Antioxidants, Oxidative Stress, and Cardiovascular Diseases

Cross-Cultural Comparisons and Prospective Cohort Studies

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Abstract

Background: Antioxidants in plant foods have been proposed to reduce the risk of cardiovascular diseases (CVD) by reducing oxidative stress. The objective was to confirm prospective studies on CVD and traditional antioxidants (beta-carotene, alpha-tocopherol), and to investigate emerging antioxidants (alpha-carotene, gamma-tocopherol, cocoa flavanols), and oxidative stress (enzyme activity of glutathione peroxidase-3) with CVD risk.

Methods: In cross-cultural studies, the Cretan (Greece) and Zutphen (The Netherlands) cohorts of Seven Countries Study were compared with respect to long-term mortality of coronary heart disease (CHD), diet, and indicators of oxidative stress. In prospective cohort studies, data were used from the Zutphen Elderly Study (~500 men aged 65-84 y), the 'Survey in Europe on Nutrition and the Elderly: a Concerted Action' (SENECA, 1168 men and women aged 70-75 y), and the Minnesota Heart Survey (270 men and women aged 26-85 y).

Results: The comparison between the Cretan and Zutphen cohorts revealed a 3-fold lower 40-year CHD mortality rate in Crete. This lower mortality in Crete was paralleled by a higher consumption of fruit, tomatoes, and olive oil, amongst others, and by a lower consumption of meat, poultry, and dairy. Consequently, the Cretan men had higher intakes of carotenoids, alpha-tocopherol, vitamin C, and dietary fiber, and lower intakes of *trans* and saturated fatty acids. This was confirmed at the age of ≥80 years, when the Cretan men had higher plasma concentrations of major dietary antioxidants (major carotenoids and alpha-tocopherol), a lower iron status, and a lower level of oxidative stress. In the Zutphen Elderly Study, the relative risk (RR) of 15-y CVD mortality for 1 standard deviation (SD) increase in dietary intake was 0.81 [95% confidence interval (CI): 0.66-0.99] for alpha-carotene and 0.80 (95% CI: 0.66-0.97) for beta-carotene. The intake of tocopherols was not related to CVD death. In SENECA, plasma concentrations of carotene (sum of alpha- and beta-carotene) were inversely related to CVD mortality (RR for 1 SD increase: 0.83; 95% CI: 0.70-1.00), but plasma concentrations of alpha-tocopherol were not. The daily use of 4 grams of cocoa, a rich source of flavanols, was related to a 3.7 mm Hg lower systolic (95% CI: –7.1 to –0.3) and a 2.1 mm Hg lower (95% CI: –4.0 to –0.2) diastolic blood pressure. The same amount of cocoa was also related to a 50% lower risk of 15-year CVD mortality (RR: 0.50; 95% CI: 0.32-0.78). Finally, in the Minnesota Heart Survey, the odds ratio of CVD mortality for the highest vs. the lowest quartile of glutathione peroxidase-3 activity was 0.42 (95% CI: 0.21-0.86). This inverse relation was confined to those with low concentrations of HDL cholesterol (odds ratio highest vs. lowest quartile: 0.17; 95% CI: 0.06-0.47).

Conclusion: The Cretan Mediterranean diet is rich in antioxidants, which may partly contribute to the low observed CHD mortality. The findings on beta-carotene support previous observational studies suggesting that this carotenoid relates to a lower CVD risk. We showed that alpha-carotene is correlated with beta-carotene in the diet, and alpha-carotene was also related to a lower CVD mortality. The results on alpha-tocopherol are in line with the outcomes of clinical trials and do not indicate a role for this vitamin in lowering CVD mortality in elderly populations.

Contents

1 Plant Foods, Antioxidants, and Cardiovascular Diseases

Plant foods, in particular fruit and vegetables, are known to contain large amounts of antioxidants. In the early 1980s, antioxidants became promising compounds in preventing or, at least, lowering the risk of cardiovascular diseases. Vitamin E, beta-carotene, and to a lesser extent vitamin C were extensively studied. Later, other carotenoids than beta-carotene and also polyphenolic antioxidants became a topic of scientific interest. This thesis comprises cross-cultural and prospective cohort studies on plant foods, antioxidants, and oxidative stress in relation to cardiovascular diseases. This introductory chapter describes what kinds of antioxidants are found in plant foods, how they are proposed to protect against cardiovascular diseases, the results of observational and intervention studies on antioxidants, and, finally, the rationale for the research in this thesis.

Plant foods and antioxidants

Plant foods, such as fruit and vegetables, whole grains, nuts and seeds, and olive oil as the principle source of fat, form key components of traditional Mediterranean diets. Diets rich in plant foods may prevent a variety of chronic diseases, including cardiovascular diseases (CVD) (1,2). Compounds that are considered to contribute to the cardiovascular beneficial effects of plant foods include dietary fiber, unsaturated fatty acids, folate, potassium, and also antioxidants.

Antioxidants are widely distributed in plant foods, in particular fruit and vegetables. Well-known antioxidants are vitamin C, vitamin E, carotenoids including beta-carotene, and polyphenols such as flavonoids. Their functions in plants are inherent to their immobility. Plants frequently have to cope with stressful environmental changes, such as drought, extreme temperatures, high levels of sunlight (radiation), and attacks by insects and pathogens (3). By synthesizing an impressive array of antioxidants, plants are able to protect themselves from these environmental threats. In general, antioxidants tend to concentrate in those parts of the plant that are most prone for oxidative damage (4). Vitamin C, for example, is in fairly high concentrations present in chloroplasts to protect the plant from reactive oxygen species that are developed during photosynthesis (4,5). Vitamin E is thought to protect plants against excess exposure to sunlight (6). Apart from absorbing light and transferring its energy to chlorophyll, carotenoids also protect plant tissues from photo-oxidative damage by losing excess of thermal energy and scavenging harmful oxygen species like triplet chlorophyll and singlet oxygen (6). Finally, phenolics, to which the flavonoids belong, possess antibacterial and antiviral effects (7).

By consuming plant foods, humans ingest antioxidants. The amount of antioxidants in the diet depends on the consumed amount and the antioxidant content of the particular plant foods. A typical diet of adults in The Netherlands provides daily roughly 80 mg of vitamin C, 13 mg of vitamin E, and about 1.3 mg of beta-carotene (8,9). There is no precise estimate of the average intake of flavonoids in The Netherlands, but it is likely to be about 180 mg per day (reference 10 and personal communication M. Bekkers).

It is thought that a mild to moderate deficiency of certain antioxidants, although in the case of the vitamins not severely enough to cause classical deficiency diseases, may increase the risk of developing CVD (11). Therefore, high intakes of antioxidants may reduce CVD risk in humans. The mode of action is proposed to be similar as in plants: by preventing oxidative damage. More specifically, antioxidants are hypothesized to inhibit the main underlying process that leads to CVD, atherosclerosis.

The oxidative modification hypothesis and its implications

Atherosclerosis is a chronic inflammatory disease of the arterial wall in which lipid accumulation is accompanied by thickening and hardening of the vessel wall (12). One of the

first steps in the process of atherosclerosis is excessive uptake of LDL cholesterol by macrophages, which then become foam cells that compose the pre-atherosclerotic fatty streaks (13).

In the late 1970s, Brown and Goldstein discovered that the rate at which macrophages in culture take up *native* LDL cholesterol is by far insufficient to load the cell with cholesterol (14). A second important observation they made was that patients totally lacking the native LDL receptor nevertheless accumulate large amounts of cholesterol in their macrophages (14). They proposed that instead of native LDL, it was some modified form of LDL that was taken up excessively by macrophages and gave rise to foam cells. In the early 1980s, the group of Steinberg found that native LDL was rapidly converted to a form that was taken up much more rapidly after it was incubated with cultured vascular endothelial cells or smooth muscle cells (15,16). Shortly there after, it was shown that the modification was basically oxidative damage of LDL (17,18). In 1989, Steinberg and colleagues published their landmark paper on the oxidative modification hypothesis of atherosclerosis (19).

The oxidation modification hypothesis did not only explain the detrimental effect of cigarette smoking on CVD (by oxidizing LDL), but also implied that antioxidants may have an important role. It was postulated that antioxidants prevent LDL from oxidation, and consequently slowdown the atherosclerotic process and reduce the risk of cardiovascular diseases. Probucol, a powerful synthetic fat-soluble antioxidant, was shown in 1986 to block the oxidation of LDL *in vitro* (20). Later, probucol was found to inhibit atherogenesis in animal models. These results, amongst others, stimulated interest to conduct epidemiologic studies and randomized controlled intervention trials on antioxidants and cardiovascular diseases. With the benefit in hindsight, the conferees at a 1991 workshop on antioxidants convened by the National Heart, Lung and Blood Institute concluded that the evidence was sufficiently strong to justify starting clinical trials (21).

While the randomized trials were ongoing and more findings from observational studies were published, antioxidants became very popular as dietary supplements. Especially vitamin E enjoyed ‘superstar status’ among the dietary supplements, particularly in the US (22). In the 1990s, about 40% of the cardiologists in the US reported to use a supplement containing vitamin E (23), a percentage that makes one wonder what these cardiologists advised their patients. Perhaps it is not surprising that the largest increase in the use of vitamin supplements in the US between 1987 and 2000 was for vitamin E (24).

Antioxidants and cardiovascular diseases: the evidence from early observational studies

Gey and colleagues published the first observational study on antioxidants and CVD. In that study, of which the first findings were published in 1987 (25) and in more detail two years later

(26), blood concentrations of antioxidants as measured in randomly selected samples of residents of 12 different European regions were cross-sectionally related to regional mortality rates of coronary heart disease at the population level. A strong inverse relation was observed for concentrations of vitamin E, whereas no associations were seen for concentrations of beta-carotene and vitamin C (26). Since then, numerous observational studies have been published. Two studies that particularly fuelled the enthusiasm to conduct large-scale trials with vitamin E were the Health Professionals' Follow-up Study (27) and the Nurses' Health Study (28). These two prospective cohort studies found that vitamin E, in particular from supplements, was related to a 40% lower risk of coronary heart disease. An indication that a high intake of carotenoids could lower coronary heart disease (CHD) risk was found in the Lipid Research Clinics' Primary Prevention Trial, where men with serum carotenoid concentrations in the top quartile had a 36% lower risk of CHD (29). In support, male health professionals in the top quintile of carotene intake had a 30% lower risk of CHD compared to those in the lowest quintile (27).

The evidence from early observational studies, in summary, suggested an inverse association between vitamin E and heart disease, possibly only at intakes that can be obtained from using vitamin E supplements. Beta-carotene could also lower heart disease risk. A similar role for vitamin C seemed unlikely given the inconsistent results from observational studies (30). However, evidence from randomized trials was needed to prove the efficacy of antioxidants in lowering CVD risk.

The proof of the pudding: randomized controlled trials

At present, a number of clinical trials reported on the efficacy of antioxidants on CVD. Most of them were randomized, double-blind, placebo-controlled trials, though differed with respect to type and dose of antioxidants, end points, duration, and study population (**Table**). The study populations participating in these trials varied, though generally consisted of subjects with established CVD, malignant disease, or who were at risk of these diseases.

