with the mitogens phytohaemagglutinin (PHA) and concanavalin A (conA) measured at baseline v. measurements made after 3 months intervention with vitamin E (●) or a placebo (○). For Spearman correlation coefficients, see result section. Proliferation was assessed by the MTT method and expressed as the absorbance at 570 nm of the formazan produced. The line shown is the plot Y=X.



Baseline values for proliferative response were not significantly correlated with age, BMI, antioxidant plasma concentrations or antioxidant vitamin intake. No differences were found between men and women or between smokers and non-smokers in baseline values.

Similar results were observed for proliferative responses with 1 mg mitogen/l (results not shown).

Discussion

In this 3-month intervention trial among apparently healthy elderly no effect was observed of 100 mg α-tocopheryl acetate on the *in vitro* proliferative response of PBMC to the mitogens PHA and ConA. Also antibody concentrations against various common antigens were not affected by vitamin E supplementation.

Aging itself, and diseases that frequently accompany aging, are thought to be factors explaining the decline in immune responsiveness with aging. Goodwin *et al.*[175] found no difference in cellular immune responsiveness between healthy and chronically ill elderly subjects. This suggests that the major determinant of decreased cellular immune function is age *per se* and not age-associated diseases. Therefore improving immune responsiveness in healthy elderly people may be of great public health importance. The present study population was selected as 'healthy' using a questionnaire on disease and medicine use, and therefore cannot be considered representative of the elderly population in general. Underlying subclinical diseases may well have been present but are not expected to have influenced the comparison between the two study groups because of randomization. Comparison within study groups of those not reporting illness during intervention with those reporting illness, did not reveal any differences in studied immune variables.

A great variability inherent to the mitogen proliferation assays has been mentioned by Meydani *et al.*[61]. To avoid inter-assay variation, isolated PBMC were stored in liquid N_2 and mitogen proliferative responses of pre-and post-intervention PBMC for each individual were measured simultaneously in the same run. The storage period of PBMC was not significantly associated with the proliferative responses.

Intra-individual variation in the mitogenic response measured at different time points may lead to regression towards the mean and could have affected our results, as we observed unexpected differences in initial values in mitogenic response between vitamin E and control groups. Baseline PHA and ConA responses in the control group were significantly lower than in the vitamin E group, while post-intervention responses

were similar in both groups and at the level of the vitamin E group at baseline. Therefore we adjusted the intervention effect (changes in proliferative response between vitamin E and control group) for initial values by means of multiple linear regression[193].

Vitamin E is thought to exert its effect on T-cell response and interleukin-2 production. Therefore an effect on humoral immune response would be primarily expected for T-cell dependent antibody responses. Ziemlanski *et al.*[187] and Meydani *et al.*[61] did not show an effect of vitamin E on total antibody (IgG, IgM, IgA, IgD) concentrations. Total IgG serum concentrations are, however, a rather crude estimate of humoral immune responsiveness. Therefore, we decided to measure specific antibodies to various common antigens like *Penicillium*, milk, wheat, and egg proteins to which most individuals are probably constantly exposed, and against which specific IgG and IgA antibodies are produced.

Taking into account the reported steady state of plasma α-tocopherol after 4-5 d following chronic administration of vitamin E[165], the lifetime of activated B-cells (4 weeks) and the half-life of antibodies in circulation (3 weeks), the intervention period of 3 months seems adequate for detecting important changes in antibody production.

Improvement of indices of the cellular and humoral immune response by vitamin E has been extensively demonstrated in animal experiments. Reviews on the subject (e.g. Meydani *et al.*[63] indicate important implications for the human situation, although this is supported by only a few studies in human populations. The first intervention study among elderly people on the effect of vitamin E on a large number of cellular and humoral immune indices was reported by Meydani *et al.*[61]. They showed increases in the proliferative response of PBMC to Con A but not to PHA after daily supplementation with 800 mg α-tocopherol for 30 d. The rise in plasma α-tocopherol in their study was 3-fold while in our study with 100 mg α-tocopheryl acetate the relative increase was 51%. The smaller increase in α-tocopherol in blood and probably also in PBMC (not measured in the present study but in the study by Meydani *et al.*[61] also 3-fold) might not have been enough to improve the *in vitro* mitogen proliferation of PBMC. This could mean that very high vitamin E doses are needed to improve mitogen-stimulated proliferation of PBMC. This is supported by another intervention in elderly subjects reported by Meydani *et al.*[196], with administration of different vitamin E doses (60, 200 and 800 mg vitamin E for 120 d), and in which a significant increase in mitogenic response to ConA was observed for the 800 mg dose only. An increased response to delayed-type hypersensitivity tests was, however, seen in all supplemented groups.

Alternatively, our results may be due partly to the relatively high baseline plasma α-tocopherol values in the present study as compared with, for example, the trial of. Meydani *et al.*[61]. Chandra[197] found, in a 1 year multivitamin supplementation (including 44 mg vitamin E) trial among healthy elderly subjects, that improvement in immunological responses (increase in natural killer cells, lymphocyte proliferation to PHA, a higher antibody response to influenza vaccine, and a lower frequency of infection-related illness) was greater among subjects who at baseline had low plasma nutrient values. Therefore we tested the intervention effect for the subjects in the lower two tertiles of the baseline plasma values revealing similar mean plasma α-tocopherol values to those in the study of Meydani *et al.*[61] but still found no effect on mitogenic response between the control and the vitamin E group. Therefore, it does not seem likely that relatively high baseline vitamin E levels are to be held responsible for not finding an effect of vitamin E supplementation on cellular immune response in the present study.

Our study in the elderly did not show improvements in specific immune indices after supplementation with 100 mg α-tocopheryl acetate for 3 months. This does not mean that relatively low doses of vitamin E might not affect other immune variables in elderly subjects (see Meydani *et al.*[196]. Studies on different doses of vitamin E and different immune variables will improve the available information on the overall possible positive effects and working mechanism and should provide necessary background information on the optimal doses. Better insight in improvement of the immune responsiveness in elderly people by vitamin E might warrant studies relating vitamin E to morbidity and mortality, especially those factors contributing to infections.

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7

Serum carotenoids, α-tocopherol and mortality risk in a prospective study among Dutch elderly*

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Abstract

Although ß-carotene has shown inverse associations with chronic diseases involving free radical damage in observational epidemiological studies less attention has been paid to five other major carotenoids also showing antioxidant activity in vitro.

We studied the associations between 7.2 year mortality and serum levels of six carotenoids, and α-tocopherol, measured in stored serum, sampled in 1991/1992 during a health survey among 638 independently living elderly subjects aged 65-85 years. Proportional hazards regression was used to estimate hazard ratios of all-cause mortality for the lowest tertiles of serum vitamins with the highest tertiles, adjusting for possible confounding effects.

During a follow-up period of 7.2 years 171 elderly died. The adjusted hazard ratios for all-cause mortality for the lowest tertiles of vitamins compared with the highest tertiles were between 1.02 and 1.73. The strongest increase in mortality risk was seen for ß-cryptoxanthin (1.52 with 95% CI: 1.00, 2.32), lutein (1.56 with 95% CI: 1.05, 2.31) and zeaxanthin (1.32, 95% CI: 0.89, 1.97) and their sum (oxygenated carotenoids: 1.73, 95% CI: 1.12, 2.67). Tests for trend were significant ($p<0.05$) for all-cause mortality risk and serum levels of total carotenoids, oxygenated carotenoids and ß-cryptoxanthin.

Our findings suggest that serum levels of individual carotenoids, particularly the oxygenated species are inversely associated with all-cause mortality and should be considered as candidates for further investigations.

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Introduction

Observational studies both on dietary intake of ß-carotene or ß-carotene rich products and serum or adipose tissue levels of ß-carotene products show inverse associations with cancer and cardiovascular disease[198]. Observational studies on the five other major carotenoids show that α-carotene intake was a better predictor for lung cancer than ß-carotene[199] and that lycopene and its main source of tomato products were inversely associated with prostate cancer[200] and myocardial infarction[201]. Lower levels of serum concentrations of lutein/zeaxanthin and ß-cryptoxanthin were observed in cases with asymptomatic atherosclerosis than in controls[39]. Plasma levels of ß-cryptoxanthin were also lower in a high than in a low CHD incidence area[202] and in cases with upper aerodigestive tract cancer than in matched controls[203]. The outcomes of these studies suggest that individual carotenoids, or the products from which they are derived, in isolation or in combination, are potentially protective against disease processes involving free radical damage[204]

In order to add to the limited epidemiological evidence we studied the association between 7.2 year all-cause mortality and the individual carotenoid components ß-carotene, α-carotene, lycopene, ß-cryptoxanthin, lutein and zeaxanthin among Dutch elderly. Furthermore, α-tocopherol (vitamin E) was included as an important antioxidant vitamin and also combined with the individual carotenoids in an antioxidant vitamin index.

Methods

Population and Design. The present study utilized data derived from a survey conducted in 1991/1992 on lifestyle and health among non-institutionalized Dutch elderly living in the city of Arnhem, aged 65-85. Mortality follow-up data were collected consecutively. The selection of this study population has been described in detail elsewhere[155].

Between October 28th 1991 and April 6th 1992 a random sample of elderly, pre-stratified on sex and age, participated in a health survey, including home interviews (n=1012) and a physical examination (n=685), including measurements of height, weight, blood pressure , electrocardiographic characteristics and spirometric function. In addition, non-fasting blood samples were taken. Of the 685 undergoing physical examination 641 donated a blood sample. For three persons the amount of serum available for the vitamin analyses was too small. This left 638 subjects for these analyses.

Written informed consent was obtained from the subjects prior to the physical examination.

Mortality follow-up. Data on death were obtained from the municipal register of Arnhem. After the collection of baseline data, information on death and migrations was reported to our department every 6 months. Survival status for those who moved from Arnhem during follow-up (n=70) was checked starting from February 1998 with the municipal authorities of the new residence. Vital status could be determined for all but one. Survival time in years began with the date of the blood collection in 1991/1992 and continued until date of death, date of checking vital status, date of loss to follow-up or 23rd of March 1999 whichever came first.

Total mortality of the 638 subjects after a mean follow-up time of 7.2 yr. was 27% (n=171; males n=108, females n=63)).

Serum antioxidant vitamins and cholesterol. Non-fasting serum samples had been stored for a mean time of six years at -80°C before vitamin analyses. Following extraction[205], concentrations of the carotenoids α-carotene, ß-carotene, lycopene, lutein, zeaxanthin and ß-cryptoxanthin and of α-tocopherol were measured in serum, by reverse-phase HPLC (adapted from Hess et al.[112] and Craft and Wise[206]). Detection after separation was carried out using two UV detectors, one for determination of carotenoids (UV. 2000) and one (UV. 1000) for determination of α-tocopherol. The coefficient of variation (CV) of pooled serum measured in duplicate in every run (n=17) was 12.3% for α-carotene, 10.2% for ß-carotene, 23.9% for lycopene, 10.7% for ß-cryptoxanthin, 14.9% for zeaxanthin, 7.2% for lutein, and 5.1% for α-tocopherol.

Total cholesterol was determined by an enzymatic method (CHOD-PAP method[207]).

Other characteristics. The interview, which was mainly pre-coded, included questions on physical activity, drinking and smoking habits, chronic diseases, use of health care, medication, supplements and personal characteristics. Physical activity was assessed by a validated questionnaire on household activities, sports and other physically active leisure time activities, developed for free-living elderly. From the responses a total activity score was calculated using an intensity code based on net energetic costs of the specific activities[208]. The presence of diseases was assessed

using a list of chronic diseases and conditions. The use of prescribed medication was asked with a reference period of three months prior to the interview. Cardiovascular disease (CVD) was considered present in subjects consulting a physician for heart disease, stroke or peripheral vascular disease 3 months prior to interview or in subjects using drugs for CVD; hypertension in subjects reporting the use of antihypertensive medication, or having a systolic blood pressure ≥ 160 mmHg and/or a diastolic blood pressure ≥ 95 mmHg; lung disease in subjects reporting chronic obstructive pulmonary disease (COPD) or asthma or the use of drugs for asthma or COPD; diabetes mellitus in subjects reporting the use of drugs for diabetes mellitus.

Alcohol consumption was coded as current drinking yes or no and for the drinkers as number of glasses per week. Pack years of cigarette smoking were calculated for present and past smokers as the number of cigarettes smoked times the number of years divided by 20. Never smokers and cigar and pipe smokers were classified as subjects with 0 pack years.

The physical examination included measurements of height, weight and blood pressure. Systolic and diastolic blood pressure were measured twice in supine position with a random-zero sphygmomanometer (Hawksley , England). The mean of the two measurements was used in the analyses.

Data analyses. Age-adjusted mean characteristics were compared between survivors and deceased by ANCOVA. Age-adjusted differences between the vital status groups were tested by using logistic regression with vital status as dependent and prevalence characteristics and age as independent variables. Plasma α-tocopherol levels were adjusted for plasma cholesterol by calculating their residuals from linear regression models with plasma cholesterol as the dependent variable[116]. For ease of interpretation, descriptives are expressed as unadjusted data.

All serum antioxidant vitamin concentrations are presented as median with their 90% ranges. Differences in vitamin concentrations between survivors and deceased were tested with the Mann-Whitney *U*-test.

Total carotenoids concentration was calculated by summing the absolute individual serum carotenoid concentrations. Oxygenated carotenoids were combined by summing serum concentrations of ß-cryptoxanthin, lutein and zeaxanthin and the hydrocarbon carotenoids were combined by summing ß-carotene, α-carotene and lycopene. An index combining both the carotenoids and α-tocopherol concentrations was made by summing individual standardized scores (Z-scores) calculated for each

log transformed vitamin by subtracting its group mean from the individual values and then dividing by the standard deviation.

Proportional hazards regression methods[209] were used to estimate the hazard ratios and corresponding 95% confidence intervals for all-cause mortality for tertiles of serum antioxidant vitamin concentrations with the highest tertile as reference. Tests for trend were performed by fitting the variables in their continuous form in the proportional hazards model. ß-Cryptoxanthin, zeaxanthin, lutein and the combination of oxygenated carotenoids were log transformed as this gave a better fit of the estimated models, as judged by the log likelihood statistic value.

The proportionality assumption was judged by visual inspection of the log-log curves of tertiles of the serum vitamin concentrations.

Models with interaction terms for gender or smoking status and the individual serum antioxidant vitamins revealed no interactions between gender or smoking status and antioxidant vitamins, so no interaction terms were included in the multivariable models. In a multivariate model adjustments were made for important confounders[210] as age, gender, pack years of cigarette smoking, alcohol consumption, serum cholesterol, body mass index, physical activity and antioxidant supplement use. Furthermore we adjusted for important predictors of total mortality; the presence of CVD, hypertension, lung disease or cancer in the multivariate models.

All analyses were performed with the statistical package SAS[194]. A p-value below 0.05 was considered as statistically significant.

Results

Male and female survivors were as expected significantly younger than the deceased (Table 7.1). Furthermore, age was significantly associated with most of the other characteristics in Table 7.1. Therefore, differences were tested after adjustment for age. Some age-adjusted differences in characteristics between survivors and deceased were more pronounced in males than females, e.g. significantly lower systolic blood pressure, higher activity scores, and a lower prevalence of lung disease and hypertension in survivors than in deceased.

Table 7.2 shows the median serum vitamin concentrations of the female and male elderly study subjects according to their survival status. The observed differences in serum levels were small but for most of the carotenoids and α-tocopherol lower in deceased than in survivors.

All serum concentrations except for lycopene, lutein and cholesterol adjusted α-tocopherol were lower ($p < 0.05$) in males than in females. Age was inversely associated with serum lycopene and (cholesterol adjusted) α-tocopherol in men; Spearman correlations of -0.15 and -0.21 respectively ($p<0.01$).

Table 7.1 Mean (SD) or prevalence (%) of baseline characteristics for male and female deceased and survivors after 7.2 years of follow-up of a Dutch cohort of elderly

	Males		Females	
	Censored n=227	Deceased n=108	Censored n=240	Deceased n=63
survival time (yr.)	7.1 (0.3)	4.1 (1.9)***	7.1 (0.3)	4.4 (1.8)***
General characteristics				
age (yr.)	72.7 (5.2)	76.0 (5.1)***	73.5 (5.6)	78.8 (5.0)***
body mass index (kg/m^2)	25.5 (3.1)	25.3 (3.0)	26.4 (4.5)	26.5 (4.3)
systolic blood pressure (mmHg)	146 (19)	152 (21)*	151 (21)	157 (22)
diastolic blood pressure (mmHg)	80 (11)	82 (11)	82 (10)	83 (12)
serum cholesterol (mmol/L)	5.9 (1.1)	6.0 (1.2)	6.6 (1.2)	6.5 (1.4)
Lifestyle characteristics				
married (%)	85	81	40	32
smokers (%)	33	34	18	10
ex-smokers (%)	56	59	24	27
never smokers (%)	11	6	58	63
pack years of cigarette smoking	21.3 (26.2)	25.2 (26.2)	5.6 (12.3)	6.1 (17.3)
alcohol consumers (%)	84	79	62	59
antioxidant supplement users (%)[a]	19	12	21	14
sporting regularly (%)	37	25	34	30
activity score	11.3 (7.7)	8.1 (7.0)**	7.0 (4.6)	5.5 (4.5)
Health characteristics[b]				
CVD present (%)	31	41	26	41
hypertension (%)	30	47*	46	60
lung disease present (%)	9	20*	9	11
cancer present (%)	2	6	3	5
diabetes mellitus (%)	2	0	4	5

[a] Antioxidant supplement use comprised vitamin C, vitamin E and multivitamin supplements

[b]See See method section

*** $p < 0.001$,** $p<0.01$ * $p <0.05$ censored and deceased significantly different after adjustment for age

The individual antioxidant carotenoids were highly correlated ($p=0.0001$) with the sum of these individual carotenoids ranging from Spearman correlations $r=0.50$ for

Table 7.2. Median concentrations of serum levels of total carotenoids, 6 specific carotenoids and α-tocopherol (μmol/L) and antioxidant vitamin index with their 90% range ( ) for male and female deceased and survivors after 7.2 year of follow-up of a Dutch cohort of elderly.