Unexpectedly, the vast majority of these trials showed that neither vitamin E (31,32), beta-carotene (31-34), nor vitamin C (32), either alone or in combination with each other (32,35), lowered the risk of cardiovascular events. These findings were similar for primary (33,36) (37,38) and secondary prevention (39,40) of CVD. Two relatively small trials reported that vitamin E (400-800 IU/day) reduced the risk of cardiovascular diseases (41,42), but their results were outweighed by the null findings of the larger trials (43). Also, in one of the two smaller trials, the 'Secondary Prevention with Antioxidants of Cardiovascular disease in Endstage renal disease' (SPACE) trial, the study population consisted specifically of patients with end-stage renal disease (41). Although such patients are known to have oxidative stress (44,45), the apparent beneficial effect of vitamin E seen in the SPACE trial was not confirmed in patients with mild-to-moderate renal insufficiency enrolled in the Heart Outcomes Prevention (HOPE) Study (46).

Table: Randomized clinical trials of antioxidant supplementation and risk of cardiovascular diseases

Study, year (reference)	Country	Design	Study population	No. participants	Men, %	Age range, y	Duration, y	End point	Antioxidant supplementation	Dosage per day	Relative risk (95% confidence interval)
Trials included in a 2003 meta-analysis (43)											
ATBC Cancer Prevention Study, 1994 (31)	Finland	2×2	Male smokers not using anticoagulants	29 133	100	50-69	6.1	CVD mortality	1. Beta-carotene 2. Vitamin E 3. Both combined	20 mg 50 IU	1.11 (1.01-1.21) 0.98 (0.89-1.07) NA
CHAOS, 1996 (42)	United Kingdom	Parallel	Subjects with angiographically proven coronary artery disease	2002	84	Mean, 62	1.4	1. Non-fatal MI alone; 2. non-fatal MI and death from CVD combined	Vitamin E	400 or 800 IU	0.23 (0.11-0.47) 0.53 (0.34-0.83)
PHS I, 1996 (33)	United States	Parallel *	Physicians free of MI, stroke, or transient cerebral ischemia; 11% was current smoker at baseline	22 071	100	40-84	12	Major CVD events	Beta-carotene	25 mg	1.00 (0.91-1.09)
CARET, 1996 (34)	United States	Parallel	Smokers, former smokers, or workers exposed to asbestos	18 314	66	45-69	6 †	CVD mortality	Beta-carotene Retinol	30 mg 25 000 IU	1.26 (0.99-1.61)
GISSI-P Trial, 1999 (48)	Italy	Parallel ‡	Subjects with previous MI, of whom 42% was current smoker at baseline	11 324	85	Mean, 59	3.5	MI, stroke, and death from CVD combined	Vitamin E	330 IU	0.98 (0.87-1.10)
SPACE, 2000 (41)	Israel	Parallel	Hemodialysis patients with CVD	196	69	40-75	1.5	MI, stroke, PAD, and unstable angina pectoris combined	Vitamin E	800 IU	0.46 (0.27-0.78)
HOPE Study, 2000 (40)	19 countries	Parallel §	Subjects with previous CVD, or with diabetes and an additional CVD risk factor	9541	73	≥55	4.5¶	MI, stroke, and death from CVD combined	Vitamin E	400 IU	1.05 (0.95-1.16)
PPP, 2001 (37)	Italy	Parallel #	Subjects free of CVD but with at least one major CVD risk factor	4495	42	Mean, 64	3.6	MI, stroke, and death from CVD combined	Vitamin E	330 IU	1.07 (0.74-1.56)
HPS, 2002 (49)	United Kingdom	Parallel	Subjects with CVD, diabetes mellitus, or hypertension	20 536	75	40-80	5	Major CHD events	Beta-carotene Vitamin E Vitamin C	20 mg 660 IU 250 mg	1.02 (0.93-1.11)

Table (cont.): Randomized clinical trials of antioxidant supplementation and risk of cardiovascular diseases

Study, year (reference)	Country	Design	Study population	No. participants	Men, %	Age range, y	Duration, y	End point	Antioxidant supplementation	Dosage per day	Relative risk (95% confidence interval)
More recently published and ongoing trials											
HOPE Study, 2004 (46)	19 countries	Parallel §	Subjects with renal insufficiency	993	87	≥55	4.5¶	MI, stroke, and death from CVD combined	Vitamin E	400 IU	1.03 (0.79-1.34)
SU.VI.MAX, 2004 (50)	France	Parallel	Apparently healthy adults, of whom 16% was current smoker at baseline	13 017	39	35-60	7.5	Major CHD events	Beta-carotene Vitamin E Vitamin C Selenium Zinc	6 mg 33 IU 120 mg 100 µg 20 mg	0.97 (0.77-1.20)
WHS, 2005 (38)	United States	Parallel	Female health professionals free of chronic diseases	39 876	0	≥45	4.1	MI, stroke, and death from CVD combined	Beta-carotene†† Vitamin E	25 mg 300 IU	1.14 (0.87-1.49)
WACS, 2007 (32)	United States	2×2×2	Female health professionals with CVD or at least 3 CVD risk factors	8171	0	≥40	9.4	MI, stroke, CABG/PTCA, and CVD mortality combined	1. Beta-carotene 2. Vitamin E 3. Vitamin C 4. Combined	25 mg 300 IU 500 mg	1.02 (0.92-1.13) 0.94 (0.85-1.04) 1.02 (0.92-1.13) No effects observed, except for fewer strokes for taking vitamins E and C (RR: 0.69; 95% CI: 0.49-0.98)
PHS II (51)	United States	2×2×2×2	Male physicians, of whom ~7500 participated in PHS I	15 000	100	≥55		Important CVD events	1. Beta-carotene 2. Vitamin E 3. Vitamin C 4. A multivitamin	25 mg 200 IU 500 mg	Follow-up ended December 2007

Abbreviations: IU, International Unit; ATBC, Alpha-Tocopherol Beta-Carotene; CVD, cardiovascular diseases; NA, not available (no interaction reported); CHD, coronary heart disease; MI, myocardial infarction; CHAOS, Cambridge Heart Antioxidant Study; CVD, cardiovascular diseases; PHS, Physicians' Health Study; CARET, Beta-Carotene and Retinol Efficacy Trial; GISSI-P, Gruppo Italiano per to Studio della Sopravvivenza nell'Infarto miocardico Prevenzione; SPACE, Secondary Prevention of Antioxidants on cardiovascular diseases in End stage renal disease; HOPE, Heart Outcomes Prevention Evaluation; PPP, Primary Prevention Project; HPS, Heart Protection Study; SU.VI.MAX, SUpplémentation en VItamines et Minéraux AntioxYdants; WHS, Women's Health Study; WACS, Women's Antioxidant and Cardiovascular Study; CABG, coronary artery bypass graft; PTCA, percutaneous transluminal coronary angioplasty.

* In the Physicians' Health Study, beta-carotene supplementation was one arm of a 2×2 factorial design for separate assessment of beta-carotene and aspirin use.

† CARET was stopped prematurely because the active treatment group was found to have an increase in lung cancer and cardiovascular mortality.

‡ In the GISSI-P Trial, vitamin E supplementation was one arm of a 2×2 factorial design for separate assessment of vitamin E and n-3 fatty acids.

§ In the HOPE Study, vitamin E supplementation was one arm of a 2×2 factorial design for separate assessment of vitamin E and ramipril use.

¶ After 4.5 y of follow-up, the follow-up on CVD events was stopped prematurely because the benefits of ramipril were conclusively shown and the effect of vitamin E was lacking.

In the PPP, vitamin E supplementation was one arm of a 2×2 factorial design for separate assessment of vitamin E and aspirin.

†† Because of the null-findings in previous trials and the potential harmful effects that these trials indicate, beta-carotene supplementation was terminated after a median follow-up of 2.1 years.

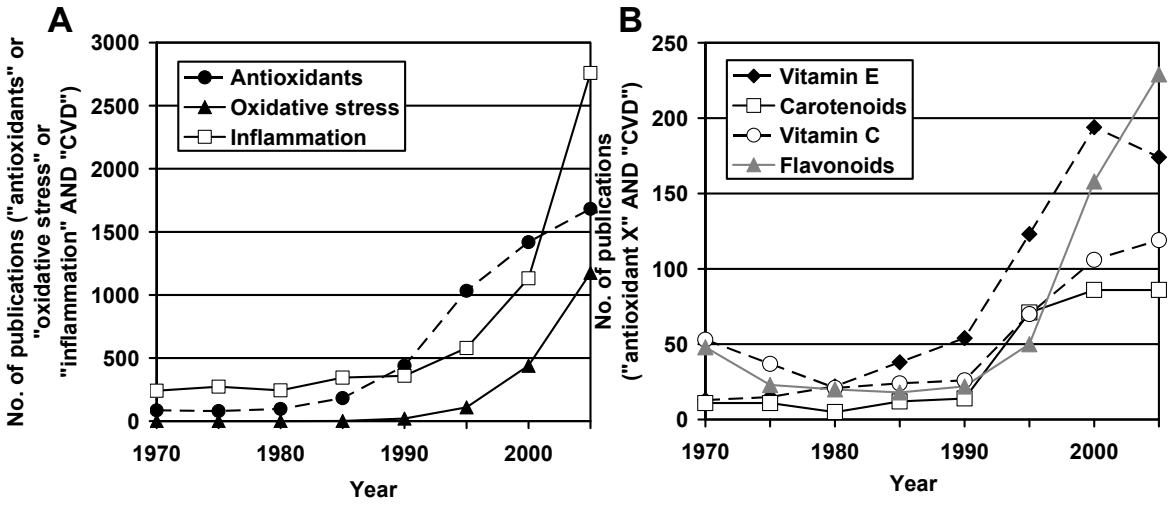

Figure: Number of publications on antioxidants, oxidative stress, inflammation (panel A) and various antioxidants (panel B) in relation to cardiovascular diseases from 1970 to 2005. Publications were those registered in PubMed (http://www.ncbi.nlm.nih.gov/entrez, accessed October 2007). Values were determined as follows: number of results from the query "antioxidants" or "oxidative stress" or "inflammation" or "antioxidant X" AND "cardiovascular diseases" AND "year Y[dp]".

Apart from the largely null results, one trial suggested that antioxidants interfere with the efficacy of statin-plus-niacin therapy in coronary heart disease patients with low HDL cholesterol concentrations (35). It was suggested that antioxidants block the increase in the protective HDL-2 and apolipoprotein A1 subfractions of HDL cholesterol (47).

The largely null-findings brought forward by the large-scale trials tempered the enthusiasm in antioxidants as preventive agents against CVD. In the popular and scientific press, antioxidants were seen as a 'myth,' a medical fairy tale (52), and it was questioned whether science was 'back to square one' (53). Especially vitamin E, the most extensively studied antioxidant, was degraded, with editorials entitled 'Is there any hope for vitamin E' (54) and '*Annus horribilis* for vitamin E' (22).

As a consequence, the annual pile of scientific publications on antioxidants and CVD changed remarkably (**Figure**). After a sharp increase in the early 1990s, the number of reports on vitamin E dropped in the last 5 years, whereas those on carotenoids leveled off. The still increasing number of research publications on antioxidants is attributable to the interest in flavonoids; the number of reports on flavonoids has sharply increased since 1990. Oxidative stress is also still a topic of great interest. Inflammation, a condition that is hard to distinguish from oxidative stress, is even more popular.

Rationale and outline of the thesis

Despite the largely null-findings of the randomized trials, continuation of scientific research on antioxidants is important and even encouraged (55). Only by continuation of antioxidant

research it is possible to clarify the contradiction in results between the observational studies and the randomized trials and to assess whether oxidative stress, the oxidation modification hypothesis, and antioxidants are relevant to human atherosclerosis.

This thesis aims to investigate the role of antioxidants and oxidative stress in the development of CVD from an epidemiological perspective. Specifically, the objective was [A] to confirm previous prospective cohort studies that found inverse associations of the traditional antioxidants beta-carotene and vitamin E with CVD risk, and [B] to investigate emerging antioxidants and oxidative stress in relation to CVD. These objectives were evaluated by cross-cultural comparisons and prospective cohort studies.

The cross-cultural studies used, in contrast to the vast majority of studies with this design, data of individuals with detailed information on possible confounding factors. In the prospective cohort studies, antioxidants in blood or diet and indicators of oxidative stress in blood were investigated. The data on dietary intake was collected repeatedly by using the dietary history method, thus representing the actual long-term intake. Chapters 2 and 3 present the cross-cultural studies in which the Cretan and Zutphen cohorts of the Seven Countries Study were compared with respect to long-term CHD mortality, diet and nutrient intake, and blood concentrations of antioxidants, fatty acids, iron status, and indicators of oxidative stress. Chapters 4 to 7 cover the prospective cohort studies. Because of the possibility that other dietary antioxidants, correlated with beta-carotene and alpha-tocopherol, may account for the inverse association in observational studies, Chapter 4 also describes associations for carotenoids and tocopherols. Chapter 5 covers a study aimed to confirm earlier published prospective cohort studies that suggested an inverse association of circulating levels of carotene and alpha-tocopherol with CVD mortality. Given the interest in cocoa as a cardioprotective food ingredient (56), Chapter 6 describes a prospective cohort study about the associations of cocoa with blood pressure and CVD mortality. The relation between a scarcely investigated antioxidant enzyme, serum glutathione peroxidase 3, and CVD mortality is described in Chapter 7. Finally, the findings of the research in this thesis are discussed and put into context in Chapter 8.

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Oxidative Stress, and Iron and Antioxidant Status in Elderly Men: Differences Between the Mediterranean South (Crete) and Northern Europe (Zutphen)

2

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Background: Oxidative stress may accelerate ageing and increase the risk of chronic diseases, such as coronary heart disease (CHD). We assessed differences in oxidative stress, and iron and antioxidant status between elderly men living in Mediterranean southern Europe (Crete, Greece) and northern Europe (Zutphen, the Netherlands).

Design: A cross-sectional study using data from two cohorts of the Seven Countries Study.

Methods: Non-fasting blood samples were drawn in 2000 from 105 men from Crete and 139 men from Zutphen, all aged 79 years or over. All assays were performed in the same laboratory.

Results: After multiple adjustments, serum levels of the markers of oxidative stress were lower in Cretan men than in men from Zutphen, as indicated by lower mean levels of hydroperoxides (33.2 versus 57.3 μmol/l; P = .005) and gamma-glutamyltransferase (20.3 versus 26.1 U/l; P = .003). The most pronounced difference in iron status was a twofold lower mean serum ferritin level in Cretan men (69.8 μg/l) compared with men from Zutphen (134.2 μg/l; P < .0001). Men from Crete had consistently higher plasma levels of major plasma antioxidants than the Zutphen men, including a nearly fourfold higher mean level of lycopene (15.3 versus 4.1 μg/100 ml; P <.0001).

Conclusions: Elderly men from Crete had consistently lower levels of the indicators of oxidative stress and iron status and higher concentrations of major antioxidants than men from Zutphen. These differences may contribute to the lower rate of CHD and total mortality that has been observed in this cohort of Cretan men.

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Introduction

Oxidative stress increases with ageing (1,2) and may be an independent risk factor for coronary heart disease(CHD) (3). Body iron stores also increase during the course of life (4), and a high iron status has been associated with an increased risk of age-related diseases, such as CHD (5). Like other transition metals, iron is thought to contribute to the development of oxidative stress by catalyzing reactions that produce oxidants. Antioxidants, on the other hand, may prevent oxidative stress by inhibiting reactions that would lead to the formation of oxidants, or by scavenging radicals.

CHD mortality rates vary substantially across Europe. In adults, CHD mortality is the highest in northern and eastern Europe, whereas much lower rates are observed in European countries surrounding the Mediterranean Sea (6,7). In the Seven Countries Study (8), CHD mortality rates were the lowest in Crete, Greece, and four times higher in Zutphen, the Netherlands. Survival rates were also greater in Crete than in Zutphen (8). The lower CHD mortality rate in Cretan men compared with those from Zutphen cannot be completely explained by differences in traditional biological risk factors because differences in serum cholesterol and blood pressure levels between these populations were small (8). This suggests that other risk factors such as oxidative stress may play a role.

At present, studies of the markers of oxidative stress, indicators of iron status, or circulating levels of antioxidants, have mostly investigated these parameters individually rather than ecologically, whereas most ecological studies lack individual data for detailed analysis. In the present study, we chose to measure indicators of oxidative stress, as well as iron and antioxidant status in the blood of elderly individuals, because they are likely to have high levels of oxidative stress. We evaluated our hypothesis that, consonant with their lower disease rates, elderly men in Crete have lower levels of the indicators of oxidative stress, a lower iron status, and higher levels of circulating antioxidants, compared with men of the same age from Zutphen, the Netherlands.

Methods

Study population

For the present study, men aged at least 79 years living on Crete (Greece) and men of the same age living in Zutphen (the Netherlands) were selected. Both cohorts consisted of participants in the Seven Countries Study (9), a prospective cohort study that started in 1958. The original cohort in Crete consisted of 686 men, of whom 165 men were still alive in 2000. Of these men, 152 were willing to participate and blood samples were drawn from 129 individuals. In 20 men, information on potential confounders was lacking, and in four men iron status could not be assessed, yielding information for 105 Cretan men for analysis (response rate 65%). The

Zutphen Elderly Study consisted of 939 men in 1985, approximately 50% of whom were first enrolled in 1960. For the examination in 2000, 240 men were still alive. Because 64 men were not willing to participate, 176 men enrolled in the study in 2000. Blood samples were taken from 146 individuals. Because information on potential confounding factors was missing on four men and iron status could not be assessed in another three, 139 men were included in the present analysis (response rate 58%). The examination on Crete was conducted between May and August 2000, and in Zutphen between March and June 2000. The study was approved by local medical ethics committees.

Blood collection

Non-fasting blood samples were collected in the morning. In Crete, blood samples were allowed to stand for 2 h at room temperature, after which plasma and serum were obtained. In Zutphen, the samples were kept cool in a box with cool elements, and plasma and serum were obtained in the afternoon. Before transport within a few days of collection to the National Institute for Public Health and the Environment (RIVM), the Netherlands, samples were stored in Zutphen at – 30ºC and in Crete at – 80ºC. Samples from Crete were transported to the Netherlands on dry ice by plane. After arriving at the RIVM, all samples were stored at – 80ºC.

Measurement of indicators of oxidative stress, iron status, and plasma antioxidants

Serum indicators of oxidative stress

We analysed two measures of in-vivo peroxidation. Total serum peroxides, primarily lipid peroxides, were measured enzymatically (OxyStat assay; Biomedica, Austria). Malondialdehyde, an end-product of lipid peroxidation, was measured by high-performance liquid chromatography (Chromsystems, Germany). Both assays were performed at the laboratories of the RIVM, Bilthoven, The Netherlands. Gamma-glutamyl-transferase (GGT), a possible marker of oxidative stress (10), was determined by a Technicon SMAC analyser (Technicon Instruments Corp., Tarrytown, New York, USA) at the Leiden University Medical Center.

Iron status

Serum iron, serum transferrin, serum ferritin and the unbound iron-binding capacity were determined at the laboratory of the RIVM by using a Hitachi 912 analyser (Roche Diagnostics, Indianapolis, Indiana, USA). The total iron-binding capacity was calculated by the sum of serum iron and unbound iron-binding capacity, and the proportion of transferrin saturation by dividing the concentration of serum iron by the total iron-binding capacity. Serum ferritin concentrations exceeding 300 µg/l in combination with a transferrin saturation exceeding 55% was taken as an indicator of iron overload (11).

Plasma antioxidant vitamins and serum endogenous antioxidants

Plasma carotenoids and tocopherols were measured by high-performance liquid chromatography methods in the laboratory of the Division of Human Nutrition (Wageningen University, the Netherlands) according to a standardized protocol. All analyses were performed under subdued yellow light to prevent photo destruction.

The endogenous antioxidants albumin, total bilirubin, and uric acid were measured in serum using a Technicon SMAC analyser at the Leiden University Medical Center. Control samples were determined during the assay for monitoring stability.

Collection of data on lifestyle, biological risk factors, and history of chronic diseases

Information on cigarette smoking, alcohol consumption, and the longest practised profession was collected by the same standardized questionnaires in Crete and Zutphen. The time spent in different physical activities was estimated in minutes per week with a validated questionnaire originally designed for retired men (12). Tertiles of physical activity were calculated based on the distribution of the total study population of 244 men. Subjects in the lowest tertile were defined as having a low physical activity. Information on whether subjects were using vitamin supplements or were on a diet prescribed by a doctor was obtained during an interview.

During a physical examination, body weight was measured according to standardized procedures. Body mass index calculated by dividing weight (kg) by the square of height (m^2), in which height measured around 1990 was used. The history of chronic diseases such as myocardial infarction, stroke, or cancer, and the presence of diabetes was ascertained by physical examinations of the subjects and by the use of questionnaires, and was verified by hospital records, information from general practitioners, and information from the previous surveys.

Statistical analysis

Analysis of covariance was used to assess possible differences in the mean values of variables between men from Crete and Zutphen, which allowed adjustment for potential confounders. For dependent variables with a positively skewed distribution, analyses were performed after natural logarithmic transformations; for these parameters, geometric means are presented. Besides adjustment for age (model 1), adjustments were made for body mass index and lifestyle factors such as smoking, alcohol consumption, physical activity, and the prevalence of chronic diseases (see subscript to Tables 2–4). All analyses were repeated after excluding subjects with a history of cardiovascular diseases, diabetes, or cancer. Means are presented with 95% confidence intervals, and reported P values are two-sided. All analyses were performed using the Statistical Analysis System, release 9.1 (SAS Institute Inc., Cary, North Carolina, USA).

Results

General characteristics

Selected characteristics of the study population are shown in Table 1. The mean age for both Cretan and Zutphen men was approximately 84 years (range 79.2–98.2). Elderly men from Crete were less likely to consume alcoholic beverages, and less often used a prescribed diet and vitamin or mineral supplements than elderly men from Zutphen. Furthermore, they had a lower body mean mass index and a lower mean C-reactive protein concentration. The proportion of men with a history of stroke and cancer was four times lower among men from Crete compared with those from Zutphen.