	Males					Females				
Serum vitamins μmol/L	Censored n=227		Deceased n=108		P[a]	Censored n=240		Deceased n=63		P[a]
Carotenoids total[b]	0.99	(0.44, 1.86)	0.90	(0.42, 1.57)	0.04	1.16	(0.54, 2.46)	1.18	(0.60, 1.95)	0.32
Antioxidant index[b]	-4.5	(-11.9, 1.49)	-5.3	(-13.6, -0.34)	0.04	-2.8	(-10.6, 3.3	-2.9	(-9.0, 2.0)	0.84
oxygenated carotenoids[b]	0.49	(0.21, 1.00)	0.44	(0.18, 0.79)	0.02	0.67	(0.28, 1.39	0.61	(0.28, 1.01)	0.18
ß-cryptoxanthin	0.20	(0.04, 0.62)	0.18	(0.03, 0.59)	0.06	0.32	(0.07, 1.01)	0.25	(0.06, 0.68)	0.21
lutein	0.25	(0.11, 0.41)	0.22	(0.10, 0.39)	0.04	0.25	(0.12, 0.51)	0.24	(0.13, 0.44)	0.46
zeaxanthin	0.032	(0.012, 0.069)	0.026	(0.012, 0.064)	0.08	0.036	(0.016, 0.073)	0.036	(0.016, 0.079)	0.81
hydrocarbon carotenoids[b]	0.45	(0.12, 1.07)	0.43	(0.13, 0.88)	0.38	0.52	(0.14, 1.29)	0.54	(0.22, 0.87)	0.80
ß-carotene	0.27	(0.09, 0.67)	0.30	(0.10, 0.57)	0.94	0.37	(0.14, 1.29)	0.36	(0.14, 0.71)	0.89
α-carotene	0.042	(0.007, 0.13)	0.039	(0.009, 0.12)	0.22	0.050	(0.014, 0.16	0.051	(0.021, 0.13)	0.45
lycopene	0.095	(0.004, 0.38)	0.074	(0.003, 0.39)	0.05	0.084	(0.005, 0.33)	0.070	(0.009, 0.28)	0.63
α-tocopherol	32.9	(21.5, 51.2)	31.3	(22.6, 52.0)	0.13	35.8	(23.4, 52.6)	34.2	(25.0, 52.1)	0.82
cholesterol adjusted. α-tocopherol[c]	34.7	(27.0, 49.8)	33.0	(24.9, 48.3)	0.02	33.9	(24.8, 50.0)	33.5	(25.8, 50.4)	0.87

[a] P for differences between serum vitamin levels of censored and deceased tested with Mann-Whitney U-test. [b] carotenoids total is the sum of the six individual carotenoids; antioxidant index is the sum of the Z-scores of the log-transformed individual carotenoids and α-tocopherol (see method section), oxygenated carotenoids is the sum of ß-cryptoxanthin, lutein and zeaxanthin, hydrocarbon carotenoids is the sum of ß-carotene, α-carotene, and lycopene. [c]See method section

Table 7.3 Hazard ratios (HR) of all-cause mortality and 95% confidence intervals (CI) of tertiles of antioxidant serum vitamin concentrations in Dutch elderly

	gender adjusted models				multivariate adjusted models[a]			
Tertiles of serum vitamins (µmol/L)	lowest	medium	highest	p for trend	lowest	medium	highest	p for trend
carotenoids total	1.38 (0.93, 2.03)[b]	1.33 (0.90, 1.96)	1.0	0.008	1.33 (0.88, 2.01)	1.28 (0.86, 1.91)	1.0	0.02
antioxidant index	1.43 (0.97, 2.11)	1.35 (0.92, 1.99)	1.0	0.05	1.28 (0.84, 1.95)	1.29 (0.86, 1.94)	1.0	0.20
oxygenated carotenoids[c]	1.62 (1.09, 2.42)	1.40 (0.93, 2.10)	1.0	0.004	1.73 (1.12, 2.67)	1.45 (0.94, 2.24)	1.0	0.006
ß-cryptoxanthin[c]	1.44 (0.97, 2.14)	1.36 (0.92, 2.01)	1.0	0.02	1.52 (1.00, 2.32)	1.18 (0.79, 1.77)	1.0	0.02
lutein[c]	1.42 (0.98, 2.05)	1.05 (0.71, 1.54)	1.0	0.03	1.56 (1.05,2.31)	1.11 (0.74, 1.67)	1.0	0.05
zeaxanthin[c]	1.40 (0.97, 2.03)	1.01 (0.69, 1.50)	1.0	0.09	1.32 (0.89, 1.97)	1.09 (0.73, 1.62)	1.0	0.18
hydrocarbon carotenoids	1.15 (0.79-1.67)	1.18 (0.81-1.71)	1.0	0.11	1.07 (0.72, 1.58)	1.14 (0.78,1.66)	1.0	0.31
ß-carotene	1.04 (0.71, 1.53)	1.33 (0.92, 1.93)	1.0		1.02 (0.68, 1.52)	1.37 (0.94, 2.01)	1.0	
α-carotene	1.19 (0.80, 1.76)	1.43 (0.98, 2.09)	1.0		1.11 (0.74, 1.67)	1.42 (0.97, 2.08)	1.0	
lycopene	1.50 (1.03, 2.18)	1.28 (0.88, 1.88)	1.0	0.07	1.22 (0.83, 1.79)	1.15 (0.78, 1.69)	1.0	0.37
cholesterol adjusted α-tocopherol	1.42 (0.96, 2.09)	1.55 (1.06, 2.27)	1.0		1.11 (0.74, 1.65)	1.35 (0.91, 1.99)	1.0	

[a] adjusted for gender, age, pack years of cigarette smoking, serum cholesterol mmol/L, body mass index, alcohol consumption, antioxidant supplement use, physical activity score, presence of CVD, lung disease, hypertension or cancer (see method section for description)

[b] Hazard ratios are given with 95% confidence intervals within parentheses

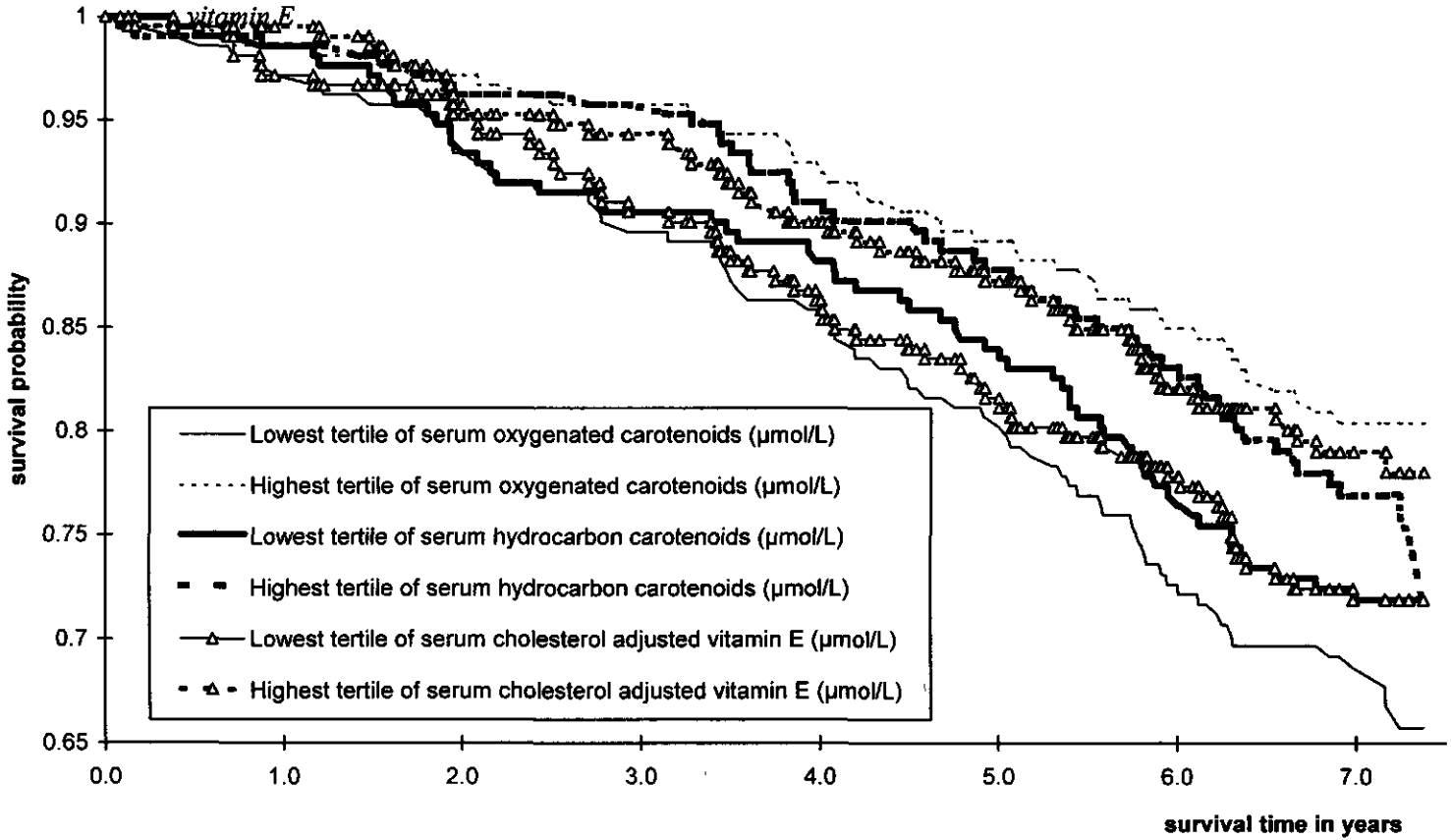
[c] The continuous log-transformed serum values were used as independent variable in the model for test of trend

lutein to r=0.80 for ß-carotene. Serum concentrations of cholesterol adjusted α-tocopherol showed a Spearman correlation of r=0.18 (p=0.0001) with total carotenoids. Among each other the highest Spearman correlations for the carotenoids were seen for ß- and α-carotene and for lutein and zeaxanthin, r=0.78 and r=0.67 respectively. Lowest correlations were observed for lycopene with the other carotenoids ranging from r=0.14 with zeaxanthin to r=0.41 for ß-carotene.

In Figure 7.1 survival curves (Kaplan-Meier) show that the subjects in the lowest tertile of oxygenated carotenoids and cholesterol adjusted α-tocopherol have a lower survival than in the highest tertile. For the hydrocarbon carotenoids this is not observed, mainly by a drop in the survival for the highest tertile after 7 years.

In Table 7.3 the hazard ratios and 95% confidence intervals (CI) for all-cause mortality are given of tertiles of the serum antioxidant vitamins. The gender adjusted hazard ratios for all-cause mortality are higher for the lowest compared to the highest tertiles for all the vitamins except ß-carotene and only significantly for the sum of oxygenated carotenoids and lycopene with hazard ratios of 1.62 (95% confidence interval =1.07, 2.45) and 1.50 (1.03, 2.18), respectively. Also the intermediate tertile of cholesterol adjusted α-tocopherol showed an increased risk of 55% on total mortality compared to the lowest tertile (hazard ratio of 1.55 (1.06, 2.27). Significant ($p<0.05$) inverse trends are observed between all-cause mortality and serum levels of total carotenoids, sum of oxygenated carotenoids, ß-cryptoxanthin, lutein and borderline for antioxidant index, zeaxanthin and lycopene. For ß-carotene and α-carotene no significantly increased risks are found for the lowest and intermediate tertile. While it seems that the intermediate tertile shows the highest risk and also the Kaplan-Meier survival curves supported this (data not shown). Therefore, no test for trend was performed for those carotenoids. After adjustment for potential confounders the hazard ratios were similar in magnitude as for the gender adjusted models. The adjusted hazard ratio for the lowest compared to the highest tertile of the sum of oxygenated carotenoids remained significantly increased (1.73, 95%CI:1.12-2.67) and became significant for ß-cryptoxanthin and zeaxanthin with hazard ratios of 1.52 (1.00,2.32) and 1.56 (1.05,2.31), respectively. The inverse associations between all-cause mortality and total serum carotenoids, oxygenated carotenoids and ß-cryptoxanthin remained significant after multivariate adjustments and became borderline significant (p=0.05) for lutein.

Figure 7.1. Kaplan-Meier estimates of survival functions for all-cause mortality of the lowest and highest tertiles of serum oxygenated and hydrocarbon carotenoids and cholesterol adjusted serum vitamin E



Discussion

In this study we observed that the sum of serum levels of six carotenoids, of oxygenated carotenoids and the individual carotenoid ß-cryptoxanthin were inversely associated with 7.2 year all-cause mortality. For the sum of oxygenated carotenoids, ß-cryptoxanthin and lutein the subjects in the lowest tertile showed a significant increase in all-cause mortality with the highest tertile as reference ranging from 56% for lutein to 73% for the sum of oxygenated carotenoids.

Discrepancies between study findings for serum levels of total carotenoids and mortality may partly be explained by differences in component carotenoids[211,212]. In our study comprising all six major carotenoids an inverse association with all-cause mortality was observed. In a prospective nested-case control study[212] including only lycopene, ß- and α-carotene, no association was found between quartiles of the sum of these carotenoids and risk of nonfatal myocardial infarction or death from coronary heart disease (CHD) among smoking and non-smoking males. In a study by Sahyoun et al.[211] it is not clear which carotenoids were included in the total carotenoids variable; they[211] found in a cohort of 725 elderly aged 60 year and over an inverse association between plasma carotenoid levels and 12 year all-cause mortality after adjustment for age, gender and serum cholesterol which was no longer significant when other potential confounders were controlled for. A nested-case control study also combining six individual carotenoids, reported significant inverse associations with upper aerodigestive tract cancer[203].

A possible protective effect of serum ß-cryptoxanthin and lutein on mortality as observed in our study is supported by three other observational studies. Howard et al.[202] found significantly lower plasma levels of ß-cryptoxanthin in a population living in an area with a high CHD incidence (Belfast) than in a population from a low CHD incidence area (Toulouse). In a cross-sectional study[39] significantly lower serum concentrations of ß-cryptoxanthin and lutein plus zeaxanthin were observed in cases with asymptomatic atherosclerosis (90th percentile of carotid intima media thickness) than in controls (below 75th percentile of carotid intima media thickness). For men with upper aerodigestive tract cancer lower levels of ß-cryptoxanthin were reported than for matched controls[203]. Although a mechanism is not specified yet, the suggestion of Howard et al.[202] that the oxygenated carotenoids ß-cryptoxanthin, lutein and zeaxanthin may be of special interest in preventing CHD is carefully supported by the results of the study of Irribarren et al.[39] and by our study. We observed an inverse trend (p=0.006) between the sum of the oxygenated carotenoids (including ß-

cryptoxanthin, lutein and zeaxanthin) and total mortality, which was higher than for the specific carotenoids. If oxygenated carotenoids would be especially important in CHD the strong association with total mortality in our study might be due to the large contribution of CHD to total mortality in Dutch elderly.

We studied all-cause mortality to cover the assumed associations of the different carotenoids on both CHD and cancer as main causes of total mortality. The possible protective effects of the carotenoids on different diseases may be due to common mechanisms like their antioxidant activity in vitro[213-215], although the effect in vivo is debated[216]. Besides a common protective mechanism of the individual carotenoids on a range of diseases, their differences in structure, metabolism, transport and tissue distribution[217,218] may explain differential effects on different diseases as hypothesized for lycopene on prostate cancer[200], lutein and zeaxanthin on degenerative macular eye disease, ß-carotene and α-carotene on specific cancers[199,203], and the oxygenated carotenoids ß-cryptoxanthin, lutein and zeaxanthin on CVD[39,202]. Unfortunately, we were not able to further explore cause-specific disease and mortality as no such data were available.

In our study ß-cryptoxanthin (26%), ß-carotene (31%) and lutein (25%) were the three main contributors to total carotenoids. In a study comparing individual carotenoids among elderly from cities in the USA and 10 European countries, it was seen that median serum concentrations of ß-carotene, lutein and ß-cryptoxanthin were mostly of similar magnitude as in our study[219]. Serum lycopene concentrations varied considerably among study populations. In our population lycopene concentrations were fourfold less than in the elderly populations from the USA, France, Ireland and Italy[202,219]. In these populations lycopene was the main contributor to serum total carotenoids. This resulted in higher absolute total carotenoid values than in our study. As lycopene is mainly derived from the intake of tomato and tomato products, differences in diet between these countries are probably responsible.

For cholesterol adjusted serum vitamin E we found lower concentrations in deceased men than in survivors. No significant increased risk or inverse association with total mortality was found after adjustment for major confounders. In line with other observational studies serum vitamin E in normal physiologic ranges is in general not strongly associated with mortality risk. The inverse associations found are usually due to intake of high-dosed vitamin supplements[50]. Our study was not influenced by vitamin E supplement use as only six subjects used vitamin E supplements and none of the elderly reported the use of specific carotenoids-containing supplements. The

antioxidant index results in our study give no support to the suggested interaction among carotenoids themselves and α-tocopherol[214,220]

The carotenoids we studied are mainly derived from the intake of fruit -and vegetables which is consistently inversely associated with CVD and cancer[221,222]. It can, of course, not be ruled out that the observed associations may be linked to other protective constituents or factors related to fruit- and vegetable intake[223]. Associations of specific carotenoids with specific diseases would be more suggestive of a possible real association with the carotenoids.

Some of the methodological points in our study to consider will be shortly discussed below. Serum concentrations were measured only once in 1991/1992 and possible changes over 6 years follow-up were not registered. We assumed in our analyses that the serum vitamin concentrations of 1991/1992 were indicative for serum concentrations during follow-up time supported by the findings that mean plasma concentrations of α-tocopherol, ß-carotene, α-carotene, lycopene, and lutein also measured in a subgroup of 77 subjects[129] in 1994 were similar with values in 1991/1992. Moreover, tertile classification of serum vitamins between the two time periods was concordant in 54-72% of the cases and for less than 10% of the cases the misclassification was more than one tertile.