Table 1: Selected characteristics of elderly men in Crete (Greece) and Zutphen (the Netherlands) in 2000

Characteristic	Crete	Zutphen	P value*
Number of men	105	139	
Demographics and risk factors			
Age, y	84.5 (4.1)	84.0 (3.5)	.33
Cigarette smoking, %	0.5	0.1	.92
Alcohol consumption, %	59.0	74.8	.009
Low physical activity, %	31.4	32.4	.88
Diet prescription, %	4.8	14.4	.01
Use of vitamin supplements, %	2.9	18.0	.0002
Body mass index, kg/m^2	24.6 (4.2)	25.9 (3.7)	.01
Systolic blood pressure, mm Hg	150.2 (21.5)	145.7 (21.5)	.11
Diastolic blood pressure, mm Hg	76.9 (10.9)	74.4 (10.2)	.06
Serum total cholesterol, mmol/L	5.05 (0.93)	5.23 (0.98)	.15
Serum HDL cholesterol, mmol/L	1.21 (0.29)	1.23 (0.32)	.65
Serum C-reactive protein, mg/L, median [IQR]	2.8 [1.4, 5.5]	3.4 [2.0, 6.0]	.03
History of chronic diseases, %			
Myocardial infarction	16 (15.2)	23 (16.5)	.78
Stroke	6 (5.7)	23 (16.5)	.01
Diabetes	10 (9.5)	17 (12.2)	.50
Cancer	7 (6.7)	29 (20.9)	.002

Values are means (standard deviation) unless otherwise indicated.
* P value for differences between Cretan and Zutphen elderly men based on unpaired t-test, Mann-Whitney U test, or Chi-square test.

Serum indicators of oxidative stress

After adjustment for potential confounders, Cretan elderly men had on average lower levels of serum hydroperoxides and GGT than elderly men in Zutphen (**Table 2**). Furthermore, the mean level of serum malondialdehyde was non-significantly lower in Cretan men, a result that persisted after the exclusion of men with chronic diseases (data not shown).

Table 2: Mean levels (95% CI) of serum indicators of oxidative stress in elderly men living in Crete and Zutphen in 2000

Indicator of oxidative stress	Age-adjusted			Multivariable-adjusted*		
	Crete (n=105)	Zutphen (n=139)	P	Crete (n=105)	Zutphen (n=139)	P
Hydroperoxides, µmol/L†	34.6 (26.3-45.3)	55.6 (43.9-70.4)	.01	33.2 (25.0-43.9)	57.3 (45.0-73.0)	.005
Malondialdehyde, mmol/L	98.4 (94.3-102.5)‡	103.2 (99.7-106.7)	.08	98.1 (94.0-102.3)‡	103.4 (99.8-107.0)	.07
Gamma-glutamyl-transferase, U/L†	20.2 (18.0-22.7)‡	26.1 (23.6-28.8)	.001	20.3 (18.0-22.9)‡	26.1 (23.5-28.9)	.003

*Adjusted for age (continuous), body mass index (continuous), smoking (yes or no), alcohol use (yes or no), physical activity (lowest tertile versus highest two tertiles), diet prescription (yes or no), history of myocardial infarction, stroke, diabetes, and cancer (all yes or no).
† Geometric means and 95%CI.
‡ Information on this variable was missing of one subject.

Iron status

Men from Crete had a lower iron status than men from Zutphen, as indicated by lower adjusted mean levels of serum iron and serum ferritin, a lower mean percentage of transferrin saturation, and a higher unbound iron-binding capacity (**Table 3**). The average serum transferrin and total iron-binding capacity did not differ between cohorts. There were few men with a possible iron overload, and its prevalence did not differ between Cretan and Zutphen men. Additional adjustment for serum C-reactive protein yielded similar results. Likewise, excluding subjects with a history of chronic diseases did not alter the results essentially.

Table 3: Mean levels (95% CI) of serum indicators of iron status in elderly men living in Crete and Zutphen in 2000

Indicator of iron status	Age-adjusted			Multivariable-adjusted *		
	Crete (n=105)	Zutphen (n=139)	P	Crete (n=105)	Zutphen (n=139)	P
Serum iron, µmol/L	15.2 (14.1-16.4)	17.6 (16.5-18.7)	.02	15.4 (14.1-16.6)	17.6 (16.5-18.6)	.01
Ferritin, µg/L	67.9 (56.6-81.3)	137.1 (117.1-160.4)	<.001	69.8 (57.9-84.1)	134.2 (114.3-157.6)	<.001
Transferrin, g/L	2.61 (2.52-2.70)	2.54 (2.46-2.61)	.21	2.62 (2.53-2.72)	2.53 (2.45-2.61)	.12
Total iron-binding capacity, µmol/L	54.7 (52.9-56.5)	53.2 (51.6-54.7)	.20	55.1 (53.2-56.9)	52.9 (51.3-54.5)	.09
Transferrin saturation, %	28.9 (26.6-31.3)	33.8 (31.7-35.9)	.003	29.0 (26.6-31.5)	33.7 (31.6-35.8)	.007
Unbound-iron binding capacity, µmol/L	39.5 (37.3-41.6)	35.5 (33.7-37.4)	.007	39.7 (37.4-41.9)	35.4 (33.4-37.3)	.005
Iron overload†, No. (%)	1 (1.0)	3 (2.2)	.61	1 (1.0)	2 (1.4)	.95

*Adjusted for age (continuous), body mass index (continuous), smoking (yes or no), alcohol use (yes or no), physical activity (lowest tertile versus highest two tertiles), diet prescription (yes or no), history of myocardial infarction, stroke, diabetes, and cancer (all yes or no).

Plasma antioxidant vitamins and serum endogenous antioxidants

Average plasma levels of the exogenous antioxidants beta-carotene, lycopene, zeaxanthin, lutein, and alpha-tocopherol were higher and that of gamma-tocopherol was lower in Cretan men than in Zutphen men (**Table 4**). Plasma beta-cryptoxanthin levels did not differ between cohorts. Plasma alpha-carotene was on average lower in Cretan men compared with men from Zutphen, despite a correlation coefficient between plasma alpha- and beta-carotene of 0.68. The most pronounced difference was a nearly fourfold higher mean level of lycopene in Cretan men. Plasma folic acid was on average higher in men from Crete. These findings were similar after excluding subjects with a history of chronic diseases. The multivariable-adjusted mean concentrations of the endogenous antioxidants albumin and total bilirubin were higher among

Table 4: Mean levels (95% CI) of plasma and serum antioxidants in elderly men living in Crete and Zutphen in 2000

	Age-adjusted			Multivariable-adjusted *		
Parameter	**Crete (n=105)**	**Zutphen (n=139)**	**P**	**Crete (n=105)**	**Zutphen (n=139)**	**P**
Plasma antioxidant vitamins						
Carotenoids (μg/100 mL)						
Beta-cryptoxanthin	9.3 (7.8-10.8)	10.4 (9.0-11.7)	.30	9.1 (7.5-10.7)	10.5 (9.2-11.9)	.20
Lycopene	15.3 (13.8-16.8)	4.1 (2.8-5.3)	<.001	15.3 (13.7-16.8)	4.1 (2.8-5.4)	<.001
Beta-carotene	26.1 (22.8-29.4)	16.3 (13.4-19.2)	<.001	25.0 (21.5-28.5)	17.1 (14.1-20.1)	.001
Alpha-carotene	1.31 (0.18-2.44)	3.12 (2.14-4.10)	.02	0.97 (0.00-2.15)	3.38 (2.37-4.39)	.003
Zeaxanthin	2.53 (2.29-2.78)	1.30 (1.09-1.51)	<.001	2.55 (2.30-2.79)	1.29 (1.08-1.50)	<.001
Lutein	22.7 (21.0-24.5)	13.6 (12.0-15.1)	<.001	22.6 (20.7-24.4)	13.7 (12.0-15.3)	<.001
Tocopherols (μg/100 mL)						
Alpha-tocopherol	1171 (1118-1224)	1052 (1006-1098)	.001	1186 (1131-1240)	1041 (994-1088)	.0002
Gamma-tocopherol	72.9 (66.5-79.3)	94.0 (88.5-99.6)	<.001	75.0 (68.4-81.6)	92.4 (86.7-98.1)	.0002
Folic acid (ng/mL)	6.44 (5.82-7.11)†	4.87 (4.46-5.31)	<.001	6.33 (5.70-7.02)†	4.92 (4.51-5.39)	.0006
Serum endogenous antioxidants						
Albumin, g/L	41.6 (41.0-42.1)†	39.5 (39.1-40.0)	<.001	41.7 (41.7-42.3)†	39.4 (38.9-39.9)	<.001
Total bilirubin, μmol/L	10.6 (9.8-11.4)	9.3 (8.6-10.0)	.02	10.6 (9.8-11.5)	9.3 (8.6-10.0)	.02
Uric acid, mmol/L	387.9 (371.7-404.6)	365.6 (351.5-379.6)	.04	386.9 (370.2-403.6)	366.3 (351.9-380.7)	.08

* Adjusted for age (continuous), body mass index (continuous), smoking (yes or no), alcohol use (yes or no), physical activity (highest tertile versus lowest two tertiles), diet prescription (yes or no), history of myocardial infarction, stroke, diabetes, and cancer (all yes or no).
† Information on this variable was missing of one subject.

Cretan men. Serum uric acid was significantly higher in men from Crete after adjustment for age, although this difference attenuated slightly after additional adjustment.

Discussion

The present study compared markers of oxidative stress, and iron and antioxidant status in elderly men in Crete (Greece) with men in Zutphen (the Netherlands). Serum levels of total peroxides and GGT were lower in men from Crete, indicating a lower level of oxidative stress compared with men from Zutphen. Cretan men also had a lower iron status compared with men in Zutphen, with the most pronounced difference being a twofold lower average serum ferritin concentration. Finally, average plasma levels of major antioxidants were higher among Cretan men, including a nearly fourfold higher mean lycopene level.

In the current study, the measurements of serum markers of oxidative stress, and iron and antioxidant status were performed at the same laboratory blinded by cohort identity of the samples, thereby excluding possible interlaboratory differences. Lifestyle factors, weight and height were assessed by using the same standardized procedures in Crete and Zutphen.

The study does have certain limitations. We attempted to ascertain the history of chronic diseases by using information from different sources, including data from general practitioners, hospital discharge information, and information from repeated medical examinations. Despite the abundant body of information, we may have underestimated the prevalence of stroke and cancer in Cretan men because of underdiagnosis. However, the results did not change materially after the exclusion of subjects with a history of chronic diseases from the analysis, indicating that confounding by chronic diseases is not a major issue.

The results of the present study may not be generalizable to younger populations. Our study population consisted of men aged 79 years and over, who differ from their younger counterparts who had died already. The findings also might not apply exactly to a future cohort of elderly men who might experience a higher survival rate. Also, because of the cross-sectional design of the study, it is not possible to draw conclusions as to whether the differences in oxidative stress and iron and antioxidant status actually contribute to the difference in CHD mortality and survival between the Cretan and Zutphen cohorts.

Oxidative stress reflects an imbalance between reactive oxygen species and the antioxidative defense mechanisms of the body, in favor of the former. It is considered to be implicated in the ageing process and the development of many chronic diseases, including CHD. As there is no single valid marker for oxidative stress, it is recommended to measure different markers of oxidative stress simultaneously. We determined three markers of oxidative stress, including two markers of peroxidation, i.e. total hydroperoxides and malondialdehyde. Serum hydroperoxides were on average 40% lower in Cretan men, whereas mean levels of malondialdehyde did not differ between Cretan and Zutphen men. The validity of malondialdehyde as a biomarker has been questioned because of its instability and lack of

specificity (13). The association of serum malondialdehyde and serum hydroperoxides with the incidence of CHD remains to be established in prospective cohort studies.

Moderately elevated levels of GGT are considered to indicate oxidative stress (10) and are shown to be an independent risk factor for hypertension (14, 15), diabetes (15,16), and cardiovascular diseases (17,18). It was recently found that atherosclerotic coronary plaques have GGT activity (19). Our observation that Cretan men have lower levels of GGT than men from Zutphen was independent of alcohol consumption, and corresponds with the results of these studies. Future studies should address whether GGT is related to cardiovascular diseases and which lifestyle and dietary factors besides alcohol modify GGT levels.

We found that elderly men living on Crete had a substantially lower iron status than those in Zutphen, including lower iron body stores. The lower iron status in the men from Crete is probably the result of a life-long lower dietary intake of iron, and may partly explain the fourfold lower mortality rate of CHD of the Cretan men compared with the men in Zutphen. However, the role of iron in the development of oxidative stress and degenerative diseases, including CHD, is controversial. Although it was first thought that high iron body stores increased the risk of CHD (5), this was not found by later published studies (20–22). According to the current evidence, the risk reduction induced by a lower body iron store is considered to be small, if present at all.

In the present study, plasma concentrations of alpha-tocopherol and major carotenoids were higher among Cretan men compared with men from Zutphen. These higher levels of antioxidants are probably the result of adherence to the traditional Mediterranean diet by the Cretan men, which is known for its abundance in fruit and vegetables and the use of olive oil. In contrast, plasma levels of alpha-carotene and gamma-tocopherol were higher in men from Zutphen. The finding for plasma alpha-carotene is unexpected given its known strong positive correlation with plasma beta-carotene. Although these differences may be caused by a different consumption of specific foods rich in alpha-carotene and gamma-tocopherol, various metabolic differences may exist between the Greek and Dutch populations. For example, it may be that carotenoids compete with each other and with vitamin E during absorption (23), and that the extent of this phenomenon may differ depending on the overall dietary context.

Average plasma levels of folic acid were higher in Cretan men. Recent published intervention studies have indicated that this vitamin improves vascular function (24,25) and reduces pulse pressure and arterial stiffness (26) by a mechanism independent of homocysteine lowering. It is suggested that folic acid may increase the bioavailability of nitric oxide, which may explain its endothelial-dependent vasodilatory effects. However, the dose of folic acid used in the intervention studies was beyond that obtained by diet, and it is unclear whether dietary folate can improve vascular function.

In conclusion, the present study shows that men aged 79 years and over from Crete have a lower level of oxidative stress, a lower iron status, and higher levels of major plasma antioxidants than men of the same age from Zutphen. These differences may contribute to the

lower CHD and total mortality rate and a higher survival that has been observed in the Cretan male population.

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Diet, Biomarkers, Major Risk Factors, and 40-Year Coronary Mortality in the Cretan and Zutphen Cohorts of the Seven Countries Study

3

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Background: We examined correlates of the difference in 40-year CHD mortality rates between a Mediterranean and a Dutch cohort, differing in habitual diet.

Methods: From 1960 through 2000 we followed two cohorts of men initially aged 40-59 years in Crete (Greece) and in Zutphen (The Netherlands). Major risk factors were assessed repeatedly in survivors. Food samples representing the average diet of each cohort in 1960 and 2000 were chemically analyzed for nutrient content. Blood samples obtained in 2000 were analyzed for biomarkers of fatty acid and antioxidant intake and indicators of oxidative stress.

Results: After 40 years follow-up, all-cause mortality rates per 1000 person-years were 25.9 in Crete and 33.3 in Zutphen. For coronary heart disease (CHD) mortality these rates were 1.8 and 8.9, respectively. Serum cholesterol, systolic blood pressure, and smoking combined explained about 30% of the cohort differences in CHD mortality. Body mass index, physical activity and resting heart rate did not help explain the cohort differences in CHD mortality. Ecologically, the Cretan Mediterranean diet was lower in *trans* and saturated fatty acids, and higher in oleic acid, fiber and antioxidants than the Zutphen diet. This was reflected in serum levels of fatty acids, antioxidants, and indicators of oxidative stress measured in 2000.

Conclusions: Thirty percent of the 5-fold lower CHD mortality rate in Crete compared to Zutphen was explained by classical CHD risk factors. The difference in diet is a likely candidate to explain the remaining difference in CHD mortality.

Submitted for publication.

Introduction

The Seven Countries Study was initiated in 1958 to test whether the incidence of coronary heart disease (CHD) differed across populations with varying dietary intakes of fatty acids or other candidate risk factors (1). The age-standardized 25-year mortality rates from CHD and all causes in Crete (Greece) belonged to the lowest of the 16 included cohorts (2). By comparison, the age-standardized mortality rate per 1000 men for CHD was 46 in Crete and 147 in the town of Zutphen (The Netherlands) and for all-cause mortality 314 and 480, respectively (2).

Many factors have been suggested to explain the low mortality rates in Crete, including classical risk factors such as serum cholesterol, blood pressure and smoking. Other factors mentioned were diet, periods of fasting as recommended by the Greek Orthodox Church (3), high levels physical activity, the exceptional climate, and even the after-lunch siesta (4).

Evidence is accumulating that the traditional Cretan Mediterranean diet lowers CHD risk. Intervention studies showed that adherence to a Mediterranean diet improved blood lipid levels and endothelial function, and lowered in vivo lipoprotein oxidation and inflammation (5-9). Observational studies noted that adherence to a Mediterranean diet is associated with a lower mortality from CHD and prolonged survival (10-13).

In the present study, we analyzed whether the differences in 40-year CHD mortality in originally middle-aged men in Crete and Zutphen could be explained by differences in classical risk factors. We chemically analyzed the habitual diets of both cohorts and explored its role in relation to CHD mortality in ecologic analyses.

Methods

Study population

The present study was based on data from the Seven Countries Study in Crete (Greece) and Zutphen (the Netherlands). At baseline examinations in 1960, the men were aged between 40-59 years. Repeated measurements among survivors took place in 1970, 1990/1991, and 2000. In Zutphen, an additional sample of men of the same age was added in 1985. This sample was, together with the survivors from 1960, used for the dietary assessment and blood sample collection that took place in 2000; all other analyses are based on the original 1960 cohort only.

Assessment of lifestyle and risk factors

Information on smoking was collected with standardized questionnaires. The percentage of cigarette smokers in Crete and Zutphen and the percentage of men who smoked only cigars or pipes in Zutphen (14) were calculated. There were no men in Crete who smoked cigars or pipes. For the present analysis, smoking was classified as either current smoking (including cigars or

pipes), former smoking (those who stopped smoking within 10 years), or never smoking (never smokers and those who stopped smoking for more than 10 years).

Physical activity in 1960 was assessed using information on job-related physical activity (1). Men were classified in three categories: little, moderate or strenuous physical activity. In 2000, physical activity was assessed using a validated questionnaire providing information about the number of minutes spent on different daily activities per week (15,16).

Throughout the study, weight and height were measured and body mass index (BMI) was calculated in kg/m^2. Systolic blood pressure was measured supine at the right arm using standardized methodology (1). Resting heart rate was derived from electrocardiograms.

Serum obtained from casual blood samples was analyzed for total cholesterol using standardized methods (1,17). In 2000, serum samples were also analyzed for concentrations of major dietary antioxidants and indicators of oxidative stress (18). We also measured the composition of fatty acids in serum cholesteryl esters by HPLC (19). Serum concentrations of folate were measured by a CEDIA kit (Roche Diagnostics, Almere, The Netherlands). High-sensitive C-reactive protein (hsCRP) concentrations were determined by a (latex) high-sensitivity kit (Roche Diagnostics, Almere, The Netherlands), and soluble intercellular adhesion molecule-1 (sIACM1) by using an ELISA (Diaclone, Sangiun, Amsterdam, The Netherlands).

Assessment of usual dietary intake

Dietary intake was estimated in a sample of 30 men in Crete between 1960 and 1965 and of 45 men in Zutphen in 1960 by using the seven-day weighed record method (20). Dietary intake was also assessed in all Zutphen men by using the cross-check dietary history method (21,22), which provides information about the *habitual* food consumption. This method was also used in the food consumption surveys conducted in Crete and Zutphen in 2000.

The dietary records in 1960 were coded in 1986 by one dietician and summarized in 15 homogeneous food groups (23). To describe the traditional Cretan Mediterranean diet, the same food grouping was used in a 1948 dietary survey independent of the Seven Countries Study using a seven-day food record and presented in Table 2 (24).

Due to aging, total energy intake had decreased between 1960 and 2000. In the analyses on food patterns, the consumption of food groups was therefore standardized for a diet of 2500 kcal.

Chemical analysis of the average diet

The nutrient content of food composites representing the average food consumption pattern of the Cretan and Zutphen men in 1960 and 2000 was chemically analyzed. For the 1960 food patterns this was done in 1987 (25) and for the 2000 food patterns in 2001. Oxalic acid was added to the composites to preserve the vitamin C content, and tertiary butyl hydroxyquinone was added to prevent fat oxidation. The food composites were homogenized and frozen at –20ºC until further analysis.

The nutrient analyses were performed with well-established procedures in 1987 and 2001. These methods were subjected to regular proficiency schemes which guarantee stable performance. The total lipid fraction was isolated and determined by a procedure described by Folch (26), after which sub-fractions of fatty acids were determined by gas chromatography (27). In the 1960 diet in Zutphen, the trans fatty acids with more than 18 carbon atoms, derived from hydrogenated fish oils, were determined by infrared spectrometry (27). N-3 fatty acids were assessed by using food composition tables (28,29) due to unavailability of some kinds of the fish on the market at the time of food collection. Protein was determined as total nitrogen, using a conversion factor of 6.25 (30). Mono- and disaccharides were determined by enzymatic methods (30), and starch was measured as previously described (31). Glucose was subsequently determined enzymatically (32). Alcohol was determined by an enzymatic assay (33). Total dietary fiber was determined by the enzymatic-gravimetric AOAC method (31). Folates were analyzed as monoglutamates by HPLC (34). The vitamin C content was quantified by a fluorimetric method (35). Carotenoids (36) and tocopherols (37) were measured by reversed phase HPLC. Flavonols were determined by HPLC methods (38,39). Total energy intake was calculated by using Atwater factors (40).

Morbidity and mortality data

Information on the prevalence of myocardial infarction and diabetes was repeatedly collected by questionnaire and by information obtained from general practitioners or hospital registries. Vital status was verified approximately every five years during 40 years of follow-up. In Zutphen, two men were lost to follow-up and none in Crete. The final causes of death were coded by one clinical epidemiologist (A.M.) according the 8th Revision of the International Classification of Diseases of the World Health Organization (ICD-8). Death from CHD was defined as codes 410-414 as the first cause of death or as sudden death (code 795) as the first cause of death combined with coronary heart disease as underlying cause of death. Survival time was calculated in years as the time from the first examination around 1960 to the date of death, loss to follow-up, or completion of the study in 2000, whichever came first.

Statistical analysis

All analyses were performed with SAS software (version 9.1, SAS Institute, Inc., Cary, North Carolina). Analysis of covariance was used to calculate mean values of and to test for mean differences between various biomarkers (18).