Misclassification by a conscious shift in diet, altering serum concentrations, after diagnosis of risk factors or chronic diseases prior to baseline measurements may have occurred but is not thought to have highly influenced our results as excluding the first year of follow-up from analyses did not markedly change the observed associations (data not shown).

Finally, loss of carotenoids or α-tocopherol by storage for 6 years at a temperature of-80° is not likely[224,225]. Furthermore, laboratory personnel was unaware of the subjects status and sampling, storage and further handling of the sera was carried out identically, so bias in comparing deceased and censored subjects was excluded.

In conclusion, this study provides information on the six major serum carotenoids and combinations of them in relation to all-cause mortality risk and showed that the inverse association is especially profound for the sum of the oxygenated carotenoids (ß-cryptoxanthin, lutein and zeaxanthin). Research into the possible protective constituents of fruits- and vegetables intake should consider individual carotenoids and/or their combinations instead of focusing primarily on ß-carotene.

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8

General discussion

"The 'antioxidant vitamin-cardiovascular disease' hypothesis is still promising but still unproven: need for randomized trials[53]".

"Are clinical trials really the answer?[226,227]"

The studies reported in this thesis were designed to contribute to the accumulating research evidence that supplementation with the antioxidant vitamin E would be beneficial for health. The studies specifically focused on the groups of smokers and elderly hypothesized to be at risk for high oxidative stress.

Two intervention trials are the core of this thesis to formally test the causal effect of vitamin E on markers of atherosclerosis and immune response (chapters 3 to 6). In addition, two observational studies among populations abstaining from antioxidant supplements are described in chapters 2 and 7. These studies were designed to investigate associations between physiological (i.e. unsupplemented) levels of vitamin E and total mortality and carotid IMT levels, as a marker of atherosclerosis.

In this concluding chapter, the main findings of our studies (summarized in Table 8.1) are discussed in relation to other scientific evidence in this area. Methodological aspects that may interfere with the interpretation of these findings are discussed. Implications and directions for future research will be presented at the end of this chapter.

Main findings in relation to other scientific literature

The studies presented in this thesis do not provide unequivocal evidence in support of a beneficial effect of vitamin E supplementation on atherosclerosis and general health among the populations of smokers and elderly. However, as Table 8.1 reveals, the results show a consistent tendency towards benefit in specific cases. The size and

strength of this tendency will be discussed relative to other scientific evidence, taking into account study design, exposure- and outcome parameters.

Table 8.1 Main findings of the studies described in this thesis

Population	vitamin E	main findings
Observational studies		
158 CVD-free male smokers (*Chapter 2*)	low plasma and dietary levels	* no associations with increased IMT
638 male and female elderly people (*Chapter 7*)	low serum levels	* increased mortality risk (NS)
Randomized double-blind placebo-controlled intervention trials		
189 male smokers (*Chapter 3*)	2 year 400 IU	* tendency of reduced increase in IMT (NS) * significant decrease of *in vitro* susceptibility of LDL to oxidation
Population as in chapter 3: by GSTM1-genotype (*Chapter 4*)	as in chapter 3	*null vs. positive GSTM1 genotype:* * significant increased change in IMT * reduced prevalence of increased IMT by vitamin E supplementation (NS)
82 elderly people (*Chapter 5*)	3 months 100 IU	* significant decrease in oxidation of LDL-linoleic acid * significant increase in lagtime of in vitro oxidation of LDL
Population as in chapter 5 (*Chapter 6*)	as in chapter 5	* no significant effect on immune response

CVD =cardiovascular disease, IMT =intima media thickness of the common carotid artery, GSTM1-genotype=glutathione S-transferase genotype, NS =not statistically significant at the p=0.05 level.

Observational studies

The cross-sectional analysis among smokers, reported in chapter 2, revealed no association between low plasma levels of cholesterol-adjusted α-tocopherol and dietary vitamin E intake with intima media thickness (IMT) levels of the common

carotid artery. In line with several other studies[76,96-101], IMT was strongly associated with classical cardiovascular disease (CVD) risk factors such as smoking (more specifically depth of inhalation), plasma cholesterol levels, blood pressure, and age. Other mainstream cross-sectional comparison studies of physiological (i.e. unsupplemented) vitamin E levels on IMT similarly revealed no associations[39,103,105]. Overall, our findings in unsupplemented populations are in line with other observational studies that have also failed to find support for the hypothesized associations except for those highly specific cases such as for erythrocyte vitamin E levels and carotid IMT among elderly[102] and for women above 55 years in age in the Atherosclerosis Risk in Community (ARIC) study[105].

The prospective study, described in chapter 7, identified lower age- and serum cholesterol adjusted serum levels of α-tocopherol among deceased men compared to survivors. After seven years of follow-up, gender adjusted mortality risk was 0.70 (95% confidence interval CI 0.48-1.04) for the highest compared to the lowest tertile of serum α-tocopherol. After adjustment for major CVD risk factors, the mortality risk increased to 0.90 (95% CI 0.61-1.35). These findings are in line with all-cause mortality risk point estimates for both supplemented and unsupplemented vitamin E studies, although studies involving vitamin E supplementation generally report stronger effects (see Table 8.2 for details). In the Nurses Health Study[49] all-cause mortality risk for vitamin E supplement users was 0.87 (95% CI 0.69-1.10), in the EPESE study[51] relative risk was RR=0.66 (95% CI 0.53-0.83) and RR=0.73 (95% CI 0.58-0.91) after CVD risk factor adjustment. In the Massachusetts study[211], mortality risk for high versus low dietary vitamin E intake was RR=0.77 (95% CI 0.51-1.18) and RR=0.99 (95% CI 0.58-1.27) for high versus low plasma levels. Despite the fact that total mortality risk is clearly a critical marker of general health, in the context of this thesis it would have been preferable to assess cause-specific CVD-mortality rates. Unfortunately, data were not available for this detailed analysis.

The intervention trials described in this thesis specifically focus on vitamin E and atherosclerosis. As the strength and consistency of prospective studies on vitamin E and CVD morbidity and mortality[49-52,141,211,228,229] have been sufficiently convincing to justify the initiation of formal intervention trials, such as reported in this thesis, these prospective studies will be discussed briefly. Overall, these studies (see Table 8.2) show a remarkably consistent (albeit not always statistically significant) pattern. Most point estimates of relative risks range between 0.60 and 0.80 implying a 20 to 40% risk reduction in cardiovascular events and mortality. This effect on cardiovascular

Table 8.2 Associations of vitamin E intake and plasma levels and relative risk (RR) of total mortality and cardiovascular disease (CVD) in prospective cohort studies

Study[literature reference] and population	vitamin E[a]	CVD events	Study period	RR (95% confidence intervals)[b]	
Health Professional Study[50]	food and	667 events[c]	1986-1990	0.79 (0.54-1.15)[c]	
39,910 US male health professionals, 40-75 yr.	supplement			0.70 (0.55-0.89)[d]	
	Q5->Q1			0.63 (0.47-0.84)	Supplement > 100 IU, >= 2 yr.
Nurses Health Study[49]	food and	552 events	1980-1988	0.95 (0.72-1.23)[c]	
87,245 US female nurses, 34-59 yr.	supplement			0.57 (0.41-0.78)[d]	
	Q5->Q1			0.59 (0.38-0.91)	Supplement >= 2yr
Basel prospective study[228]	plasma	132 deaths	1971-1985	No association	Data not quantitated
2,974 men, mean age 51 yr.					
Finnish Mobile Clinic Study[141]	food	244 deaths	1966-1984	0.68 (0.42-1.11)	men (trend p=0.01))
5,133 Finnish males and females,30-69 yr.	T3->T1			0.35 (0.14-0.88)	women (trend p< 0.01)
IOWA Women's Health Study[52]	food and	242 deaths	1986-1992	0.38 (0.18-0.80)[c]	
34,486 postmenopausal women,55-69 yr.	supplement			1.09 (0.67-1.77)[d]	
	Q5->Q1				
EPESE[51]	supplement	3490 total deaths	1984-1993	0.73 (0.58-0.91)[e]	all-cause
11,178 elderly, 67-105 yr.		1101 CHD deaths		0.59 (0.37-0.93)[e]	CHD
Meyer et al[238]	supplement	100 events	1985-1991	0.21[e]	crude RR
2,313 Quebec city men					
Zutphen study[239]	food	42 events	1970-1985	1.64 (0.54-4.97)[c]	
552 men aged 50-69 yr.	T3 -> T1				
Massachusetts[211]	food and	155 total deaths	1981-1993	0.83 (0.54-1.27)[c]	all-cause
725 elderly > 60 yr.	plasma	75 CHD deaths		0.75 (0.41-1.39)[c]	CHD
	Q5->Q1			1.07 (0.62-1.86)[f]	all-cause
				1.51 (0.68-3.37)[f]	CHD
Rotterdam study[229]	food	124 events	1990-1996	1.07 (0.67-1.73)[c]	
4,802 elderly males and females, 55-95 yr.	T3-> T1				

[a] Q5->Q1: comparison of subjects in the highest quintile of vitamin E intake or plasma level with those in the lowest quintile.
T3->T1: comparison of subjects in the highest tertile of vitamin E intake or plasma level with those in the lowest quintile.

[b] RRs are given adjusted for multiple cardiovascular disease risk factors unless stated otherwise [c] intake from foods (no supplements), [d] intake from supplements only, [e] comparison of vitamin E supplement users with those using no supplements, [f] plasma levels of vitamin E

risk reduction is clearly more pronounced for long-term users of high dosed vitamin E supplements.

Intervention trials of vitamin E and markers of atherosclerosis

Among both smokers (chapter 3) and elderly (chapter 5) vitamin E in LDL and plasma increased significantly upon supplementation with 400 IU for 2 years and 100 IU for 3 months, respectively. In both studies supplementation reduced the susceptibility of low density lipoprotein (LDL) cholesterol to in vitro oxidation. This finding is consistent with the vast majority of other vitamin E supplementation trials with doses starting from as low as 25 IU[44,45,124,147,148,230]. Assessment of the clinical relevance of these findings is however difficult. Despite the plausibility of a pivotal role for LDL oxidation in the onset and progression of atherosclerosis, the validity of the in vitro Cu^{2+}-induced oxidation of LDL as a marker of in vivo oxidation and increased CVD risk is far from unambiguous (see paragraph on methodological consideration). Yet, the in vitro LDL-oxidation measure has considerably progressed the understanding of determinants of LDL's susceptibility to oxidation such as several antioxidants, fatty acid composition of LDL[231] (chapter 5) and density of LDL[232,233]. This is confirmed by our finding (chapter 5) that the oxidation susceptibility of LDL depends on the ratio of α-tocopherol to linoleic acid in LDL.

The progression of carotid IMT is recognized as a good marker for CVD risk (see methodological considerations: use of surrogate endpoints for atherosclerosis). The trial among smokers described in chapter 3 revealed a non-significant 47% reduction in carotid IMT in a randomized trial after 2 years supplementation with 400 IU vitamin E. This is in line with results from the only comparable study that is presently available (presented by J. Salonen at the 1999 Conference of the European Society of Cardiology (ESC) in Barcelona). That placebo controlled trial applied a two by two factorial design with 200 mg vitamin E and 500 mg slow release vitamin C for three years. Smokers receiving only vitamin E showed a non-significant reduction in carotid IMT progression very similar to our result of 47%. This finding appeared not to generalize to the population of women or to non-smokers. The observed significant reduction in carotid IMT progression in the group receiving both vitamin E and C led the investigators to the conclusion that slow release vitamin C supplementation is additionally required to enable beneficial effects of vitamin E. Support for this contention can be found in in-vitro studies that have identified regenerating effect of vitamin C on oxidized vitamin E. Assessment of the clinical

Table 8.3 Effect of vitamin E supplementation on clinical endpoints of cardiovascular disease in primary and secondary large- scaled randomized double-blind placebo-controlled trials.

Study[literature reference]	Population, design and study period	Supplementation[a]	events	RR (95% confidence intervals)[b]
Alpha-Tocopherol	29,1333male smokers,50-69 yr.	50 mg vitamin E		**Vitamin E vs. no vitamin E[c]**
Beta-Carotene	primary prevention trial	20 mg ß-carotene	3570 total deaths	1.02 (0.91-1.05)
Prevention Study[54]	2*2 factorial		1239 IHD-deaths	0.95 (0.85-1.06)
	1985-1993		110 hemorrhagic stroke deaths	1.50 (1.04-2.2)
			123 ischemic stroke deaths	0.84 (0.59-1.19)
			251 other CVD deaths	1.06 (0.75-1.50)
ATBC[240]	22,269 male smokers, free of CHD	See above		**Vitamin E vs. no vitamin E**
			1983 new cases of angina pectoris	0.91 (0.83-0.99)
ATBC[55]	1862 male smokers, with previous MI	See above		**Vitamin E vs. placebo**
			424 major coronary events:	0.90 (0.67-1.22)
			190 nonfatal-MI	0.62 (0.41-0.96)
			234 CHD-mortality	1.33 (0.86-2.05)
ATBC[237]	27,271 male smokers, with no MI history	See above		**Vitamin E vs. no vitamin E**
			2111 major coronary event:	0.96 (0.88-1.04)
			1204 nonfatal-MI	
			907 fatal CHD	0.92 (0.81-1.05)
CHAOS[56,241-243]	Secondary prevention	537 mg vitamin E		**Vitamin E vs. placebo**
	2002 CHD-patients	(n=546)	62 total deaths	1.25 (0.76-2.04)
		268 mg vitamin E	105 CV-deaths + nonfatal MI	0.53 (0.34-0.83)
		(n=489)	55 nonfatal MI	0.23 (0.11-0.47)
			50 CV-deaths	1.18 (0.62-2.27)

Study[literature reference]	Population, design and study period	Supplementation[a]	events	RR (95% confidence intervals)[b]	
GISSI-Prevenzione[57]	11,324 survivors MI Secondary prevention 2*2 factorial 1993-1999	300 mg vitamin E 1 g n-3 poly unsaturated fatty acids		**vitamin E vs. no vitamin E**	
			Total mortality	0.92 (0.82-1.04)	
			Death+nonfatal MI+stroke	0.95 (0.86-1.05)	
			CV-deaths+nonfatal MI+stroke	0.98 (0.87-1.10)	
			CV deaths	0.94 (0.81-1.10)	
				vitamin E vs. placebo	
			Total mortality	0.86 (0.72-1.02)	
			Death+nonfatal MI+stroke	0.89 (0.77-1.03)	
			CV-deaths+nonfatal MI+stroke	0.88 (0.75-1.04)	
			CV deaths	0.80 (0.65-0.99)	
HOPE[58]	9,541 men and women at increased CVD risk 2*2 factorial 1995-1999	400 mg vitamin E 10 mg ramipril (ACE-inhibitor)		**vitamin E**	**no vitamin E[d]**
			nonfatal MI + stroke	16%	15.4%
			CHD-deaths	7.1%	6.8%
			Stroke	4.4%	3.8%

CVD = cardiovascular disease, IHD = ischaemic heart disease, MI = myocardial infarction, CHD = coronary heart disease, CV = cardiovascular

[a] In this Table 1 mg vitamin E =1 mg dl-α-tocopheryl acetate= 1 IU vitamin E

[b] RRs are given adjusted for multiple cardiovascular risk factors unless stated otherwise. In factorial designed studies comparisons are made for subjects receiving vitamin E vs. no vitamin E unless there is a statistical interaction between supplementation groups. Both vitamin E vs. no vitamin E and vitamin E vs. placebo are given if they show markedly differences in outcome.

[c] Crude relative risks (RR) and 95% CI calculated from mortality rates given in Albanes et al. [54] where 95% CI is calculated as $e^{\ln(RR) \pm 1.96\ SE}$.

[d] No significant differences between percentage of events between vitamin E and those not receiving vitamin E. Not published results as presented at the 1999 Congress of the European Society of Cardiology in Barcelona.

relevance of a non-significant reduction in IMT progression as found for vitamin E supplementation in smoking men in our trial (chapter 3) and the trial by Salonen et. al (1999 ESC-congress in Barcelona) is complicated by ambivalent findings from large intervention trials on vitamin E and clinical hard-endpoints. Detailed information on results from these trials can be found in Table 8.3. In short, significant beneficial effects on non-fatal but not fatal MI have been reported for 50 IU vitamin E supplementation among smokers with previous MI[55] and for 400 and 800 IU in CVD patients[56]. MI survivors showed no reduction upon 300 mg vitamin E on the combined endpoint of death, non-fatal MI and non-fatal stroke but did show significant reductions in cardiovascular deaths especially sudden deaths[57]. Similarly, results from the HOPE trial[58] among 9,541 patients at high risk for cardiovascular events (presented at 1999 ESC-congress in Barcelona) showed no reduction in incidence of MI, strokes, or cardiovascular deaths after 4.5 year supplementation with 400 IU vitamin E. However, the vitamin E part of this trial will be continued to assess longer term effects of vitamin E on vascular disease[58]. All together, the ambivalent results from these trials do not yet justify a sound conclusion on the effects of vitamin E in the prevention of CVD. Overall, they have failed to identify a beneficial effect of vitamin E in doses ranging from 50 to 400 IU and follow-up periods up to 8 years.

To further assess the relevance of the non-significant reduction in progression of IMT among smokers as observed in our main study, we hypothesized that possible beneficial effects of vitamin E on atherosclerosis may be more pronounced in specific sub-populations at multiple risk for oxidative stress. Therefore, the results of the main trial described in chapter 3 were further differentiated by genetic predisposition. Specifically, smokers with the null- genotype for glutathione S-transferase μ (GSTM1), thus lacking the detoxifying GSTM1 enzyme were compared to smokers with the positive genotype (chapter 4). Indeed, vitamin E supplementation reduced the progression of specific IMT sites among those with the GSTM1-null genotype, but not for those with the positive genotype. At present there are no comparable studies on GSTM1 polymorphism and atherosclerosis and its interaction with vitamin E. If further studies confirm our results, GSTM1 genotype may importantly improve the predictive value of the relation between smoking and CVD. Furthermore, mechanistically it may provide more insight into the potential role of vitamin E on CVD risk for populations subject to increased oxidative stress.

Intervention trial of vitamin E and immune response

The results from the intervention described in chapter 6 showed no effect of 100 IU vitamin E supplementation on the humoral and cellular immune response among elderly. This may partly be due to the relatively low vitamin E dose applied and the selection of a healthy elderly population. This is supported by concurrent vitamin E intervention trials among healthy elderly[61,196] reporting significant positive effects for doses of 800 mg vitamin E applied for 30 days[61] and doses of 200 mg (but not 60 or 800 mg) applied for 4.5 months[196]. Another trial on immunological parameters[69] among healthy elderly applying supplementation of 50 and 100 mg for six months, only showed beneficial effects for 100 mg vitamin E among those elderly in a sub-optimal state of health. New trials investigating the effect of vitamin E on reduction of intensity and incidence of infection are currently ongoing and should hopefully provide insight into the clinical relevance of vitamin E induced improvements on immune response. These results have to be awaited before recommendations on increased intake of vitamin E among healthy elderly people would be justified.

Methodological considerations

Epidemiological studies are susceptible to several forms of bias. Precision and validity of the studies presented in this thesis will be discussed to the extent that they might have influenced the interpretation of the results. Specific attention will be paid to the use of surrogate endpoints or markers for atherosclerosis and CVD risk, study power, selection bias, confounding and information bias.

Use of surrogate endpoints for atherosclerosis

The use of surrogates of clinical manifestations of CVD such as oxidation of LDL (chapter 3 and 5) and progression of carotid IMT (chapters 2, 3 and 4) provides valuable information on mechanisms and/or biological efficacy of preventive interventions among humans without the need for very large-scale studies or long-term follow-up. Such more efficient study designs would in turn allow for more flexibility in the testing of different doses and duration. That information would provide extremely useful input into the design of large-scale trials on vitamin E and CVD endpoints.

The disadvantage of some intermediate endpoints is that they are often applied prior to unequivocal confirmation of their predictive value for the clinical endpoint purport to measure. At later state, they may then turn out not to be as valid a marker

as originally anticipated. The in-vitro susceptibility of LDL to oxidation[28] was used in the trials among elderly (chapter 5) and smokers (chapter 6). Although some studies have revealed increased in-vitro susceptibility of LDL to oxidation among CVD patients[31,32], just as many studies fail to demonstrate such confirmation[36,39-41]. Also in our vitamin E trial among smokers (described in chapter 3) the 2 year change in susceptibility to oxidation of LDL was not associated with the change in carotid IMT. Yet, the hypothesized role of oxLDL in the atherosclerotic process still finds strong support and a marker that accurately mimics the situation of in vivo LDL oxidation would be of great importance. At the moment auto-antibodies against oxLDL are being applied as an in vivo marker for oxidation of LDL and associations with cardiovascular events have been promising[30,33-35], but not conclusive.

Progression of the intima media thickness (IMT) of the common carotid artery as measured by B-mode ultrasound constitutes a good marker for atherosclerosis. It is a noninvasive, harmless and highly reproducible[89,90] method that can be applied to general populations in large-scale epidemiological studies. Reproducibility data of the IMT measurements, as applied in chapters 2 to 4, showed between- and within variation lower than 5% for the combined left and right CCA far and near wall[90]. The mean difference (SD) between observers was 0.006 mm (0.03) and 0.006 (0.02) mm within observers. Measurement error of IMT tended to increase with increasing levels of IMT[90], which is in line with reports by others[128]. The reproducibility study[90] among subjects with an increased IMT (> 1.1 mm) also revealed that the between-observer variation was clearly larger than the within-observer variation. For that reason, in our IMT trial (described in chapters 3 and 4) an effort was made to have the same ultrasonographer scanning the subjects before and after the two year intervention. The use of a combination of sites at the common carotid artery such as near wall and far wall at the right and left site was used in estimating progression of IMT in the studies described in chapter 2, 3 and 4. Such combination of sites (assuming similar precision and validity in measurement for individual sites) will reduce the variability considerably, leading to increased precision[132].

The validity of using IMT as a predictive marker for CVD is supported by an increasing number of studies showing positive associations between absolute levels of IMT with generalized atherosclerosis[91], and with peripheral-[92], cerebro- and cardiovascular disease[93-95]. Moreover, the clinical relevance of progression of IMT as a marker for CVD has been reported[117]. This allows for direct comparison to other studies in the (qualitative) assessment of the clinical relevance of our intervention effect of 0.014 mm reduction in progression of CCA-IMT (chapter 2) and 0.022 mm

reduction among smokers with the GSTM1-0 genotype (chapter 4). The 8.8 years follow-up of the cholesterol lowering atherosclerosis study[117] reported a relative risk of 3.1 (95% CI [2.1-4.5]) on any coronary event for every 0.03 mm/year progression in IMT Also, in a cross sectional study Bots et al.[95] reported a relative risk of 1.34 for stroke and 1.25 for MI for every additional difference of 0.16 mm in CCA-IMT after adjustment for major cardiovascular risk factors.

Together, these findings support our use of measurement of progression carotid IMT as a valid and reproducible marker for progression of atherosclerosis. Moreover, the extent of reduction in progression of carotid IMT may indicate substantial clinical relevance.

Study power

Despite the fact that our intervention trial on vitamin E showed approximately the anticipated relevant 50% reduction in progression of IMT among general smokers (chapter 2) and even stronger in smokers with GSTM-1 null genotype (chapter 3), this result did not reach statistical significance. Study power problems warrant cautious interpretation of the lack of a significant effect. It turned out that the spontaneous 2-year progression of 0.030 mm in the placebo group and the absolute difference in progression between vitamin E and placebo group was smaller than was anticipated on the basis of the limited information available at the start of the trial[77,126]. Also, the fact that high IMT baseline values, such as in our high risk groups of lifelong male smokers, have higher levels of measurement error associated with them[128] has further contributed to the lower than expected power of our study.

Selection bias

"Selection bias are distortions that result from procedures used to select subjects and from factors that influence study participation"[234]. The common element of such biases is that the relation between exposure and disease is different for those who participate and those who should be theoretically eligible for study, including those who do not participate.

Such selection bias is not thought to be a problem in the observational studies described in chapters 2 and 7. It is not conceivable that the selection (and self-selection) was related to the association between plasma levels of vitamins and IMT levels or total mortality during seven year follow-up. Selective dropout was not a problem in the double-blind randomized vitamin E intervention trials described in

chapters 3 to 6 as such drop out proved equally distributed across vitamin E and placebo groups.

On the other hand, it is quite likely that the elderly and smokers participating in the described studies constitute a selective population, which may have consequences for the generalizability or external validity of the findings. In general, people participating in health surveys or intervention trials may be more health conscious than the eligible non-responders. The effect of a selective and possibly more healthy population of smokers (chapter 2 to 4) may have reduced the strength of reported effects, particularly if the hypothesized effects are stronger among higher risk sub-populations. The results of a non-response study comparing 534 non-responders with 73 responders revealed no significant differences in age, history or presence of CVD, physical activity, demographic characteristics and subjective health score (chapter 2). However, non-responders in this study differed from responders mainly in their smoking behavior. Another selection problem among lifelong smokers may be due to natural selection. Such natural selection may have influenced the generalizability of our findings to the total population of smokers. On the other hand, especially peripheral vascular disease is hypothesized to be an especially relevant disease among healthier smokers as those with other risk factors such as hypercholesterolaemia or hypertension are likely to have diseased at an earlier age[235].

In conclusion, for the intervention trials described in this thesis the influence of bias on internal validity is unlikely because of an appropriate double-blind randomized placebo-controlled study design. However, our study population of elderly and smokers may constitute a healthy sub-populations of the respective source populations, affecting generelazibility (external validity). If this has affected the results of our intervention trials it is conceivable to have possibly attenuated the findings.

Information bias

Observational studies as described in chapters 2 and 7 are sensitive to information bias causing misclassification of subjects according to exposure or outcome. In our studies potential problems regarding differential misclassification, when the proportion of subjects misclassified on exposure depends on disease status or vice versa, were dealt with either prior to (chapter 2) or within the data analysis (chapter 7). Specifically, in the study on increased IMT (chapter 2), differential misclassification of subjects on smoking characteristics and vitamin E levels was prevented by studying only healthy CVD-free smokers thus excluding those smokers that might have altered their

smoking or dietary habits as a consequence of CVD or CVD medication use. In the prospective study on antioxidants and total mortality (chapter 7) differential misclassification cannot be fully ruled out as prior to baseline measurements subjects may have consciously changed their diet, altering their serum concentrations of antioxidants, in response to a diagnosis of risk factors or chronic disease However, as excluding the first years of follow-up did not markedly change the observed associations, this is unlikely to have markedly influenced the results of our study.

In the prospective study on total mortality (chapter 7) serum levels of vitamin E were classified in tertiles. Serum vitamin levels were measured only once at baseline and nondifferential misclassification of the tertiles of serum vitamin E may have occurred. Bias from independent nondifferential misclassification is usually in the direction of the null value, which is diluting the possible effect[234]. However, when more categories are involved the bias may actually be away from the null value. We checked the seriousness of this effect by measuring the serum vitamin E levels in a sub-sample of our population of elderly halfway through the follow-up and classifying them into tertiles again. Tertile classification between baseline and halfway through was concordant in 72% of the cases and for only 5% of the cases the misclassification was more than one tertile. Hence, such misclassification will not have seriously influenced our results.

In conclusion, information bias was appropriately addressed in the studies described in this thesis and is unlikely to have played a substantial role in the interpretation of the reported findings.

Confounding

The effect of an exposure on outcome may be biased by a confounding factor when this factor is a risk factor for the outcome and is associated with the exposure under study in the source population and is not in itself an intermediate step in the causal path between the exposure and the outcome[234]. In the observational and intervention studies described in this thesis potential confounding was appropriately addressed during the data analysis phase by adjusting for potential confounders in multivariate analyses.

Lipid profile is a potential confounder in the observational studies (chapters 2 and 7) on plasma vitamin E and increased IMT and total mortality, respectively. After all, plasma levels of vitamin E are highly correlated with plasma cholesterol levels. To account for this potential collinearity problem, in the multivariate analyses plasma vitamin E was adjusted for plasma cholesterol by calculating their residuals from

linear regression models with plasma cholesterol as the dependent variable. In the trials on vitamin E and immune response (chapter 6) and vitamin E and progression of IMT (chapters 3 and 4) initial values on the outcome variables of cellular immune response and IMT were found to differ between vitamin E and placebo groups. To guard against potential confounding on the effect measurements, initial values were adjusted for in the multivariate analyses.

In retrospect, this unexpected issue would preferably have been addressed in the design phase rather than the analysis phase, by randomizing within levels of initial values (block randomization)[234]. In the IMT trial (chapter 3) baseline differences might have been prevented against at some additional costs arising from extra IMT measurements prior to randomization and prolonged recruitment period. In the immune response trial (chapter 6) the likelihood of fully successful randomization might similarly have been increased by a further increase in the number of subjects[234]. However, in this study the issue of block randomization is slightly more complex due to a technical restriction in the analytical phase. To reduce the inter-assay variation inherent to proliferative response of immune cells, each subject's pre- and post-intervention samples need to be analyzed simultaneously in a single run. Hence, block randomization on pre-intervention sample values was infeasible, as it would introduce considerable inter-assay variation that, in turn, would seriously undermine the efficiency of the block randomization.

Overall, we believe that effects of confounding were adequately accounted for in the data-analysis phase of our studies. However, to be fully confident about this, in future applications it would be recommendable to anticipate on these unexpected effect during the design and resourcing phases (time and money) of these studies, whenever feasible.

Conclusion and implications

Based on new understanding of the potential mechanistic role of oxidants in development and clinical expression of coronary heart disease, in 1991, a group of leading experts in the field sat together in a workshop to discuss the role and directions for future trials on antioxidants in the prevention of human atherosclerosis[154]. Consensus conclusion of this workshop was that "the available evidence justified clinical trials of natural antioxidants (associated with no increase in risk) but that clinical trials with antioxidant drugs (which might carry deleterious side effects) should be deferred until more knowledge is available". Today, over 8 years later, from the accumulating studies including our own, it has to be concluded that

there is still limited evidence in favor of beneficial effects of vitamin E supplementation on immune response and atherosclerosis in smokers and elderly.

Our studies did not show an improvement in immune response among elderly upon vitamin E supplementation. In other studies[61,69,196] such beneficial effects have been reported for doses higher than that applied in our study and in populations of less healthy elderly. Overall, the results are still mixed and the outcomes of ongoing trials on the effect of vitamin E supplementation on incidence and severity of infectious diseases will have to be awaited before sound conclusions are justified regarding the desirability of a change in recommendation for vitamin E intake in elderly populations.

The other trials described in this thesis are similarly not unequivocally supportive for a general beneficial effect of vitamin E supplementation on atherosclerosis among smokers and elderly. Modest support for a beneficial effect was only found in the very specific case of a hypothesized low antioxidant defense among smokers lacking the detoxifying enzyme activity of GSTM-1. The observed beneficial effects of vitamin E supplementation on oxidation susceptibility found in both smokers and elderly is very difficult to interpret as the relevance of these findings to the in vivo situation has yet to be established.

The evidence that vitamin E may reduce risk of coronary heart disease mainly comes from prospective cohort studies and is reasonably strong and consistent. However, these results find only limited support from (recent) primary and secondary randomized trials. There may be a role in prevention of non-fatal myocardial infarction in those with coronary heart disease[55,56]. Several randomized trials including a vitamin E arm are still ongoing and the results will be available in the near future. Whether these trials will add to the inconsistency of results or will provide conclusive results regarding the potential beneficial effect of vitamin E will have to be awaited.

Future directions

Vitamin E and cardiovascular disease

Except for the specific cases of smokers with the GSTM1-0 polymorphism (this thesis, chapter 4) or for nonfatal MI among CVD patients, the hypothesis of a beneficial effect of vitamin E on CVD in general is not supported by the studies in this thesis nor by currently available results from large primary and secondary prevention trials. Several prevention trials on vitamin E and clinical endpoints are ongoing and will hopefully shed more light on the inconsistent results found so far. At the moment,

prior to the availability of these results initiation of new clinical trials on vitamin E and CVD endpoints is not warranted.

In the meantime, future research may more meaningfully be directed towards the concept of biomarkers[236] of lipid peroxidation and oxidative damage to the vessel walls to establish the significance and optimal intake of vitamin E. Furthermore, synergistic effects of other nutrients, such as the hypothesized regenerating effect of vitamin C on oxidized vitamin E, can be studied.

After all, the role of in vivo LDL lipid peroxidation in atherosclerosis is widely accepted and the importance of vitamin E as an in vivo antioxidant strongly suggests a possible role of vitamin E in the atherosclerotic process. This role is probably more subtle and specific than hypothesized at the start of our studies and other large clinical trials. A major complication in finding a biomarker for LDL lipid peroxidation in vivo is that it occurs within the vessel wall. Considerable resource (time and money) should be invested for further improvement of measurement and identification of new and better markers. Serious candidates that need further development and validation are isoprostanes (general marker for lipid peroxidation) and levels of circulating antibodies against oxidized LDL.

Research should also be directed towards further validation and refinement of the measurement of IMT progression and other easy to administer relevant markers for preclinical atherosclerosis suitable to administer in large prevention trials. In this respect, next to ultrasound measurements recent advances in imaging technology have revealed promising methods such as positron emission tomography, magnetic resonance imaging and ultrafast computed tomography which are potential methods to identify early functional and structural vascular changes[237]. Such measures can not only play a particularly important role in the validation of new biomarkers applied in future 'short-term' human experiments but also provide a way to test potential preventive agents on a preclinical stages of atherosclerosis in high risk asymptomatic subjects.

All together these directions should provide the platform from which to decide the necessity and design of future large-scale clinical trials on optimal vitamin E levels, and ideal combinations with synergistic nutrients and in groups that may particularly benefit from vitamin E supplementation.

Vitamin E and immune response

The study described in this thesis does not provide support for a beneficial effect of vitamin E on parameters of the immune response in elderly people. However, studies

applying higher doses and in elderly with a sub-optimal health status suggest that such effects may be real. This, together with the clinical relevance of age-induced decline of immune parameters warrants future trials. Actually, some of such trials on the effect of supplementation of vitamin E on incidence and severity of respiratory infections in elderly are already ongoing (Graat, Wageningen University The Netherlands and Meydani, Tufts University USA). These well-designed and large-scale studies will provide more definite evidence on the role of vitamin E supplementation on infectious diseases.

References

1. Diplock AT. Will the 'good fairies' please prove to us that vitamin E lessens human degenerative disease? *Free Radic Res* 1997;26:565-583.
2. Mason KE. The first two decades of vitamin E. *Fed Proc* 1977;36:1906-1910.
3. Farrell PM. Human health and disease. Deficiency states, pharmacological effects, and nutrient requirements. **In:** *Vitamin E. A comprehensive treatise*.Machlin LJ (ed). New York and Basel, Marcel Dekker, INC., 1980, pp 520-620.
4. Goldstein JL, Brown MS. The low-density lipoprotein pathway and its relation to atherosclerosis. *Ann Rev Biochem* 1977;46:897-930.
5. Brown MS, Goldstein JL. Scavenger cell receptor shared. *Nature* 1985;316:680-1:
6. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol: Modifications of LDL that increase its atherogenicity. *New Eng J Med* 1989;320:915-924.
7. Witztum JL. The oxidation hypothesis of atherosclerosis. *Lancet* 1994;344:793-795.
8. Tengerdy RP. Effect of vitamin E on immune response. **In:** *Vitamin E. A comprehensive treatise*.Machlin LJ (ed). New York and Basel, Marcel Dekker, INC, 1980, pp 429-444.
9. Meydani M. Vitamin E. *Lancet* 1995;345:170-175.
10. Traber MG, Packer L. Vitamin E: beyond antioxidant function. *Am J Clin Nutr* 1995;62:1501S-1509S.
11. Weksler ME, Hutteroth TH. Impaired lymphocyte function in aged humans. *J Clin Invest* 1974;53:99-104.
12. Burns EA, Lum LG, Seigneuret MC, Giddings BR, Goodwin JS. Decreased specific antibody synthesis in old adults: decreased potency of antigen-specific B cells with aging. *Mech Ageing Dev* 1990;53:229-241.
13. Makinodan T. Patterns of age-related immunologic changes. *Nutr Rev* 1995;53:27S-34S.
14. Gutteridge JCM, Halliwell B. Oxidative stress. **In:** *Antioxidants in nutrition, health, and disease*. Oxford New York Tokyo, Oxford University Press, 1994, pp 90-110.
15. Cohn W, Gross P, Grun H, Loechleiter F, Muller DPR, Zulauf M. Tocopherol transport and absorption. *Proc Nutr Soc* 1992;51:179-188.
16. Reccommended dietary allowances, 10th ed. *NRC-National Academy of Sciences* 1989;p. 285.
17. Diplock AT. Safety of antioxidant vitamins and beta-carotene. *Am J Clin Nutr* 1995;62:1510S-1516S.
18. Garewal HS, Diplock AT. How 'safe' are antioxidant vitamins? *Drug Safety* 1995;13:8-14.
19. Bendich A, Machlin LJ. Safety of oral intake of vitamin E. *Am J Clin Nutr* 1988;48:612-619.
20. Meydani SN, Meydani M, Blumberg JB, Leka LS, Pedrosa M, Diamond R, Schaefer EJ. Assessment of the safety of supplementation with different amounts of vitamin E in healthy older adults. *Am J Clin Nutr* 1998;68:311-318.
21. Tsai AC, Kelley JJ, Peng B, Cook N. Study on the effect of megavitamin E supplementation in man. *Am J Clin Nutr* 1978;31:831-837.
22. Farrell PM, Bieri JG. Megavitamin E supplementation in man. *Am J Clin Nutr* 1975;28:1381-1386.