For each cohort, Kaplan-Meier survival probabilities covering 40 years of follow-up were used to plot the distributions of time to death from CHD and all-causes. The log-rank test was used to test differences in survival between cohorts. Cox models were used to explain the difference in CHD mortality between Crete and Zutphen, estimated as the proportionate change in the regression coefficient for cohort after addition of serum cholesterol, systolic blood

pressure, smoking, BMI, heart rate and physical activity to the model (41). Statistical tests were based on two-sided probability and P-values less than 5% indicated statistical significance.

Comparisons of diet between cohorts were ecologic, employing in Crete the independent set of observations in 1948, a subset of participants in 1960 and the surviving cohort in 2000, whereas in Zutphen diet data were available in all participants in 1960 and among survivors in 2000. Chemical analysis of composite diets for 1960 and 2000 in Crete and Zutphen were presented.

Results

Risk and lifestyle factors

In 1960, the cohort in Crete consisted mostly of farmers (75%) and in Zutphen of blue-collar workers (49%), white-collar workers (15%), professionals (18%), and small-business owners (15%).

In 1960, the Cretan cohort had a more beneficial risk profile than the cohort of Zutphen (**Table 1**), as indicated by lower mean values of serum total cholesterol, systolic blood pressure, smoking, BMI, and resting heart rate (for all P <.0001). During follow-up, the differences in these risk factors shrunk. In 1990 and 2000, blood pressure values were higher in Crete than in Zutphen.

In 1960, a high level of physical activity was observed for 62% of the Cretan men and 11% of the Zutphen men. In 2000, when the men were between 80 and 99 years old, the age-adjusted time spent on moderate-to-vigorous physical activities was twice as high in Crete (9.2 hours/week in Crete versus 4.3 hours/week in Zutphen, P = .0006). The lower heart rate in Crete compared to Zutphen was consistent with greater physical activity in Crete. Both the Cretan and Zutphen men were moderate alcohol users (**Table 2**).

Food consumption patterns and nutrient intakes

Based on information collected in 1948 and 1960, the traditional Cretan food pattern was characterized by high intake of bread and cereals, legumes, fruit, tomatoes, and olive oil and low intake of fish, dairy and meat products (**Table 2**). The Zutphen diet in 1960 included high amounts of potatoes, dairy and meat products, solid fats, and sugar. Survivors in Crete largely kept their traditional food pattern until 2000, although the consumption of bread and tomatoes decreased and the meat consumption doubled. In Zutphen, the consumption of bread, potatoes and solid fats decreased substantially, but a twofold increase was observed in fish consumption and a four-fold increase in fruit and orange juice consumption.

In 1960 the diet of both cohorts was high in fat, but the fatty acid composition differed markedly (**Table 3**). Whereas the Cretan diet was low in saturated and *trans* fatty acids and high in the oleic acid, the diet in Zutphen was high in saturated, *trans* and linoleic acid. In 1960 the

Table 1: Risk and lifestyle factors in the Cretan and Zutphen cohorts of the Seven Countries Study from 1960 to 2000

	Crete				Zutphen			
Risk factor	**1960**	**1970**	**1991**	**2000**	**1960**	**1970**	**1990**	**2000**
Number examined (response rate %)	686 (98)	611 (95)	256 (67)	152 (94)	878 (81)	646 (84)	235 (75)	76 (72)
Age, yrs	49.0 (5.5)	58.9 (5.4)	77.8 (4.7)	84.6 (4.3)	49.5 (5.5)	59.1 (5.4)	76.0 (4.5)	84.6 (3.6)
Serum total cholesterol, mmol/l	5.34 (1.07)	6.03 (1.11)	5.75 (1.10)	5.03 (0.96)	6.09 (1.15)	6.18 (1.10)	6.09 (1.12)	5.42 (1.02)
Systolic BP, mm Hg	137.0 (20.0)	137.2 (23.4)	162.9 (22.9)*	150.7 (21.6)	144.4 (19.8)	147.0 (21.2)	152.8 (20.4)	151.2 (21.0)
Body mass index, kg/m^2	22.9 (3.0)	24.1 (3.5)	24.6 (3.6)	24.1 (4.1)	24.0 (2.7)	25.1 (2.7)	25.0 (3.2)	25.1 (3.2)
Resting heart rate, beats/min†	61.8 (11.7)	NA	67.8 (13.6)	71.5 (11.8)	72.6 (12.7)	NA	75.7 (14.7)	73.8 (11.1)
Cigarette smoking, %	57.3	59.2	17.0	10.6	74.5	52.7	28.3	14.5
Only cigar or pipe smoking, %	0	0	0	0	14.3	21.1	9.7	0
Prevalence of diabetes, %	0.6	2.8	4.3	8.5	0.9	2.9	15.0	15.8
Prevalence of myocardial infarction, %	0.2	0.8	5.0	12.7	1.3	6.6	22.2	19.4

Abbreviation: NA, not available.

* Due to missing values on systolic blood pressure based on n=188.

†Information on heart rate in Crete was obtained for 642 men in 1960, 214 men in 1991, and 107 men in 2000, and in Zutphen for 812 men in 1960.

intake of the n-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid was low in both diets. Furthermore, the intake of dietary fiber, alpha-tocopherol and vitamin C was higher in Crete than in Zutphen, whereas the intake of flavonols was higher in Zutphen.

In 2000, the diets of both cohorts contained less energy than earlier. In Crete, the energy from fat was still above 40%, whereas that in Zutphen had decreased to 36%. Also, in Zutphen the intake of *trans* fatty acids had decreased to an amount comparable with Crete. Dietary fiber intake had decreased in Crete to an amount comparable with Zutphen. The intake of folate, total carotenoids, lycopene, alpha-tocopherol, vitamin C was higher in Crete, whereas Zutphen men still had a higher intake of flavonols, and also of beta-carotene.

Biomarkers of nutrient intake

In 2000 the difference in fatty acid composition of cholesteryl esters in serum between the cohorts reflected the difference in dietary fatty acid composition (**Table 4**). The men in Crete had higher proportions of oleic acid and lower proportions of linoleic acid and lower proportions of the different n-3 fatty acids than the men in Zutphen. In general, mean serum concentrations of the antioxidants were higher in Crete, including a nearly fourfold higher lycopene concentration.

Table 2: Average daily food consumption per person in Crete (1948, 1960-1965, and 2000) and Zutphen (1960 and 2000), standardized for a 2500 kcal diet

	Crete			Zutphen		
Daily dietary intake	**1948**	**1960-1965**	**2000**	**1960**	**1960**	**2000**
Population*	128 families	30 men	152 men	45 men	878 men	122 men
Assessment method	7-day record	7-day record	Dietary history	7-day record	Dietary history	Dietary history
Period of assessment	Sept-Oct	Sept-Oct	July-Aug	May-July	May-July	March-June
Energy intake, kcal†	2547	2820	2220	2756	3107	2019
Daily food intake per 2500 kcal						
Bread, g	231	336	141	229	212	158
Cereals, g	111	26	95	15	14	31
Potatoes, g	159	168	84	229	275	132
Legumes, g	44	26	59	1	9	9
Vegetables, g	254	169	185	206	162	157
tomatoes, g	112	110	64	19	3	7
Fruit and orange juice, g	258	410	317	74	90	305
oranges, g	NA	65	97	NA	25	36
orange juice, g	NA	NA	19	NA	NA	40
Meat, g	31	31	60	125	89	122
Poultry, g	5	13	17	0	0	17
Fish, g	25	16	31	11	16	26
Snails, g	7	0	2	0	0	0
Eggs, g	7	23	13	25	27	18
Milk products, g	32	208	200	416	428	417
yoghurt, g	0	41	38	25	32	57
Cheese, g	9	11	21	28	25	35
Edible fats, g	83	84	79	71	72	47
olive oil, g	80	84	78	0	0	1
Sugar products, g	15	18	16	76	66	64
Ethanol, g	4	10	14	3	3	14

Abbreviation: NA, not available.

* In 1948 in Crete, dietary intake per capita as estimated in 128 families is shown (24). Otherwise, dietary intake in men participating in the Seven Countries Study is presented.

† Total energy was based on food composition tables (Crete 1948, and Zutphen dietary history in 1960) or chemical analyses in equivalent food composites (Crete 1960-1965 and 2000, and Zutphen food record in 1960 and dietary history in 2000).

Table 3: Chemical analyses of the nutrient content of food composites representing the average daily consumption in the Cretan and Zutphen cohorts of the Seven Countries Study in 1960 and 2000

	Crete				Zutphen			
	1960 (food record)		2000 (dietary history)		1960 (food record)		2000 (dietary history)	
Dietary factor	Daily intake	En%	Daily intake	En%	Daily intake	En%	Daily intake	En%
Energy and energy-delivering nutrients								
Total energy, kcal	2975	100.0	2220	100.0	2810	100.0	2019	100.0
Protein, g	90	12.1	71	12.8	92	13.1	75	14.9
Carbohydrates, g	331	44.5	220	39.6	281	40.0	220	44.2
Fat, g	135	40.8	108	43.8	145	46.4	81	36.1
Saturated fatty acids	29	8.8	28	11.4	62	19.9	38	17.0
Trans fatty acids	0.6	0.2	1.4	0.6	26	8.3	1.1	0.5
Monounsaturated fatty acids	86	26.0	66	26.8	42	13.5	27	12.0
Oleic acid	79	24.0	63	25.5	30	9.6	24	10.7
Polyunsaturated fatty acids	13	3.9	11	4.5	21	6.7	15	6.7
n-6								
Linoleic acid, C18:2n-6	11	3.3	8.1	3.3	13	4.2	13	5.8
n-3								
Alpha-linolenic acid, C18:3n-3	1.2	0.4	1.1	0.4	2.2	0.7	1.4	0.6
Fish fatty acids EPA + DHA*	0.09	<0.1	0.16	<0.1	0.09	<0.1	0.19	<0.1
Fiber, folate, and antioxidants								
Dietary fiber, g	44		26		26		23	
Folate, µg	NA		198		NA		153	
Carotenoids, µg	NA		4450		NA		3857	
Beta-carotene	NA		730		NA		1927	
Lycopene	NA		1723		NA		193	
Alpha-tocopherol, mg	22.0		19.7		8.8		9.6	
Beta- and gamma-tocopherol, mg	6.8		4.6		12.1		8.1	
Vitamin C, mg	140		153		112		76	
Flavonols, mg	16		16		33		24	

* The intake of EPA (eicosapentaenoic acid, C20:5) and DHA (docosehexaenoic acid, C22:6) were estimated by using food composition tables (28).

Serum markers of oxidative stress, inflammation, and endothelial function

Mean gamma-glutamyltransferase activity and serum concentrations of hydroperoxides and malondialdehyde were lower in Cretan compared to Zutphen men, although the latter finding reached only borderline significance. Mean concentrations of hsCRP and sICAM1 were also lower in Cretan men.

All-cause and coronary heart disease mortality

After 40 years of follow-up, the all-cause mortality rate per 1000 person-years was 25.9 in Crete and 33.3 in Zutphen. For CHD mortality these rates were 1.8 in Crete and 8.9 in Zutphen. Kaplan-Meier survival curves for CHD and all-cause mortality differed significantly between cohorts ($P < .0001$; **Figure 1**). The median survival time for all-cause mortality was 32 years in Crete (95% CI: 31 to 33 years), and 26 years in Zutphen (95% CI: 24 to 27 years).