23. Palinski W, Rosenfeld ME, Ylä-Herttuala S, Gurtner GC, Socher SS, Butler SW, Parthasarathy S, Crew TE, Steinberg D, Witztum JL. LDL undergoes oxidative modification in vivo. *Proc Natl Acad Sci USA* 1989;86:1372-1376.
24. Ylä-Herttuala S, Palinski W, Butler SW, Picard S, Steinberg D, Witztum JL. Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL. *Arterioscler Thromb* 1994;14:32-40.
25. Kugiyama K, Kerns SA, Morrisett JD, Roberts R, Henry PD. Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low density lipoproteins. *Nature* 1990;344:160-162.
26. Triau JE, Meydani SN, Schaefer EJ. Oxidized low density lipoprotein stimulates prostacyclin production by adults human vascular endothelial cells. *Arteriosclerosis* 1988;8:810-818.
27. Ylä-Herttuala S, Palinski W, Rosenfeld ME, Parthasarathy S, Carew TE, Butler S, Witztum JL, Steinberg D. Evidence for the presence of oxidatively modified LDL in atherosclerotic lesions of rabbit and man. *J Clin Invest* 1989;84:1086-1095.
28. Esterbauer H, Striegl G, Puhl H, Rotheneder M. Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Rad Res Comms* 1989;6:67-75.
29. Halliwell B. Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. *Br J Exp Pathol* 1989;70:737-757.
30. Maggi E, Chiesa R, Melissano G, Castellano R, Astore D, Grossi A, Finardi G, Bellomo G. LDL oxidation in patients with severe carotid atherosclerosis. A study of in vitro and in vivo oxidation markers. *Arterioscler Thromb* 1994;14:1892-1899.
31. Regnström J, Nilsson J, Tornvall P, Landou C, Hamsten A. Susceptibility to LDL oxidation and coronary atherosclerosis in man. *Lancet* 1992;339:1183-1186.
32. Halevy D, Thiery J, Nagel D, Arnold S, Erdmann E, Hofling B, Cremer P, Seidel D. Increased oxidation of LDL in patients with coronary artery disease is independent from dietary vitamins E and C. *Arterioscler Thromb Vasc Biol* 1997;17:1432-1437.
33. Salonen JT, Ylä-Herttuala S, Yamamoto R, Butler S, Korpela H, Salonen RM, Nyyssönen K, Palinski W, Witztum JL. Autoantibody against oxidised LDL and progression of carotid atherosclerosis. *Lancet* 1992;339:883-887.
34. Chiesa R, Melissano G, Castellano R, Astore D, Marone EM, Grossi A, Maggi E, Finardi G, Casasco A, Bellomo G. In search of biological markers of high-risk carotid artery atherosclerotic plaque: enhanced LDL oxidation. *Ann Vasc Surg* 1998;12:1-9.
35. Orchard TJ, Virella G, Forrest KY, Evans RW, Becker DJ, Lopes VM. Antibodies to oxidized LDL predict coronary artery disease in type 1 diabetes: a nested case-control study from the Pittsburgh Epidemiology of Diabetes Complications Study. *Diabetes* 1999;48:1454-1458.
36. Croft KD, Dimmitt SB, Moulton C, Beilin LJ. Low density lipoprotein composition and oxidizability in coronary disease. Apparent favourable effect of beta blockers. *Atherosclerosis* 1992;97:123-130.
37. Virella G, Virella I, Leman RB, Pryor MB, Lopes VM. Anti-oxidized low-density lipoprotein antibodies in patients with coronary heart disease and normal healthy volunteers. *Int J Clin Lab Res* 1993;23:95-101.
38. van de Vijver LP, Steyger R, van Poppel G, Boer JM, Kruijssen DA, Seidell JC, Princen HM. Autoantibodies against MDA-LDL in subjects with severe and minor atherosclerosis and healthy population controls. *Atherosclerosis* 1996;122:245-253.
39. Iribarren C, Folsom AR, Jacobs DR, Gross MD, Belcher JD, Eckfeldt JH. Association of serum vitamin levels, LDL susceptibility to oxidation, and autoantibodies against

MDA-LDL with carotid atherosclerosis. A case control study. *Arterioscler Thromb Vasc Biol* 1997;17:1171-1177.

40. van de Vijver LP, Kardinaal AF, van Duyvenvoorde W, Kruijssen DA, Grobbee DE, van Poppel G, Princen HM. LDL oxidation and extent of coronary atherosclerosis. *Arterioscler Thromb Vasc Biol* 1998;18:193-199.
41. Leinonen JS, Rantalaiho V, Solakivi T, Koivula T, Wirta O, Pasternack A, Alho H, Lehtimaki T. Susceptibility of LDL to oxidation is not associated with the presence of coronary heart disease or renal dysfunction in NIDDM patients. *Clin Chim Acta* 1998;275:163-174.
42. Leinonen JS, Rantalaiho V, Laippala P, Wirta O, Pasternack A, Alho H, Jaakkola O, Ylä-Herttuala S, Koivula T, Lehtimaki T. The level of autoantibodies against oxidized LDL is not associated with the presence of coronary heart disease or diabetic kidney disease in patients with non-insulin-dependent diabetes mellitus. *Free Radic Res* 1998;29:137-141.
43. Jessup W, Rankin SM, DeWhalley CV, Hoult RS, Scott J, Leake DS. α-tocopherol consumption during LDL oxidation. *Biochem J* 1990;265:399-405.
44. Princen HM, van Duyvenvoorde W, Buytenhek R, van der Laarse A, van Poppel G, Gevers-Leuven J, van Hinsbergh VW. Supplementation with low doses of vitamin E protects LDL from lipid peroxidation in men and women. *Arterioscler Thromb Vasc Biol* 1995;15:325-333.
45. Jialal I, Fuller CJ, Huet BA. The effect of α-tocopherol supplementation on LDL oxidation. A dose-response study. *Arterioscler Thromb Vasc Biol* 1995;15:190-198.
46. Manson JE, Gaziano JM, Jonas MA, Hennekens CH. Antioxidants and cardiovascular disease: A review. *J Am Coll Nutr* 1993;12:426-432.
47. Stampfer MJ, Rimm EB. Epidemiologic evidence for vitamin E in prevention of cardiovascular disease. *Am J Clin Nutr* 1995;62:1365S-1369S.
48. Tribble DL. AHA Science Advisory. Antioxidant consumption and risk of coronary heart disease: emphasis on vitamin C, vitamin E, and beta-carotene: A statement for healthcare professionals from the American Heart Association. *Circulation* 1999;99:591-595.
49. Stampfer MJ, Hennekens CH, Manson JE, Colditz GA, Rosner B, Willett WC. Vitamin E consumption and the risk of coronary disease in women. *N Engl J Med* 1993;328:1444-1449.
50. Rimm EB, Stampfer MJ, Ascherio A, Giovannucci E, Colditz GA, Willett WC. Vitamin E consumption and the risk of coronary heart disease in men. *N Engl J Med* 1993;328:1450-1456.
51. 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* 1996;64:190-196.
52. 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* 1996;334:1156-1162.
53. Hennekens CH, Gaziano JM, Manson JE, Buring JE. Antioxidant vitamin-cardiovascular disease hypothesis is still promising, but still unproven: the need for randomized trials. *Am J Clin Nutr* 1995;62:1377S-1380S.
54. Heinonen OP, Albanes D. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *N Engl J Med* 1994;330:1029-1035.

55. Rapola JM, Virtamo J, Ripatti S, Huttunen JK, Albanes D, Taylor PR, Heinonen OP. Randomised trial of alpha tocopherol and beta carotene supplements on incidence of major coronary events in men with previous myocardial infarction. *Lancet* 1997;349:1715-1720.
56. Stephens NG, Parsons A, Schofield PM, Kelly F, Cheeseman K, Mitchinson MJ, Brown MJ. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet* 1996;347:781-786.
57. GISSI-Prevenzione Investigators. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. *Lancet* 1999;354:447-455.
58. Kleinert S. HOPE for cardiovscular disease prevention with ACE-inhibitor ramipril. *Lancet* 1999;354:841.
59. The HOPE (Heart Outcomes Prevention Evaluation) Study: the design of a large, simple randomized trial of an angiotensin-converting enzyme inhibitor (ramipril) and vitamin E in patients at high risk of cardiovascular events. The HOPE study investigators. *Can J Cardiol* 1996;12:127-137.
60. Manson JE, Gaziano JM, Spelsberg A, Ridker PM, Cook NR, Buring JE, Willett WC, Hennekens CH. A secondary prevention trial of antioxidant vitamins and cardiovascular disease in women. Rationale, design, and methods. The WACS Research Group. *Ann Epidemiol* 1995;5:261-269.
61. Meydani SN, Barklund MP, Liu S, Meydani M, Miller RA, Cannon JG, Morrow FD, Rocklin R, Blumberg JB. Vitamin E supplementation enhances cell-mediated immunity in healthy elderly subjects. *Am J Clin Nutr* 1990;52:557-563.
62. Goetzel EJ. Vitamin E modulates the lipoxygenation of arachidonic acid in leukocytes. *Nature* 1980;288:183-185.
63. Meydani SN, Wu DY, Santos NS, Hayek MG. Antioxidants and immune response in aged persons: overview of present evidence. *Am J Clin Nutr* 1995;62:1462S-1476S.
64. Beharka AA, Wu D, Han SN, Meydani SN. Macrophage prostaglandin production contributes to the age-associated decrease in T cell function which is reversed by the dietary antioxidant vitamin E. *Mech Ageing Dev* 1997;93:59-77.
65. Goodwin JS, Garry PJ. Relationship between megadose vitamin supplementation and immunological function in a healthy elderly population. *Clin Exp Immunol* 1983;51:647-653.
66. Chavance M, Herbeth B, Fournier C, Janot C, Vernhes G. Vitamin status, immunity and infections in an elderly population. *Eur J Clin Nutr* 1989;43:827-835.
67. Payette H, Rola-Pleszczynski M, Ghadirian P. Nutrition factors in relation to cellular and regulatory immune variables in a free-living elderly population. *Am J Clin Nutr* 1990;52:927-932.
68. Meydani SN, Meydani M, Blumberg JB, Leka LS, Siber G, Loszewski R, Thompson C, Pedrosa MC, Diamond RD, Stollar BD. Vitamin E supplementation and in vivo immune response in healthy elderly subjects. *JAMA* 1997;277:1380-1386.
69. Pallast EG, Schouten EG, de Waart FG, Fonk HC, Doekes G, von Blomberg BM, Kok FJ. Effect of 50- and 100-mg vitamin E supplements on cellular immune function in noninstitutionalized elderly persons. *Am J Clin Nutr* 1999;69:1273-1281.
70. Miller ER, Appel LJ, Jiang L, Risby TH. Association between cigarette smoking and lipid peroxidation in a controlled feeding study. *Circulation* 1997;96:1097-1101.
71. Morrow JD, Frei B, Longmire AW, Gaziano JM, Lynch SM, Shyr Y, Strauss WE, Oates JA, Roberts LJ. Increase in circulating products of lipid peroxidation (F2-isoprostanes) in smokers. Smoking as a cause of oxidative damage. *N Engl J Med* 1995;332:1198-1203.

72. Brown KM, Morrice PC, Duthie GG. Vitamin E supplementation suppresses indexes of lipid peroxidation and platelet counts in blood of smokers and nonsmokers but plasma lipoprotein concentrations remain unchanged. *Am J Clin Nutr* 1994;60:383-387.
73. Mezzetti A, Lapenna D, Pierdomenico SD, Calafiore AM, Costantini F, Riario-Sforza G, Imbastaro T, Neri M, Cuccurullo F. Vitamins E, C and lipid peroxidation in plasma and arterial tissue of smokers and non-smokers. *Atherosclerosis* 1995;112:91-99.
74. Cade JE, Margetts BM. Relationship between diet and smoking. Is the diet of smokers different? *J Epidemiol Comm Health* 1991;45:270-272.
75. Zondervan KT, Ocké MC, Smit HA, Seidell JC. Do dietary and supplementary intakes of antioxidants differ with smoking status? *Int J Epidemiol* 1996;25:70-79.
76. Howard G, Wagenknecht LE, Burke GL, Diez-Roux A, Evans GW, McGovern P, Nieto FJ, Tell GS. Cigarette smoking and progression of atherosclerosis. *JAMA* 1998;279:119-124.
77. Salonen RM, Salonen JT. Progression of carotid atherosclerosis and its determinants: a population-based ultrasonography study. *Atherosclerosis* 1990;81:33-40.
78. Halliwell B, Gutteridge JCM. Free radicals, ageing, and disease. **In:** *Free radicals in biology and medicine*. Oxford, Clarendon Press, 1993, pp 454-458.
79. Rodriguez-Martinez MA, Ruiz-Torres A. Homeostasis between lipid peroxidation and antioxidant enzyme activities in healthy human aging. *Mech Ageing Dev* 1992;66:213-222.
80. Rondanelli M, Melzi dG, Anesi A, Ferrari E. Altered oxidative stress in healthy old subjects. *Aging* 1997;9:221-223.
81. Young EA. Evidence relating selected vitamins and minerals to health and disease in the elderly population in the United States: introduction. *Am J Clin Nutr* 1982;36:979-985.
82. Schneider EL, Vining EM, Hadley EC, Farnham SA. Recommended dietary allowances and the health of the elderly. *New Engl J Med* 1986;314:157-160.
83. Guemouri L, Artur Y, Herbeth B, Jeandel C, Cuny G, Siest G. Biological variability of superoxide dismutase, glutathione peroxidase, and catalase in blood. *Clin Chem* 1991;37:1932-1937.
84. Ceballos PI, Trivier JM, Nicole A, Sinet PM, Thevenin M. Age-correlated modifications of copper-zinc superoxide dismutase and glutathione-related enzyme activities in human erythrocytes. *Clin Chem* 1992;38:66-70.
85. Garcia-Arumi E, Andreu AL, Lopez-Hellin J, Schwartz S. Effect of oxidative stress on lymphocytes from elderly subjects. *Clin Sci* 1998;94:447-452.
86. Pigeolet E, Remacle J. Alterations of enzymes in ageing human fibroblasts in culture. V. Mechanisms of glutathione peroxidase modification. *Mech Ageing Dev* 1991;58:93-109.
87. Powell JT, Edwards RJ, Worell PC, Franks PJ, Greenhalgh RM, Poulter NR. Risk factors associated with the development of peripheral arterial disease in smokers: a case control study. *Atherosclerosis* 1997;129:41-48.
88. Higenbottam T, Shipley MJ, Rose G. Cigarettes, lung cancer, and coronary heart disease: the effects of inhalation and tar yield. *J Epidemiol Comm Health* 1982;36:113-117.
89. Kanters SDJM, Algra A, van Leeuwen MS, Banga JD. Reproducibility of in vivo carotid intima-media thickness measurements: a review. *Stroke* 1997;28:665-671.
90. Smilde TJ, Wollersheim H, VanLangen H, Stalenhoef AFH. Reproducibility of ultrasonographic measurements of different carotid and femoral artery segments in

healthy subjects and in patients with increased intima-media thickness. *Clin Sci* 1997;93:317-324.