Plots of the log-minus-log survival function against log-time showed a decrease in parallelism in curves between cohorts with increasing follow-up for mortality (**Figure 2**). Therefore, follow-up time was split at 30 years. Baseline serum cholesterol, systolic blood pressure and smoking explained for about 30% of the cohort differences in CHD mortality for both 30 and 40 year of follow-up (**Table 5**). BMI, physical activity and resting heart rate did not contribute additionally either to the prediction of CHD death or to explaining the difference in death rates between the cohorts. Separate models for the last 10 years showed that the difference in all-cause mortality between cohorts was no longer present. However, serum cholesterol, systolic blood pressure and smoking still contributed to the difference in CHD mortality between the cohorts. In explaining the differences in mortality rates, models using the baseline risk factors were superior compared to time-dependent models.

Discussion

In the present study, the 40-year all-cause mortality rate was substantially lower in Crete compared to Zutphen. This was partly explained by a lower CHD mortality rate in Crete, which was 1/5 that in Zutphen. In the 1960s the Cretan men had lower average serum total cholesterol and systolic blood pressure levels and a lower prevalence of smokers. The difference in CHD mortality could partly be explained by baseline levels of these risk factors. A substantial part of the residual difference in CHD mortality may be explained by the baseline difference in diet between Crete and Zutphen.

The strengths of the present study include the nearly complete mortality follow-up from middle-age onwards, and the comprehensive long-term assessment of risk factors and diet. Also, rather than using food composition tables like many studies, we directly analyzed the nutrient content of the diet in food composites representing the average food consumption pattern of the men in Crete and Zutphen.

Table 4: Serum fatty acid composition, serum antioxidants and vitamins, and markers of oxidative stress, inflammation and endothelial function in 2000 at the age 80-99 years in the Cretan and Zutphen cohorts of the Seven Countries Study

	Age-adjusted			Multivariable-adjusted *		
Serum variable	**Crete**	**Zutphen**	**P**	**Crete**	**Zutphen**	**P**
Number	105	139		105	139	
Fatty acid composition, %						
C18:1 (n-9)	29.3 (28.7-29.9)	16.1 (15.8-16.6)	<.0001	29.2 (28.6-29.8)	16.1 (15.6-16.7)	<.0001
C18:2 (n-6)	41.1 (40.1-42.0)	53.6 (52.8-54.5)	<.0001	41.1 (4.0-42.1)	53.6 (52.8-54.5)	<.0001
C18:3 (n-3)	0.41 (0.39-0.44)	0.57 (0.55-0.60)	<.0001	0.41 (0.38-0.43)	0.58 (0.55-0.60)	<.0001
C20:5 (n-3)	0.84 (0.71-0.97)	1.22 (1.11-1.34)	<.0001	0.85 (0.72-0.99)	1.21 (1.09-1.33)	.0003
C22:6 (n-3)	0.59 (0.53-0.65)	0.71 (0.65-0.76)	.004	0.60 (0.54-0.66)	0.70 (0.64-0.75)	.03
Others	27.8 (27.2-28.4)	27.8 (27.3-28.3)	.92	27.8 (27.2-28.5)	27.7 (27.2-28.3)	.81
Antioxidants and folic acid						
Total carotenoids (µg/100 ml)	77.3 (70.7-83.9)	48.7 (42.9-54.5)	<.0001	75.5 (68.5-82.4)	50.1 (44.1-56.0)	<.0001
Beta-carotene (µg/100 ml)	26.1 (22.8-29.4)	16.3 (13.4-19.2)	<.0001	25.0 (21.5-28.5)	17.1 (14.1-2.1)	.001
Lycopene (µg/100 ml)	15.3 (13.8-16.8)	4.1 (2.8-5.3)	<.0001	15.3 (13.7-16.8)	4.1 (2.8-5.4)	<.0001
Alpha-tocopherol (µg/100 ml)	1244 (1189-1299)	1146 (1098-1194)	.009	1261 (1204-1317)	1134 (1085-1182)	.001
Folic acid (ng/ml)	7.5 (6.8-8.2)	5.5 (4.9-6.1)	<.0001	7.4 (6.6-8.1)	5.6 (5.0-6.3)	.0007
Markers of oxidative stress, inflammation, and endothelial function						
Malondialdehyde (mmol/l)	98.4 (94.3-102.5)	103.2 (99.7-106.7)	.08	98.1 (94.0-102.3)	103.4 (99.8-107.0)	.07
Hydroperoxides (µmol/l)	34.6 (26.3-45.3)	55.6 (43.9-70.4)	.01	33.2 (25.0-43.9)	57.3 (45.0-73.0)	.005
Gamma-glutamyl transferase (U/l)	20.2 (18.0-22.7)	26.1 (23.6-28.8)	.001	20.3 (18.0-22.9)	26.1 (23.5-28.9)	.003
hsCRP (mg/l)	2.7 (2.3-3.2)	3.6 (3.2-4.0)	.004	2.8 (2.4-3.2)	3.6 (3.2-3.9)	.01
sI-CAM1 (ng/ml)	626 (587-665)	708 (674-742)	.002	622 (581-663)	710 (675-746)	.002

Abbreviations: hsCRP, high-sensitive C-reactive protein; sICAM-1, soluble intercellular adhesion-molecule.

* Adjusted for age, BMI, smoking (yes/no), alcohol consumption (yes/no), physical activity, diet prescription (yes/no), and prevalence of diabetes (yes/no), myocardial infarction (yes/no), stroke (yes/no), or cancer (yes/no).

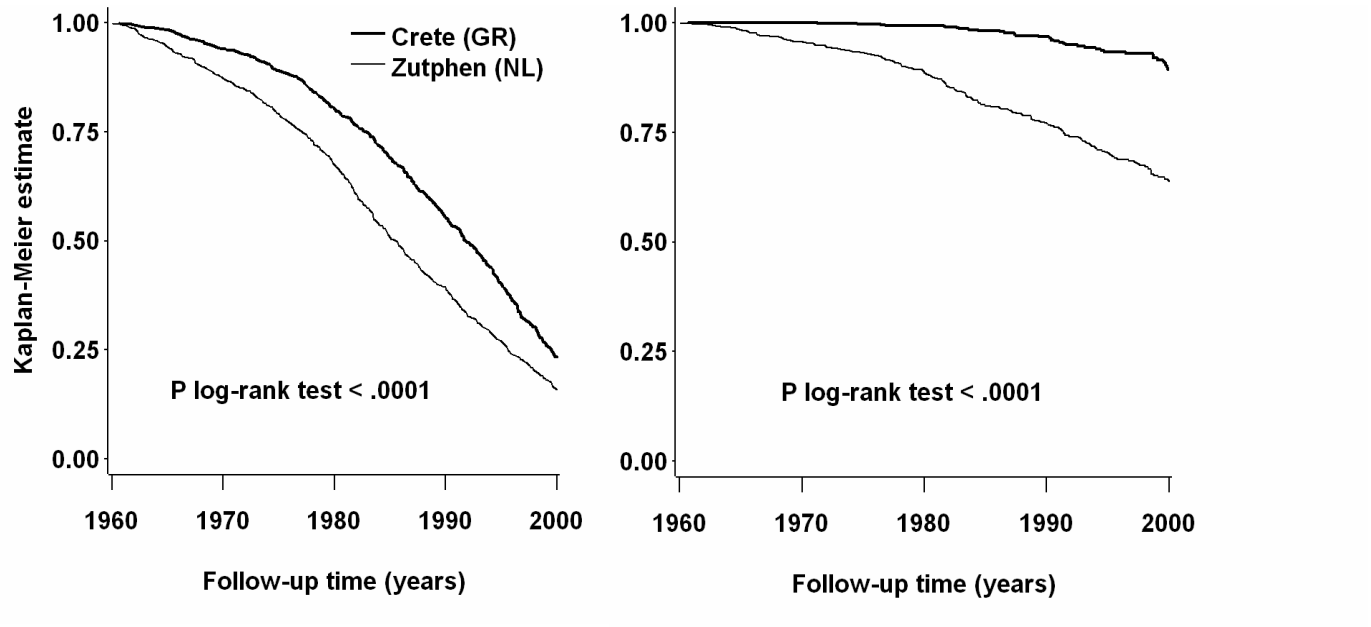

Figure 1: Kaplan-Meier survival curves for 40-year all-cause (left) and CHD mortality (right) in the Cretan and Zutphen cohorts of the Seven Countries Study.

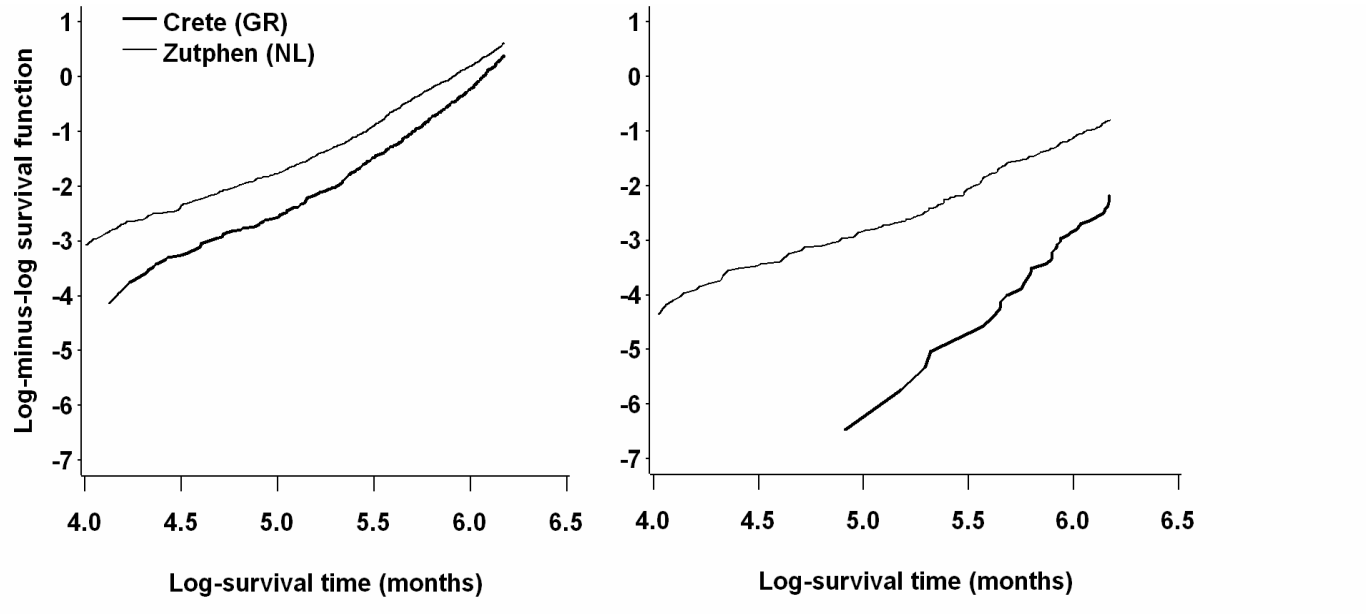

Figure 2: Log-minus-log survival curves for 40-year all-cause (left) and CHD mortality (right) in the Cretan and Zutphen cohorts of the Seven Countries Study.