91. Grobbee DE, Bots ML. Carotid artery intima-media thickness as an indicator of generalized atherosclerosis. *J Intern Med* 1994;236:567-573.
92. Allan PL, Mowbray PI, Lee AJ, Fowkes FG. Relationship between carotid intima-media thickness and symptomatic and asymptomatic peripheral arterial disease. The Edinburgh Artery Study. *Stroke* 1997;28:348-353.
93. Crouse JR, Craven TE, Hagaman AP, Bond MG. Association of coronary disease with segment-specific intimal- medial thickening of the extracranial carotid artery. *Circulation* 1995;92:1141-1147.
94. Adams MR, Nakagomi A, Keech A, Robinson J, McCredie R, Bailey BP, Freedman SB, Celermajer DS. Carotid intima-media thickness is only weakly correlated with the extent and severity of coronary artery disease. *Circulation* 1995;92:2127-2134.
95. Bots ML, Hoes AW, Koudstaal PJ, Hofman A, Grobbee DE. Common carotid intima-media thickness and risk of stroke and myocardial infarction: the Rotterdam Study. *Circulation* 1997;96:1432-1437.
96. Heiss G, Sharett AR, Barnes R, Chambless LE, Szklo M, Alzola C. Carotid atherosclerosis measured by B-mode ultrasound in populations: Associations with Cardiovascular risk factors in the ARIC study. *Am J Epidemiol* 1991;134:250-256.
97. Joensuu T, Salonen RM, Winblad I, Korpela H, Salonen JT. Determinants of femoral and carotid artery atherosclerosis. *J Intern Med* 1994;236: 79-84:79-84.
98. Wagenknecht LE, D'Agostino R, Savage PJ, O'Leary DH, Saad MF, Haffner SM. Duration of diabetes and carotid wall thickness; The Insulin Resistance Atherosclerosis Study (IRAS). *Stroke* 1997;28:999-1005.
99. Dempsey RJ, Moore RW. Amount of smoking independently predicts carotid artery atherosclerosis severity. *Stroke* 1992;23:693-696.
100. Tell GS, Polak JF, Ward BJ, Kittner SJ, Savage PJ, Robbins J. Relation of smoking with carotid artery wall thickness and stenosis in older adults. The Cardiovascular Health Study. *Circulation* 1994;90:2905-2908.
101. Howard G, Burke GL, Szklo M, Tell GS, Eckfeldt J, Evans G, Heiss G. Active and passive smoking are associated with increased carotid wall thickness. The Atherosclerosis Risk in Communities Study. *Arch Intern Med* 1994;154:1277-1282.
102. Bonithon-Kopp C, Coudray C, Berr C, Touboul PJ, Feve JM, Favier A, Ducimetiere P. Combined effects of lipid peroxidation and antioxidant status on carotid atherosclerosis in a population aged 59-71 y: The EVA Study. *Am J Clin Nutr* 1997;65:121-127.
103. Azen SP, Qian D, Mack WJ, Sevanian A, Selzer RH, Liu CR, Liu CH, Hodis HN. Effect of supplementary antioxidant vitamin intake on carotid arterial wall intima-media thickness in a controlled clinical trial of cholesterol lowering. *Circulation* 1996;94:2369-2372.
104. Salonen JT, Nyyssönen K, Parviainen M, Kantola M, Korpela H, Salonen RM. Low plasma beta-carotene, vitamin E and selenium levels associated with accelerated carotid atherogenesis in hypercholesterolemic Eastern Finnish men. Abstracts of the 33rd annual conference on cardiovascular disease epidemiology and prevention. *Circulation* 1993;87:678.
105. 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. *Circulation* 1995;92:2142-2150.

106. Touboul PJ, Prati P, Scarabin PY, Adrai V, Thibout E, Ducimetiere P. Use of monitoring software to improve the measurement of carotid wall thickness by B-mode imaging. *J Hypert* 1992;10 (Suppl.):S37-S41.
107. Rose G, Blackburn H, Gillum RF, Prineas RJ. Cardiovascular survey methods. World Health Organisation Monograph series. World Health Organisation. Geneva. 1982;56:149-152.
108. Feyerabend C, Bryant AE, Jarvis MJ, Russell MAH. Determination of cotinine in biological fluids of non-smokers by packed column gas-liquid chromatography. *J Pharm Pharmacol* 1986;38:917-919.
109. Ocké MC, Bueno de Mesquita B, Pols M, Smit H, van Staveren WA, Kromhout D. The Dutch EPIC food frequency questionnaire. II Relative validity and reproducibility for nutrients. *Int J Epidemiol* 1997;26:S49-S58.
110. Stichting Nederlandse Voedingsstoffenbestand. Dutch Nutrient Data Base 1997. The Hague, Voorlichtingsbureau voor de Voeding. Voorlichtingsbureau voor de Voeding. The Hague.
111. Brubacher G, Vuilleumier JP. Vitamin C. **In:** *Clinical Biochemistry. Principles and methods*.Curtis HCh, Roth M (eds). Berlin, Walter de Gruyter, 1974, pp 992-997.
112. Hess D, Keller HE, Oberlin B, Bonfanti R, Schuep W. Simultaneous determination of retinol, tocopherols, carotenes and lycopene in plasma by means of high performance liquid chromatography on reversed phase. *Internat J Vit Nutr Res* 1991;61:232-238.
113. Rose G, McCartney P, Reid DD. Self-administration of a questionnaire on chest pain and intermittent claudication. *Br J Prev Soc Med* 1977;31:42-48.
114. Demacker PNM, Hijmans AG, Vos-Jansen HE, van 't Laar A, Jansen AP. A study of the use of polyethylene glycol in estimating cholesterol in high-density lipoprotein. *Clin Chem* 1980;26:1775-1779.
115. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972;18:499-502.
116. Willett W. Nutritional epidemiology. Monographs in Epidemiology and Biostatistics. Oxford University Press. New York,Oxford. 1990;15:261-263.
117. Hodis HN, Mack WJ, LaBree L, Selzer RH, Liu CR, Liu CH, Azen SP. The role of carotid arterial intima-media thickness in predicting clinical coronary events. *Ann Intern Med* 1998;128:262-269.
118. Stepney R. Are smokers' self-reports of inhalation a useful measure of smoke exposure. *J Epidemiol Comm Health* 1982;36:109-112.
119. Frei B, Forte TM, Ames BN, Cross CE. Gas phase oxidants of cigarette smoke induce lipid peroxidation and changes in lipoprotein properties in human blood plasma. Protective effects of ascorbic acid. *Biochem J* 1991;277:133-138.
120. Scheffler E, Wiest E, Woehrle J, Otto I, Schulz I, Huber L, Ziegler R, Dresel HA. Smoking influences the atherogenic potential of low-density lipoprotein. *Clin Invest* 1992;70:263-268.
121. Diaz MN, Frei B, Vita JA, Keaney JF. Mechanisms of disease: antioxidants and atherosclerotic heart disease. *N Engl J Med* 1997;337:408-416.
122. Harats D, Ben-Naim M, Dabach Y, Hollander G, Stein O, Stein Y. Cigarette smoking renders LDL susceptible to peroxidative modification and enhanced metabolism by macrophages. *Atherosclerosis* 1989;79:245-252.
123. Sasaki A, Kondo K, Sakamoto Y, Kurata H, Itakura H, Ikeda Y. Smoking cessation increases the resistance of low-density lipoprotein to oxidation. *Atherosclerosis* 1997;130:109-111.

124. Princen HMG, VanPoppel G, Vogelezang C, Buytenhek R, Kok FJ. Supplementation with vitamin E but not β-carotene in vivo protects LDL from lipid peroxidation in vitro; effect of cigarette smoking. *Arterioscler Thromb* 1992;12:554-562.
125. Smilde TJ, VandenBerkmortel FWPJ, Boers GHJ, Wollersheim H, de Boo T, VanLangen H, Stalenhoef AFH. Carotid and femoral artery wall thickness and stiffness in patients at risk for cardiovascular disease, with special emphasis on hyperhomocysteinemia. *Arterioscl Thromb Vasc Biol* 1998;18:1958-1963.
126. Probtsfield JL, Byington RP, Egan DA, Espeland MA, Margitic SE, Riley WA, Furberg CD. Methodological issues facing studies of atherosclerotic change. *Circulation* 1993;87:74-81.
127. Hodis HN, Mack WJ, LaBree L, Selzer RH, Liu C, Alaupovic P, Kwong FH, Azen SP. Reduction in carotid artery wall thickness using lovastatin and dietary therapy: A randomized, controlled clinical trial. *Ann Intern Med* 1996;124:548-556.
128. Stenslandbugge E, Bonaa KH, Joakimsen O. Reproducibility of ultrasonographically determined intima media thickness is dependent on arterial wall thickness: the Tromso study. *Stroke* 1997;28:1972-1980.
129. de Waart FG, Moser U, Kok FJ. Vitamin E supplementation in elderly lowers the oxidation rate of linoleic acid in LDL. *Atherosclerosis* 1997;133:255-263.
130. Pessah-Rasmussen H, Stavenow L, Seidegard J, Solem JO, Israelsson B. Lack of glutathione transferase activity in intermittent claudication. *Int Angiol* 1990;9:70-74.
131. Rebbeck TR. Molecular epidemiology of the human glutathione *S*-transferase genotypes GSTM1 and GSTT1 in cancer susceptibility. *Cancer Epidemiol Biomarkers Prev* 1997;6:733-743.
132. Bots ML, de Jong PT, Hofman A, Grobbee DE. Left, right, near or far wall common carotid intima-media thickness measurements: associations with cardiovascular disease and lower extremity arterial atherosclerosis. *J Clin Epidemiol* 1997;50:801-807.
133. Brockmöller J, Kerb R, Drakoulis N, Nitz M, Roots I. Genotype and phenotype of glutathione *S*-transferase class μ isoenzymes μ and θ in lung cancer patients and controls. *Cancer Res* 1993;53:1004-1011.
134. Pessah-Rasmussen H, Seidegard J, Stavenow L, Solem JO, Lindblad B, Xu CB. Glutathione transferase activity in human vessels and in cultured arterial smooth muscle cells. *Int Angiol* 1993;12:348-354.
135. Mooney LVA, Bell DA, Santella RM, van Bennekum AM, Ottman R, Paik M, Blaner WS, Lucier GW, Covey L, Young TL, Cooper TB, Glassman AH, Perera. Contribution of genetic and nutritional factors to DNA damage in heavy smokers. *Carcinogenesis* 1997;18:503-509.
136. Montauban-van-Swijndregt AD, The SH, Gussenhoven EJ, Lancee CT, Rijsterborgh H, de Groot E, van der Steen AF, Bom N, Ackerstaff RG. An in vitro evaluation of the line pattern of the near and far walls of carotid arteries using B-mode ultrasound. *Ultrasound Med Biol* 1996;22:1007-1015.
137. Howard G, Burke GL, Evans GW, Crouse JR, Riley W, Arnett D, de Lacy R, Heiss G. Relations of intimal-medial thickness among sites within the carotid artery as evaluated by B-mode ultrasound. ARIC Investigators. Atherosclerosis Risk in Communities. *Stroke* 1994;25:1581-1587.
138. Rosfors S, Hallerstam S, Jensen UK, Zetterling M, Carlstrom C. Relationship between intima-media thickness in the common carotid artery and atherosclerosis in the carotid bifurcation. *Stroke* 1998;29:1378-1382.

139. Gey KF, Puska P, Jordan P, Moser UK. Inverse correlation between plasma vitamin E and mortality from IHD in cross-cultural epidemiology. *Am J Clin Nutr* 1991;53:326S-334S.
140. Riemersma RA, Wood DA, Macintyre CCA, Elton RA, Gey KF, Oliver MF. Risk of angina pectoris and plasma concentrations of vitamins A, C, and E and carotene. *Lancet* 1991;337:1-5.
141. Knekt P, Reunanen A, Jarvinen R, Seppanen R, Heliovaara M, Aromaa A. Antioxidant vitamin intake and coronary mortality in a longitudinal population study. *Am J Epidemiol* 1994;139:1180-1189.
142. 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 atherosclerosis. *JAMA* 1995;273:1849-1854.
143. Regnström J, Nilsson J, Moldeus P, Ström K, Båvenholm P, Tornvall P, Hamsten A. Inverse relation between the concentration of low-density lipoprotein vitamin E and severity of coronary artery disease. *Am J Clin Nutr* 1996;63:377-385.
144. Kok FJ, deBruijn AM, Vermeeren R, Hofman A, vanLaar A, deBruin M, Hermus RJJ, Valkenburg HA. Serum selenium, vitamin antioxidants, and CV mortality: a 9-year follow-up study in the Netherlands. *Am J Clin Nutr* 1987;45:462-468.
145. Hense HW, Stender M, Bors W, Keil U. Lack of an association between serum vitamin E and myocardial infarction in a population with high vitamin E levels. *Atherosclerosis* 1993;103:21-28.
146. Kardinaal AFM, Kok FJ, Ringstad J, Gomez-Aracena J, Mazaev VP, Kohlmeier L, Martin BC, Aro A, Kark JD, Delgado-Rodrigues M, Riemersma RA, van 't Veer P, Huttunen JK, Martin-Moreno JM. Antioxidants in adipose tissue and risk of myocardial infarction: the EURAMIC study. *Lancet* 1993;342:1379-1384.
147. Dieber-Rotheneder M, Puhl H, Waeg G, Striegl G, Esterbauer H. Effect of oral supplementation with d-α-tocopherol on the vitamin E content of human LDL and resistance to oxidation. *J Lipid Res* 1991;32:1325-1332.
148. Belcher JD, Balla J, Jacobs DR, Gross M, Jacob HS, Vercellotti GM. Vitamin E, LDL, and endothelium. Brief oral vitamin supplementation prevents oxdized LDL-mediated vascular injury in vitro. *Arterioscler Thromb* 1993;13:1779-1789.
149. Esterbauer H, Jurgens G, Quehenberger O, Koller E. Autoxidation of human LDL: loss of PUFAs and vitamin E and generation of aldehydes. *J Lipid Res* 1987;28:495-509:
150. Bonanome A, Pagnan A, Biffanti S, Opportuno A, Sorgato F, Dorella M, Maiorino M, Ursini F. Effect of dietary monounsaturated and polyunsaturated fatty acids on the susceptibility of plasma LDL to oxidative modification. *Arterioscl Thromb* 1992;12:529-533.
151. Thomas MJ, Thornburg T, Manning J, Hooper K, Rudel LL. Fatty acid composition of low-density lipoprotein influences its susceptibility to autoxidation. *Biochemistry* 1994;33:1828-1834.
152. Schmuck A, Fuller CJ, Devaraj S, Jialal I. Effect of aging on susceptibility of low-density lipoproteins to oxidation. *Clin Chem* 1995;41:1628-1632.
153. Stulnig TM, Jürgens G, Chen Q, Moll D, Schönitzer D, Jarosch E, Wick G. Properties of low density lipoproteins relevant to oxidative modifications change paradoxically during aging. *Atherosclerosis* 1996;126:85-94.
154. Steinberg D. Antioxidants in the prevention of human atherosclerosis. Summary of the proceedings of a national heart, lung and blood institute workshop: september 5-6, 1991, Bethesda, Maryland. *Circulation* 1992;85:2338-2344.

155. van den Hombergh CE, Schouten EG, van Staveren WA, van Amelsvoort LG, Kok FJ. Physical activities of noninstitutionalized Dutch elderly and characteristics of inactive elderly. *Med Sci Sports Exerc* 1995;27:334-339.
156. Himber J, Bühler E, Moll D, Moser UK. Low density lipoprotein for oxidation and metabolic stusies. Isolation from small volumes of plasma using a tabletop centrifuge. *Int J Vit Nutr Res* 1995;65:137-142.
157. Frei B, Gaziano JM. Content of antioxidants, performed lipid hydroperoxides, and cholesterol as predictors of the susceptibility of human LDL to metal ion-dependent and -independent oxidation. *J Lipid Res* 1993;34:2135-2145.
158. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959;37:911-917.
159. Raederstorff D, Meier CA, Moser U, Walter P. Hypothyroidism and thyroxin substitution affects the n-3 fatty acid composition of rat liver mitochondria. *Lipids* 1991;26:781-787.
160. Haller J, Löwik M, Ferry M, Ferro-Luzzi A. Nutritional status: blood vitamins A, E, B6, B12, folic acid and carotene. *Eur J Clin Nutr* 1991;45(suppl 3):63-82.
161. Berliner JA, Navab M, Fogelman AM, Frank JS, Demer LL, Edwards PA, Watson AD, Lusis AJ. Atherosclerosis: Basic Mechanisms; oxidation, inflammation, and genetics. *Circulation* 1995;91:2488-2496.
162. Verlangieri AJ, Bush MJ. Effects of d-α-tocopherol supplementation on experimentally induced primate atherosclerosis. *J Am Coll Nutr* 1992;11:131-138.
163. Kleinveld HA, Hak-Lemmers HLM, Hectors MPC, DeFouw NJ, Demacker PNM, Stalenhoef AFH. Vitamin E and fatty acid intervention does not attenuate the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbits. *Arterioscler Thromb Vasc Biol* 1995;15:290-297.
164. van Staveren WA, Deurenberg P, Burema J, de Groot LCPGM, Hautvast JGAJ. Seasonal variation in food intake, pattern of physical activity and change in body weight in a group of young adult Dutch women consuming self-selected diets. *Int J Obesity* 1986;10:133-145.
165. Dimitrov NV, Meyer C, Gilliland D, Ruppenthal M, Chenoweth W, Malone W. Plasma tocopherol concentrations in response to supplemental vitamin E. *Am J Clin Nutr* 1991;53:723-729.
166. Croft KD, Williams P, Dimmitt S, Abu-Amsha R, Beilin LJ. Oxidation of LDL: effect of antioxidant content, fatty acid composition and intrinsic phopholipase activity on susceptibility to metal ion-induced oxidation. *Biochim Biophys Acta* 1995;1254:250-256.
167. Reaven P, Parthasarathy S, Grasse BJ, Miller E, Almazan F, Mattson FH, Khoo JC, Steinberg D, Witztum JL. Feasibility of using an oleate-rich diet to reduce the susceptibility of LDL to oxidative modification in humans. *Am J Clin Nutr* 1991;54:701-706.
168. Reaven PD, Grasse BJ, Tribble DL. Effects of linoleate-enriched and oleate-enriched diets in combination with α-tocopherol on the susceptibility of LDL and LDL subfractions to oxidative modification in humans. *Arterioscler Thromb* 1994;14:557-566.
169. Kishimoto S, Tomino S, Mitsuya H, Fujiwara H, Tsuda H. Age-related decline in the in vitro and in vivo syntheses of anti-tetanus toxoid antibody in humans. *J Immunol* 1980;125:2347-2352.
170. Wayne SJ, Rhyne RL, Garry PJ, Goodwin JS. Cell-mediated immunity as predictor of morbidity and mortality in subjects over 60. *J Gerentol* 1990;45:45M-48M.

171. Roberts-Thomson IC, Whittingham S, Youngchaiyud U, Mackay IR. Ageing, immune response, and mortality. *Lancet* 1974;ii:368-370.
172. Gardner ID. The effect of aging on susceptibility to infection. *Rev Infect Dis* 1980;2:801-810.
173. Murasko DM, Weiner P, Kaye D. Association of lack of mitogen-induced lymphocyte proliferation with increased mortality in the elderly. *Aging Immunol Infect Dis* 1988;1:1-6.
174. Goodwin JS. Decreased immunity and increased morbidity in the elderly. *Nutr Rev* 1995;53:41S-46S.
175. Goodwin JS, Searles RP, Tung SK. Immunological responses of a healthy elderly population. *Clin Exp Immunol* 1982;48:403-410.
176. Corwin LM, Shloss J. Role of antioxidants on the stimulation of the mitogenic response. *J Nutr* 1980;110:2497-2505.
177. Likoff RO, Guptill DR, Lawrence LM, McKay CC, Mathias MM, Nockels CF, Tengerdy RP. Vitamin E and aspirin depress prostaglandins in protection of chickens against Escherichia coli infection. *Am J Clin Nutr* 1981;34:245-251.
178. Meydani SN, Meydani M, Verdon CP, Shapiro AA, Blumberg JB, Hayes KC. Vitamin E supplementation supresses prostaglandin E1(2) synthesis and enhances the immune response of aged mice. *Mech Ageing Dev* 1986;34:191-201.
179. Hayek MG, Meydani SN, Meydani M, Blumberg JB. Age differences in eicosanoid production of mouse splenocytes: effects on mitogen-induced T-cell proliferation. *J Gerentol* 1994;49:197B-207B.
180. Tengerdy RP, Heinzerling RH, Brown GL, Mathias MM. Enhancement of the humoral immune response by vitamin E. *Int Arch Allergy* 1973;44:221-232.
181. Tengerdy RP, Mathias MM, Nockels CF. Effect of vitamin E on immunity and disease resistance. **In:** *Vitamins, nutrition and cancer*.Prasad JS (ed). Basel, Karger, 1984, pp 123-133.
182. Corwin LM, Shloss J. Influence of vitamin E on the mitogenic response of murine lymphoid cells. *J Nutr* 1980;110:916-923.
183. Corwin LM, Gordon RK, Shloss J. Studies on the mode of action of vitamin E in stimulating T-cell mitogenesis. *Scand J Immunol* 1981;14:565-571.
184. Larsen HJ, Tollersrud S. Effect of dietary vitamin E and selenium on the phytohaemagglutinin response of pig lymphocytes. *Research in Veterinary Science* 1981;31:301-305.
185. Bendich A, Gabriel E, Machlin LJ. Dietary vitamin E requirement for optimum immune responses in the rat. *J Nutr* 1986;116:675-681.
186. Reddy PG, Morrill JL, Minocha HC, Stevenson JS. Vitamin E is immunostimulary in calves. *Journal of Dairy Science* 1987;70:993-999.
187. Ziemlanski S, Wartanowicz M, Klos A, Raczka A, Klos M. The effects of ascorbic acid and α-tocopherol supplementation on serum proteins and immunoglobulin concentrations in the elderly. *Nutr Int* 1986;2:1-5.
188. Houba R, van Run P, Heederik D, Doekes G. Wheat antigen exposure assessment for epidemiologic studies in bakeries using personal sampling and inhibition ELISA. *Clin Exp Allergy* 1996;26:154-163.
189. Zock JP, Doekes G, Heederik D, van Zuylen M, Wielaard P. Airborne dust antigen exposure and specific IgG response in the potato processing industry. *Clin Exp Allergy* 1996;26:542-548.
190. Doekes G, Kaal MJH, van Ieperen-van Dijk AG. Allergens of *Pityrosporum ovale* and *Candida Albicans*. II Physicochemical characterization. *Allergy* 1993;48:401-408.

191. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferatin and cytotoxicity assays. *J Immunol Methods* 1983;65:55-63.
192. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modification to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* 1997;1986:271-277.
193. Chambless LE, Roeback JR. Methods for assessing difference between groups in change when initial measurement is subject to intra-individual variation. *Statistics in Medicine* 1993;12:1213-1237.
194. Anonymous*SAS Istitute Inc. SAS/STAT User's Guide.* Cary, NC, SAS Institute Inc., 1996,
195. Gey FK. Optimum plasma levels of antioxidant micronutrients; Ten years of antioxidant hypothesis on atherosclerosis. *Bibl Nutr Dieta* 1994;51:84-99.
196. Meydani SN, Leka L, Loszewski R. Long term vitamin E supplementation enhances immune response in healthy elderly. *FASEB J* 1994;8:A272, p1574.
197. Chandra RK. Effect of vitamin and trace-element supplementation on immune responses and infection in elderly subjects. *Lancet* 1992;340:1124-1127.
198. van Poppel G. Epidemiological evidence for ß-carotene in prevention of cancer and cardiovascular disease. *Eur J Clin Nutr* 1996;50:S57-S61.
199. Ziegler RG, Colavito EA, Hartge P, McAdams MJ, Schoenberg JB, Mason TJ, Fraumeni JF. Importance of α-carotene, β-carotene, and other phytochemicals in the etiology of lung cancer. *J Natl Cancer Inst* 1996;88:612-615.
200. Giovannucci E, Clinton SK. Tomatoes, lycopene, and prostate cancer. *Proc Soc Exp Biol Med* 1998;218:129-139.
201. Kohlmeier L, Kark JD, Gomezgracia E, Martin BC, Steck SE, Kardinaal AFM, Ringstad J, Thamm M, Masaev V, Riemersma R, Martinmoreno JM, Huttunen JK, Kok FJ. Lycopene and myocardial infarction risk in the euramic study. *Am J Epidemiol* 1997;146:618-626.
202. Howard AN, Williams NR, Palmer CR, Cambou JP, Evans AE, Foote JW, Marques-Vidal P, McCrum EE, Ruidavets JB, Nigdikar SV, Rajput-Williams J, Thurnham DI. Do hydroxy-carotenoids prevent coronary heart disease? A comparison between Belfast and Toulouse. *Int J Vitam Nutr Res* 1996;66:113-118.
203. Nomura AMY, Ziegler RG, Stemmermann GN, Chyou PH, Craft NE. Serum micronutrients and upper aerodigestive tract cancer. *Cancer Epidemiol Biomarkers Prev* 1997;6:407-412.
204. Martinez-Cayuela M. Oxygen free radicals and human disease. *Biochimie* 1995;77:147-161.
205. Cantilena LR, Nierenberg DW. Simultaneous analysis of five carotenoids in human plasma by isocratic high performance liquid chromatography. *J Micronutr Anal* 1989;6:127-145.
206. Craft NE, Wise SA. Optimization of an isocratic high-performance liquid chromatographic separation of carotenoids. *J Chrom* 1992;589:171-176.
207. Siedel J, Hagele EO, Ziegenhorn J, Wahlefeld AW. Reagent for the enzymic determination of serum total cholesterol with improved lipolytic efficiency. *Clin Chem* 1983;29:1075-1079.
208. Voorrips LE, Ravelli AC, Dongelmans PC, Deurenberg P, van Staveren WA. A physical activity questionnaire for the elderly. *Med Sci Sports Exerc* 1991;23:974-979.
209. Kleinbaum DG: *Survival analysis. A self-learning text.* Springer-Verlag New York, Inc, 1997,

210. Brady WE, Mares-Perlman J, Bowen P, Stacewicz-Sapuntzakis M. Human serum carotenoid concentrations are related to physiologic and lifestyle factors. *J Nutr* 1996;126:129-137.
211. Sahyoun NR, Jacques PF, Russell RM. Carotenoids, vitamins C and E, and mortality in an elderly population. *Am J Epidemiol* 1996;144:501-511.
212. Evans RW, Shaten BJ, Day BW, Kuller LH. Prospective association between lipid soluble antioxidants and coronary heart disease in men - The Multiple Risk Factor Intervention Trial. *Am J Epidemiol* 1998;147:180-186.
213. Miller NJ, Sampson J, Candeias LP, Bramley PM, Rice-Evans CA. Antioxidant activities of carotenes and xanthophylls. *FEBS Lett* 1996;384:240-242.
214. Edge R, McGarvey DJ, Truscott TG. The carotenoids as anti-oxidants-a review. *J Photochem Photobiol B* 1997;41:189-200.
215. Stahl W, Nicolai S, Briviba K, Hanusch M, Broszeit G, Peters M, Martin HD, Sies H. Biological activities of natural and synthetic carotenoids: induction of gap junctional communication and singlet oxygen quenching. *Carcinogenesis* 1997;18:89-92.
216. Rice-Evans CA, Sampson J, Bramley PM, Holloway DE. Why do we expect carotenoids to be antioxidants in vivo? *Free Rad Res* 1997;26:381-398.
217. Kaplan LA, Lau JM, Stein EA. Carotenoid composition, concentrations, and relationships in various human organs. *Clin Physiol Biochem* 1990;8:1-10.
218. Peng YM, Peng YS, Childers JM, Hatch KD, Roe DJ, Lin Y, Lin P. Concentrations of carotenoids, tocopherols, and retinol in paired plasma and cervical tissue of patients with cervical cancer, precancer, and noncancerous diseases. *Cancer Epidemiol Biomarkers Prev* 1998;7:347-350.
219. Haller J, Weggemans RM, Lammi-Keefe C, Ferry M. Changes in the vitamin status of elderly Europeans: plasma vitamins A, E, B-6, B-12, folic acid and carotenoids. SENECA Investigators. *Eur J Clin Nutr* 1996;50:S32-S46.
220. Palozza P, Krinsky NI. ß-carotene and α-tocopherol are synergistic antioxidants. *Arch Biochem Biophys* 1992;297:184-187.
221. Ness AR, Powles JW. Fruit and vegetables, and cardiovascular disease: a review. *Int J Epidemiol* 1997;26:1-13.
222. Steinmetz KA, Potter JD. Vegetables, fruit, and cancer prevention: a review. *J Am Diet Assoc* 1996;96:1027-1039.
223. Martini MC, Campbell DR, Gross MD, Grandits GA, Potter JD, Slavin JL. Plasma carotenoids as biomarkers of vegetable intake: the University of Minnesota Cancer Prevention Research Unit Feeding Studies. *Cancer Epidemiol Biomarkers Prev* 1995;4:491-496.
224. Craft NE, Brown ED, Smith-JC J. Effects of storage and handling conditions on concentrations of individual carotenoids, retinol, and tocopherol in plasma. *Clin Chem* 1988;34:44-48.
225. Comstock GW, Alberg AJ, Helzlsouer KJ. Reported effects of long term freezer storage on concentrations of retinol, β-carotene, and α-tocopherol in serum or plasma summarized. *Clin Chem* 1993;39:1075-1078.
226. Block G. Are clinical trials really the answer? *Am J Clin Nutr* 1995;62:1517S-1520S.
227. Steinberg D. Clinical trials of antioxidants in atherosclerosis: are we doing the right thing? *Lancet* 1995;346:36-38.
228. Gey KF, Stahelin HB, Eichholzer M. Poor plasma status of carotene and vitamin C is associated with higher mortality from ischemic heart disease and stroke: Basel prospective study. *Clin Investig* 1993;71:3-6.

229. Klipstein-Grobusch K, Geleijnse JM, den Breeijen JH, Boeing H, Hofman A, Grobbee DE, Witteman JC. Dietary antioxidants and risk of myocardial infarction in the elderly: the Rotterdam Study. *Am J Clin Nutr* 1999;69:261-266.
230. Porkkala-Sarataho EK, Nyyssönen K, Kaikkonen JE, Poulsen HE, Hayn EM, Salonen RM, Salonen JT. A randomized, single-blind, placebo-controlled trial of the effects of 200 mg alpha-tocopherol on the oxidation resistance of atherogenic lipoproteins. *Am J Clin Nutr* 1998;68:1034-1041.
231. Kleinveld HA, Naber AHJ, Stalenhoef AFH, Demacker PNM. Oxidation resistance, oxidation rate, and extent of oxidation of human LDL depend on the ratio of oleic acid content to linoleic acid content: studies in vitamin E deficient subjects. *Free Rad Biol Med* 1993;15:273-280.
232. de Graaf J, Hak LH, Hectors MP, Demacker PN, Hendriks JC, Stalenhoef AF. Enhanced susceptibility to in vitro oxidation of the dense low density lipoprotein subfraction in healthy subjects. *Arterioscler Thromb* 1991;11:298-306.
233. Tribble DL, Krauss RM, Lansberg MG, Thiel PM, van den Berg JJM. Greater oxidative susceptibility of the surface monolayer in small dense LDL may contribute to differences in copper-induced oxidation among LDL density subfraction. *J Lipid Res* 1995;36:662-671.
234. Rothman KJ, Greenland S. Precision and validity in epidemiologic studies. **In:** *Modern epidemiology*. Philadelphia, Lippincott-Raven Publishers, 1998, pp 115-134.
235. Fowkes FGR, Dunbar JT, Lee AJ. Risk factor profile of nonsmokers with peripheral arterial disease. *Angiology* 1995;46:657-662.
236. Halliwell B. Establishing the significance and optimal intake of dietary antioxidants: The biomarker concept. *Nutrition Reviews* 1999;57:104-113.
237. Raitakari OT. Imaging of subclinical atherosclerosis in children and young adults. *Ann Med* 1999;31 (suppl.):33-40.
238. Meyer F, Bairati I, Dagenais GR. Lower ischemic heart disease incidence and mortality among vitamin supplement users. *Can J Cardiol* 1996;12:930-934.
239. Keil SO, Hertog MG, Feskens EJ, Kromhout D. Dietary flavonoids, antioxidant vitamins, and incidence of stroke: the Zutphen study. *Arch Intern Med* 1996;156:637-642.
240. Rapola JM, Virtamo J, Haukka JK, Heinonen OP, Albanes D, Taylor PR, Huttunen JK. Effect of vitamin E and beta carotene on the incidence of angina pectoris; a randomized, double-blind, controlled trial. *JAMA* 1996;275:693-698.
241. Walsh GP, Purvis JA, Vallely SR, Young IS, Morris SD, Yellon DM, Leake DS, Ogunyankin K, Fath-Ordoubadi F. Vitamin E and coronary heart disease [letter]. *Lancet* 1996;347:1689-1691.
242. Mitchinson MJ, Stephens NG, Parsons A, Bligh E, Schofield PM, Brown MJ. Mortality in the CHAOS trial [letter]. *Lancet* 1999;353:381-382.
243. Ness A, Smith GD. Mortality in the CHAOS trial. *Lancet* 1999;353:1017-1018.

Summary

This thesis addresses the hypothesis that the lipid soluble antioxidant vitamin E may be beneficial for several health aspects, possibly by counteracting negative effects of lipid peroxidation. More specifically the effect of vitamin E is studied in two vitamin E intervention trials on atherosclerosis and immune response (chapters 3 to 6) and two observational studies on atherosclerosis and total mortality among populations abstaining from antioxidant supplements (chapters 2 and 7). The studies focus on smokers and elderly hypothesized to be at risk for increased oxidative stress.

Vitamin E may exert its protective effect on atherosclerosis by protecting the low density lipoproteins against lipid peroxidation. Oxidative modification of low density lipoprotein cholesterol may render it more atherogenic than native LDL. Lipid peroxidation may also negatively influence immune response by structurally changing the membranes of cells involved in the immune response. Furthermore, vitamin E may exert its immune-enhancing effect by decreasing the production of immuno-suppressors in elderly, as they contribute to the age-associated dysregulation of immune response.

In **chapter 2** a cross-sectional study design was used to study the associations between vitamin E intake and plasma levels, smoking characteristics and the common carotid intima media thickness (IMT) measured by B-mode ultrasound as a marker of atherosclerosis. The 158 male lifelong CVD-free smoking subjects, aged 50 years and over, had a mean carotid IMT of 0.93 (SD=0.15) mm. In this group of life-long smokers, not using any vitamin supplements, no associations were found between carotid IMT and dietary intake of vitamin E or between carotid IMT and serum levels of vitamin E. Self-reported depth of inhalation of smoke into the lungs was the only smoke characteristic significantly associated with carotid IMT. Age-adjusted carotid IMT was significantly 0.053 mm (SD=0.022, $p<0.05$) thicker (6%) among deep inhalers compared to non-or moderate inhalers. This difference decreased to 0.036 mm (SD=0.021, p=0.08) (4%) when adjusted for other CVD risk factors. Risk factors for CVD such as age, blood pressure, LDL- and HDL cholesterol were independent predictors of carotid IMT and explained 34% of the variance in carotid IMT.

In **chapter 3** the effect of a daily dose of 364 mg (400 IU) vitamin E was studied on the 2-year change of the carotid IMT. The study was designed as a randomized placebo-controlled double-blind intervention trial among 218 male lifelong smokers from the general population aged 50 to 75 year with a mean number of 43 (SD=8) smoking years. The baseline mean carotid IMT was 0.95 mm (SD=0.16). In the placebo group the spontaneous 2-year change ('progression') in carotid IMT was 0.026 mm (95% confidence interval (CI) 0.005; 0.047, p=0.02) after adjustment for baseline IMT. After adjustment for traditional CVD risk factors a non-significant reduced progression of carotid IMT of 47% (p=0.34) was observed in the vitamin E group compared to the 0.030 mm (95% CI 0.009-0.050, p=0.006) progression in the placebo group. Vitamin E supplementation significantly reduced the in vitro susceptibility of LDL to Cu^{2+} oxidation. In the vitamin E group there were no significant associations between changes of carotid IMT and changes in lagtime.

To further assess the relevance of the non-significant reduction in carotid IMT progression, as observed in the trial described in chapter 3, these results were differentiated by genetic predisposition in **chapter 4**. Specifically, smokers with the null genotype for GSTM1 (GSTM1-*0*), who thus lack the detoxifying enzyme activity of glutathione S-transferase μ, were compared to smokers with the positive genotype (GSTM1-*1*). The results were adjusted for baseline IMT and major CVD risk factors. In the placebo group smokers with GSTM1-*0* showed a significant 2-year carotid IMT progression of 0.045 mm (95% CI 0.016-0.074, p=0.002) compared to only 0.012 mm (95% CI 0.020-0.043, p=0.47) for those with the GSTM1-*1* genotype. Among subjects in the placebo group, only for the carotid posterior wall IMT progression was significantly ($p< 0.05$) more increased in the GSTM1-*0* group than in the GSTM1-*1* group. Compared to the placebo group, vitamin E supplementation did not reduce the carotid IMT progression for those with the GSTM1-*1* genotype. However, for those with the GSTM1-*0* genotype vitamin E supplementation reduced the proportion of smokers with increased carotid IMT progression by 62% (95% CI -4%-86%, p=0.06) at the left posterior wall and by 73% (95% CI 26%-90%, p=0.01) at the left anterior wall.

In **chapter 5** the effect of 100 mg vitamin E on the in vitro susceptibility of LDL to oxidation was studied in a three months randomized double-blind placebo controlled trial in 83 apparently healthy elderly, aged 67 to 85 years. The susceptibility of LDL

to oxidation was measured by the disappearance of linoleic acid from LDL, a major substrate for lipid peroxidation, after a 5 h in vitro Cu-oxidation of LDL isolated from plasma. Vitamin E supplementation resulted in a significant two-fold decrease in percentage of oxidized linoleic acid in LDL of 10.4% compared to a decrease of 4.6% in the control group. Moreover, within the vitamin E group this protective effect was even more marked in subjects who started with a low baseline level of the α-tocopherol to linoleic acid ratio.

In the trial described in chapter 5 also the effects of 100 mg vitamin E on indices of the cellular and humoral immune response in elderly subjects (**chapter 6**) were studied. After adjustment for initial values, three months vitamin E supplementation had no significant effect on the cellular immune response measured by the in vitro mitogenic stimulation of peripheral blood mononuclear cells to the mitogens concanavalin A and phytohemagglutinin both at concentrations of 1 and 5 mg/ml. Similarly, no significant effect of vitamin E supplementation was found on humoral immune response measured by immunoglobulin (Ig)G, IgG4, and IgA antibody concentrations raised against various common antigens.

Finally, in the prospective study described in **chapter 7** the association between 7.2 year all-cause mortality and serum levels of carotenoids and vitamin E were studied among 638 independently living elderly aged 65 to 85 years. After adjustments for CVD risk factors no significant increased mortality risk was found for the lowest compared to the highest tertile of serum α-tocopherol (vitamin E) (hazard ratio 1.11, 95% CI 0.74; 1.65).

In **chapter 8** these main findings are discussed in relation to other scientific evidence. Furthermore, methodological aspects that may have interfered with the interpretation of the findings are discussed. Finally implications and directions for future research are given. Precision and validity of the studies presented in the thesis were discussed to the extent that they may have influenced the interpretation of the results. Specific attention was paid to the use of surrogate endpoints or markers of atherosclerosis, study power and selection bias, confounding and information bias. In short, the interpretation of the beneficial effects of vitamin E found on in vitro measurement of susceptibility of LDL to oxidation as a marker of atherosclerosis is difficult as the validity of this marker for the in vivo situation is unclear. The validity and

reproducibility of progression of carotid IMT as a marker for atherosclerosis is supported by an increasing number of studies thus justifying our use of this marker. Despite the fact that the vitamin E intervention among smokers approximately showed the anticipated 50% reduction in IMT progression and even stronger in smokers with the GSTM1-null genotype, this result did not reach statistical significance. The spontaneous progression of IMT in the placebo group was less than anticipated causing problems with the power of the study. This warrants cautious interpretation of the lack of a significant effect. Selection bias, information bias or confounding were appropriately addressed in the described studies either in the design phase or in the analyses of the described studies and are not believed to have played a substantial role in the interpretation of the reported findings.

In conclusion, except for a modest effect in the highly specific case of smokers with GSTM1-*0* polymorphism, the studies presented in this thesis do not provide evidence in support of a beneficial effect of vitamin E supplementation on atherosclerosis and immune response among the populations of smokers and elderly. Large primary and secondary prevention trials show modest and inconsistent results for the effect of vitamin E on cardiovascular disease. Results from some ongoing trials are awaited. Further improvement of measurement and identification of new and better markers of lipid peroxidation and oxidative damage to the vessel wall is warranted and this may help to establish the significance and optimal intake of vitamin E. A beneficial effect of vitamin E on immune response in elderly has been found in other trials with higher doses than used in our trial described in chapter 6. Large trials investigating the effect of vitamin E on incidence and severity on infectious disease are currently ongoing. These studies will provide more definite evidence on the clinical relevance of improvement of the age-induced decline of the immune response.

Samenvatting

In dit proefschrift wordt de hypothese bestudeerd dat het vetoplosbare antioxidant vitamine E een gunstig effect heeft op (aspecten van) gezondheid. Hiertoe werden twee interventie studies (hoofdstuk 3 tot en met 6) en twee observationele studies (hoofdstuk 2 en 7) uitgevoerd. De interventie onderzoeken richten zich op het effect van vitamine E op aderverkalking (atherosclerose) en immuun respons. In de observationele onderzoeken is de associatie onderzocht tussen vitamine E en atherosclerose en totale sterfte in populaties die geen antioxidant voedingssupplementen gebruikten. Het onderzoek is uitgevoerd bij rokers en ouderen waarvan verwacht wordt dat ze een extra risico hebben op verhoogde oxidatieve stress.

Oxidatieve verandering van de lage dichtheid lipoproteïnen (LDL) die cholesterol vervoeren in het bloed, kan ertoe leiden dat deze deeltjes meer atherogeen worden dan het gewone LDL. Door de mogelijke bescherming die vitamine E biedt tegen lipide peroxidatie kan het een gunstig effect hebben op atherosclerose. Lipide peroxidatie kan ook een negatieve invloed hebben op de immuun respons, onder andere door het veroorzaken van structurele veranderingen in de membranen van de cellen die betrokken zijn bij de immuun respons. Daarnaast zou vitamine E de immuun respons in ouderen kunnen verbeteren doordat het de productie vermindert van immuun onderdrukkende stoffen die bijdragen aan de leeftijdsgerelateerde vermindering van de immuun respons.

In een dwarsdoorsnede onderzoek beschreven in **hoofdstuk 2** is geen associatie gevonden tussen vitamine E inneming en plasma niveau enerzijds en de dikte van de vaatwand van de halsslagader anderzijds. Deze vaatwanddikte is een marker voor atherosclerose die werd gemeten met behulp van B-mode ultrasound. Het onderzoek is uitgevoerd onder 158 mannelijke rokers van 50 jaar en ouder, die geen vitamine preparaten gebruikten en niet eerder aan hart- en vaatziekten (HVZ) hadden geleden. De gemiddelde vaatwanddikte van de halsslagader van deze rokers was 0,93 (SD=0,15) mm. Bekende risicofactoren voor HVZ, zoals leeftijd, bloeddruk, LDL en

HDL-cholesterol bleken onafhankelijke determinanten te zijn en verklaarden samen 34% van de variantie in vaatwanddikte van de halsslagader verklaarden.

In **hoofdstuk 3** is het effect bestudeerd van een dagelijkse dosis van 364 mg (400 IU) vitamine E op de tweejaarlijkse verandering (progressie) in vaatwanddikte van de halsslagader. Dit onderzoek is opgezet als een gerandomiseerde placebo gecontroleerde dubbelblinde interventie-studie onder 218 mannelijke rokers (50-75 jaar), die gemiddeld al 43 jaar rookten (SD=8). De gemiddelde vaatwanddikte aan het begin van de studie was 0,95 mm (SD=0,16). Vitamine E suppletie verminderde de toename van vaatwanddikte met 47% (niet significant, p=0,34) ten opzichte van de significante spontane toename in de placebo groep van 0,030 mm (95% BTBHI 0,009-0,050, p=0,006). Deze resultaten waren gecorrigeerd voor beginwaarden van vaatwanddikte en bekende risicofactoren voor HVZ. Twee jaar suppletie met vitamine E leidde wel tot een significante reductie in de gevoeligheid van LDL voor *in vitro* koper oxidatie. Deze reductie bleek echter niet samen te hangen met de veranderingen in vaatwanddikte van de halsslagader.

In **hoofdstuk 4** zijn de resultaten van hoofdstuk 3 verder uitgesplitst naar genetische predispositie. Rokers met verminderde activiteit van het ontgiftende enzym glutathion *S*-transferase μ (genotype GSTM1-*0*) zijn vergeleken met rokers met het positieve genotype (GSTM1-*1*). De resultaten zijn gecorrigeerd voor beginwaarden van vaatwanddikte van de halsslagader en de belangrijkste risicofactoren voor HVZ. Rokers met het GSTM1-*0* genotype die geen extra vitamine E kregen (placebo groep) lieten een significante 2 jaarlijkse toename in vaatwanddikte zien van 0,045 mm (95% betrouwbaarheidsinterval (btbhi) 0,016-0,074, p=0,002) vergeleken met slechts 0,012 mm (95% btbhi 0,020-0,043, p=0,47) toename bij niet gesupplementeerde rokers met het GSTM1-*1* genotype. Dit verschil in progressie van de wanddikte van de halsslagader tussen niet gesupplementeerde rokers met het GSTM1-*0* en GSTM1-*1* genotype was alleen significant (p<0,05) voor de achterwand van de halsslagader. Ten opzichte van de placebogroep, bleek vitamine E suppletie de toename van de vaatwanddikte van de halsslagader niet te verminderen bij rokers met het GSTM1-*1* genotype. Echter, onder rokers met het GSTM1-*0* genotype bleek vitamine E suppletie de proportie rokers met toegenomen vaatwanddikte significant te verminderen met 62% (95% btbhi -4%-86%, p=0,06) voor de linker achter wand en met 73% (95% btbhi 26%-90%, p=0,01) voor de linker voorwand van de halsslagader.

In **hoofdstuk 5** is het effect bestudeerd van suppletie met 100 mg vitamine E op de gevoeligheid van LDL voor *in vitro* koper oxidatie. Dit onderzoek is uitgevoerd als een drie maanden durende gerandomiseerde dubbelblinde placebo gecontroleerde interventie onder 83 ogenschijnlijk gezonde ouderen in de leeftijdsgroep van 67 tot 85 jaar. De oxidatiegevoeligheid van LDL is gemeten aan de hand van de oxidatie van linolzuur, het belangrijkste substraat in LDL voor lipide peroxidatie. Vitamine E suppletie resulteerde in een significante vermindering van het percentage geoxideerd linolzuur in LDL van 10,4% vergeleken met 4,6% in de placebo groep

Als onderdeel van de trial onder oudere respondenten, beschreven in hoofdstuk 5, is ook het effect bestudeerd van 100 mg vitamine E suppletie op indicatoren voor cellulaire en humorale immuun respons (**hoofdstuk 6**). Na correctie voor beginwaarden bleek vitamine E suppletie geen effect te hebben op de cellulaire immuun respons. Deze werd gemeten door *in vitro* stimulatie van bij de immuun respons betrokken cellen met de mitogenen concanavaline A en phytohemagglutinine in concentraties van 1 en 5 mg/ml. De humorale immuun respons werd bestudeerd aan de hand van de concentraties van de immunoglobulines (Ig)G, IgG4, en IgA in reactie op verschillende veel voorkomende antigenen. Ook de humorale immuun respons werd niet beïnvloed door vitamine E suppletie.

Tenslotte is in een prospectieve studie onder 638 zelfstandig wonende ouderen in de leeftijdsgroep van 65 tot 85 jaar de associatie bestudeerd tussen sterfte gedurende 7.2 jaar follow-up en serum concentraties van carotenoïden en vitamine E (**hoofdstuk 7**). Na correctie voor risico factoren voor HVZ is geen verhoogd mortaliteitsrisico gevonden voor het laagste vergeleken met het hoogste tertiel van voor cholesterol gecorrigeerde serum α-tocopherol (vitamine E) niveaus (hazard ratio 1.11, 95% btbhi 0,74; 1,65).

In **hoofdstuk 8** worden de belangrijkste resultaten bediscussieerd in relatie tot andere wetenschappelijke bevindingen. Bovendien wordt aandacht besteed aan methodologische factoren die de interpretatie van onze resultaten zouden kunnen beïnvloeden, zoals het gebruik van surrogaat-eindpunten voor atherosclerose, het statistisch onderscheidingsvermogen (power) van onze onderzoeken, selectie bias,

informatie bias en confounding. Tenslotte worden implicaties en richtingen voor toekomstig onderzoek aangegeven.

Onze onderzoeken tonen niet aan dat suppletie met vitamine E belangrijke gunstige effecten zou hebben op atherosclerose en immuun response bij rokers en ouders. Het gunstige effect dat gevonden wordt voor *in vitro* metingen van oxidatiegevoeligheid van LDL is moeilijk te interpreteren omdat de validiteit van deze marker voor de *in vivo* oxidatie niet overtuigend is aangetoond. Een toenemend aantal studies toont aan dat de door ons gebruikte vaatwanddikte van de halsslagader een veelbelovende (valide en reproduceerbaar) marker is voor atherosclerose. Ondanks het feit dat de vitamine E interventie bij rokers leidde tot de vooraf verwachte 50% vermindering in toename van de vaatwanddikte was dit resultaat niet statistisch significant. De spontane progressie van de vaatwanddikte in de placebo groep was minder dan verwacht, wat de statistische power van onze studie aanzienlijk verminderde. Om deze reden is terughoudendheid geboden in de interpretatie van het niet-significante effect. Andere methodologische factoren zoals selectie bias, informatie bias en confounding hebben geen rol van betekenis gespeeld in de interpretatie van de gerapporteerde resultaten.

Concluderend, geven de onderzoeken in dit proefschrift geen ondersteuning voor een gunstig effect van vitamine E suppletie op atherosclerose en immuun respons bij rokers of bij ouderen. De betekenis van het GSTM1 genotype in dit proces vraagt nog om verdere bevestiging in toekomstig onderzoek. Onze resultaten zijn grotendeels in lijn met grote primaire en secundaire preventie trials die evenzeer bescheiden en inconsistente resultaten laten zien voor het effect van vitamine E op hart- en vaatziekten. Resultaten van andere nog lopende trials moeten worden afgewacht. Prioriteit dient gegeven worden aan onderzoek naar goede markers voor lipide peroxidatie en vaatwandbeschadiging om duidelijker het belang en de optimale inneming van vitamine E te kunnen vaststellen.
Een gunstig effect van vitamine E op de immuun respons bij ouderen is wel gevonden in andere trials met een hogere dosis voor vitamine E dan onze dosis in hoofdstuk 6. Op dit moment lopen nog grote trials naar het effect van vitamine E op de incidentie en ernst van infecties. Deze onderzoeken zullen meer uitsluitsel geven over de klinische relevantie van verbetering door vitamine E van de leeftijdsafhankelijke vermindering van de immuun respons.

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Vertrouw maar op de liefde
Die je voelt
Zoveel als jij nu voelt hou ik
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Frouwkje

About the author

Frouwkje Geertruida de Waart was born on 17 February 1966 in Utrecht, the Netherlands. In 1984 she completed secondary school (VWO-B) at the Chr. Lyceum in Zeist. She started to study Mathematics at the State University of Utrecht, but after one year she changed to study Human Nutrition in the former Wageningen Agricultural University. Her majors were in epidemiology, physiology and biochemistry. As part of her university education she spent six months in Bogor, Indonesia to study the association between plasma vitamin A and iron concentrations in pregnant women. Another six months were spent in Beijing, China to study body composition in Chinese female factory workers. At the former department of Epidemiology and Public Health, Wageningen Agricultural University she studied the association between moderate alcohol consumption and cardiovascular mortality in Dutch civil servants. In November 1991, she obtained her M.Sc. degree in Human Nutrition.

From February 1992 until April 1993, she was appointed as a teaching associate at the former Department of Epidemiology and Public Health, Wageningen Agricultural University. At the same department, in April 1993 she initiated her Ph.D.-project described in this thesis. In 1993 she was registered as a M.Sc. in epidemiology by the Netherlands Epidemiological Society.

Since September 1999, she works as an epidemiologist at the Regional Health Service-Nieuwe Waterweg Noord, Vlaardingen.

List of Publications

International journals

Suharno D, West CE, Muhilal, Logman MH, de Waart FG, Karyadi D, Hautvast JG. Cross-sectional study on the iron and vitamin A status of pregnant women in West Java, Indonesia. *Am J Clin Nutr* 1992;56:988-993.

de Waart FG, Li R, Deurenberg P. Comparison of body composition assessments by bioelectrical impedance and by anthropometry in premenopausal Chinese women. *Br J Nutr* 1993;69:657-664.

van Dusseldorp M, Poortvliet EJ, de Waart FG, Kok FJ, Alexandrov AA, Mazaev V, Katan MB. Anti-oxidant vitamin status of Russian children and elderly. *Eur J Clin Nutr* 1996;50:195-196.

de Waart FG, Portengen L, Doekes G, Verwaal CJ, Kok FJ. Effect of 3 months vitamin E supplementation on indices of the cellular and humoral immune response in elderly subjects. *Br J Nutr* 1997;78:761-774.

de Waart FG, Moser U, Kok FJ. Vitamin E supplementation in elderly lowers the oxidation rate of linoleic acid in LDL. *Atherosclerosis* 1997;133:255-263.

Pallast EG, Schouten EG, de Waart FG, Fonk HC, Doekes G, von Blomberg BM, Kok FJ. Effect of 50- and 100-mg vitamin E supplements on cellular immune function in noninstitutionalized elderly persons. *Am J Clin Nutr* 1999;69:1273-1281.

Grievink L, de Waart FG, Schouten EG, Kok FJ. Serum carotenoids, α-tocopherol and lung function among Dutch elderly. *Am J Resp Crit Care Med* 1999. (In press.)

de Waart FG, Smilde TJ,Wollersheim H, Stalenhoef AFH, Kok FJ. Smoking characteristics, antioxidant vitamins and carotid artery wall thickness among life-long smokers. *J Clin Epidemiol.* (Accepted).

de Waart FG, Schouten EG , Stalenhoef AFH , Kok FJ. Serum carotenoids, α-tocopherol and mortality risk in a prospective study among Dutch elderly. *Int J Epidemiol* (Provisionally accepted).

de Waart FG, Smilde TJ, Wollersheim H, Demacker PNM, Stalenhoef AFH, Kok FJ. Effect of vitamin E supplementation on 2-year progression of carotid intima media thickness in lifelong male smokers. (Submitted)

de Waart FG, Kok FJ, Smilde TJ, Hijmans A, Wollersheim H, Stalenhoef AFH. Glutathione *S*-transferase M1 genotype in smokers mediates effect of vitamin E on atherosclerosis. (Submitted).