Table 5: Hazard ratios (95% confidence intervals) for coronary heart disease mortality for risk factors measured at baseline, by duration of follow-up*

	Coronary heart disease mortality		
Variable	**0-30 year**	**0-40 year**	**30-40 year**
Age, per y	1.10 (1.07-1.13)	1.08 (1.05-1.11)	1.02 (0.97-1.08)
Serum cholesterol – per mmol/l	1.33 (1.17-1.51)	1.28 (1.15-1.43)	1.15 (0.91-1.44)
Systolic BP – per 20 mm Hg	1.35 (1.19-1.54)	1.34 (1.19-1.51)	1.29 (0.97-1.70)
Tobacco smoking			
Current smokers vs. non-smoker†	1.44 (0.92-2.24)	1.24 (0.86-1.79)	0.96 (0.49-1.90)
Recent ex-smokers vs. non-smokers†	0.94 (0.50-1.77)	0.93 (0.56-1.79)	1.00 (0.42-2.41)
Cohort – Zutphen vs. Crete			
Adjusted for age	8.83 (5.27-14.79)	5.43 (3.79-7.80)	2.49 (1.44-4.30)
Adjusted for age, serum cholesterol, systolic blood pressure, and smoking	6.41 (3.78-10.85)	4.09 (2.81-5.94)	2.11 (1.17-3.82)

Abbreviation: BP, blood pressure.
* Hazard ratios were adjusted for all other mentioned variables, unless otherwise indicated.
† Non-smokers were defined as never smokers and long-term ex-smokers (for details see text).

The study has also limitations. Although data on classical risk factors were available for all individual cohort members, those on nutrient intake were only available as average values for each cohort. Therefore, the ecologic estimates of whether the difference in dietary intake between the two cohorts explained the lower mortality rates in Crete should be judged cautiously.

Our objective was to evaluate to what extent classical risk factors explained differences in CHD mortality and to explore the role of diet herein. The results of our analyses using repeatedly collected data of individuals showed that about 30% of the difference in CHD mortality could be explained by differences in baseline serum cholesterol, blood pressure and the prevalence of smoking, with no further explanation arising from BMI, physical activity and resting heart rate as covariates. The results for serum cholesterol, systolic blood pressure and smoking were stronger for baseline data than for repeatedly collected data used in time-dependent models. This suggests that the predictive power of these risk factors is larger for middle age than for old age.

Differences in diet may explain a substantial part of the residual difference in CHD mortality between Crete and Zutphen. In 1960 there were large differences in the intake of fatty acids, dietary fiber and antioxidants. The intakes of saturated and trans fatty acids were 11 and 8% of energy lower and

the intake of dietary fiber 18g/day was higher in Crete. Also the intake of antioxidants, including carotenoids, alpha tocopherol and vitamin C was higher. These differences became smaller during follow-up but were still present in 2000.

Only a part of the difference in CHD mortality between Crete and Zutphen could be explained by traditional risk factors such as serum cholesterol, blood pressure, and smoking. This is in accord with the results of the Lyon Diet Heart Study (42). In this trial, a large effect of a Mediterranean diet enriched with alpha-linolenic acid was observed on CHD events, even though serum cholesterol, blood pressure, and BMI remained unaffected. Taken together, these findings suggest that other mechanisms are involved in lowering CHD mortality. Our findings of lower levels of oxidative stress, inflammation, and a more favorable endothelial function in Cretan men at old age suggest that these are important candidate mechanisms. Intervention studies have shown that the Mediterranean diet is likely to favorably influence these novel risk factors (5-9).

Based on the results of the Cretan cohort of the Seven Countries Study in the 1960s and of the study performed on Crete by the Rockefeller Foundation in 1948 (24), it is possible to characterize the traditional Cretan Mediterranean diet. This diet has olive oil as its principal source of fat and is rich in whole-meal bread and cereals, legumes, fruit and tomatoes and low in fish, poultry, meat and dairy products. The main characteristics of this diet were kept by the survivors in Crete in 2000. However, this traditional dietary pattern is rapidly disappearing from Crete and this is likely to have detrimental effects on the health status of the population of Crete (43).

We conclude from the findings of the present study that the difference in CHD mortality between Crete and Zutphen can partly be explained by differences in baseline serum cholesterol, blood pressure and smoking. It is likely that another part of the difference in CHD mortality is explained by the traditional Mediterranean diet. This diet is a prototype of a healthy diet with its low content of saturated and *trans* fatty acids and its high content of dietary fiber and antioxidants.

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Both Alpha- and Beta-Carotene, but Not Tocopherols and Vitamin C, Are Inversely Related to 15-Year Cardiovascular Mortality in Dutch Elderly Men

4

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The role of beta-carotene, alpha-tocopherol, and vitamin C in the prevention of cardiovascular diseases (CVD) is controversial. Prospective studies on gamma-tocopherol and carotenoids other than beta-carotene are sparse. We assessed relations between the intake of different carotenoids, alpha- and gamma-tocopherol, and vitamin C with 15-y CVD mortality in elderly men who participated in the Zutphen Elderly Study. Information on diet and potential confounding factors was collected in 1985, 1990, and 1995. In 1985, 559 men (mean age ~72 y) free of chronic diseases were included in the current analysis. After 15 y of follow-up, comprising 5744 person-years, 197 men had died from CVD. After adjustment for age, smoking, and other potential lifestyle and dietary confounders, relative risks (RR) (95% CI) of CVD death for a 1-SD increase in intake were 0.81 (0.66–0.99) for alpha-carotene and 0.80 (0.66–0.97) for beta-carotene. Carrots were the primary source of alpha- and beta-carotene and their consumption was related to a lower risk of death from CVD (adjusted RR, 0.83; 95% CI: 0.68–1.00). Intakes of carotenoids other than alpha- and beta-carotene were not associated with CVD mortality, nor were vitamin C and alpha- and gamma-tocopherol. In conclusion, dietary intakes of alpha-carotene and beta-carotene are inversely associated with CVD mortality in elderly men. This study does not indicate an important role for other carotenoids, tocopherols, or vitamin C in lowering the risk of CVD death.

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Introduction

During the past 25 y, there has been considerable interest in the role of antioxidants in the prevention of cardiovascular diseases (CVD). In particular, vitamin E (1) and beta-carotene (2) received much attention and, to a lesser extent, also vitamin C.

Findings from in vitro studies indicate that these antioxidants directly scavenge reactive oxygen and nitrogen species (3). In addition, depletion of vitamin E in humans leads to increased susceptibility of LDL to in vitro oxidation (4) and a reduction of LDL in vitro oxidation is observed after supplementation of alpha-tocopherol (5–8,9). Although there are indications that support a similar role for vitamin C (10), the evidence is equivocal (8). One intervention study, however, suggests that this antioxidant improves endothelial function (11) and a cross-sectional observational study in persons free of CVD found inverse associations of vitamin C with circulating markers of inflammation and endothelial function (12). Finally, in addition to their role as scavengers of free oxygen and nitrogen species, observational studies on carotenoids suggest that they also may have anti-inflammatory properties (13,14) and may improve endothelial function (14).

Early reports of prospective cohort studies have suggested that a high intake of vitamin E (15–17), carotene (15), fruit and vegetables rich in carotene (18), or carotenoids (17) lowers the risk of coronary heart disease. In sharp contrast, large-scale trials conducted in high-risk populations have not shown protective effects of these antioxidants against CVD (19,20). Likewise, intervention studies on the progression of atherosclerosis have in general not shown an effect of antioxidant supplementation either (21). Meanwhile, findings from observational studies continue to show inverse associations of dietary intake (22,23) or tissue levels (24,25) of especially beta-carotene with CVD risk.

The precise reason for the contradictory results between observational studies and large-scale intervention trials is yet unclear. One of the possibilities is that other carotenoids and tocopherols account for the inverse associations of beta-carotene and alpha-tocopherol with CVD in observational studies. Prospective studies on dietary carotenoids other than beta-carotene have only been published sparingly (22) and those on specifically dietary gamma-tocopherol are lacking. Therefore, we evaluated whether dietary intakes of 6 different carotenoids, alpha- and gamma-tocopherol, and also vitamin C were related to CVD mortality in elderly men living in the town of Zutphen, the Netherlands.

Materials and Methods

Study population. The Zutphen Elderly Study is a prospective population-based cohort study and is a continuation of the Zutphen Study, the Dutch contribution to the Seven Countries Study (26). Baseline data were collected between March and June in 1985 and repeated measurements were carried out in the same months in 1990 and 1995 (27). In 1985, 555 men of the original

cohort who were still alive and a random sample of 711 other men of the same age and also living in Zutphen were selected. Of these 1266 men aged 65–84 y, 939 participated (response 74%) in the study. Information on dietary intake in 1985 was collected from 876 men. Because men with known CVD, diabetes, and cancer may have changed their dietary habits, we excluded prevalent disease cases at baseline, yielding a study population of 559 men. In 1985 and 1990, the study was approved by the Medical Ethics Committee of Leiden University Medical Centre, the Netherlands, and in 1995 and 2000 by the Medical Ethics Committee of the Netherlands Organization for Applied Scientific Research.

Dietary assessment

Information on habitual food consumption was collected in 1985, 1990, and 1995 by using a cross-check dietary history method adapted to Dutch conditions (28). All subjects were interviewed about their dietary habits during the month preceding the interview. Experienced dieticians conducted the interviews, which took place at the subjects' homes in the presence of the person who usually prepared the hot meal. The habitual consumption of foods per day was estimated with a checklist and verified by estimating the quantities of foods bought per week for the whole family. In case this verification raised uncertainties about the consumption of a particular food, the participant was asked for more details.

Based on the information of the dietary histories in 1985, 1990, and 1995, the intake of nutrients and energy was calculated using the Netherlands food composition table from 1987–1988, 1989–1990, and 1996, respectively. The 2001 version of this table includes detailed information on the content of carotenoids, tocopherols, and folate in foods (29). Therefore, this release was used to calculate the intake of these nutrients in each survey year. Apart from the individual tocopherol levels, this food composition table also provides alpha-tocopherol equivalents (alpha-TE) in foods according to the formula alpha-tocopherol intake × 1.00 + beta-tocopherol × 0.40 + gamma-tocopherol × 0.10 + delta-tocopherol × 0.01 (29) [this conversion to a-TE differs from the conversion that was commonly used in the US, i.e. alpha-TE = mg alpha-tocopherol × 1.0 + mg beta-tocopherol × 0.5 + mg gamma-tocopherol × 0.1 + mg delta-tocopherol × 0.03 + mg alpha-tocotrienol × 0.3 + mg beta-tocotrienol × 0.05 (30).] The intake of *trans* fatty acids in every survey year was calculated by using previously collected information on the content of these fatty acids in foods consumed by the Zutphen elderly (31).

Collection of risk factor and morbidity data

During a medical exam, weight and height were measured while subjects wore light underwear. BMI was calculated by dividing weight by height squared (kg/m^2). Information on cigarette smoking, socioeconomic status, prescribed diet by a physician, and the use of aspirin, antihypertensive medication, and anticoagulants was collected using a questionnaire. All subjects were also asked whether they used (yes or no) multivitamin supplements or vitamin C or E supplements. Physical activity of walking, cycling, hobbies, sports, and gardening was

estimated in min/wk by using a validated questionnaire originally designed for retired men (32). In addition, socioeconomic status was assessed based on longest occupation. The highest occupational status included professionals, managers, and teachers.

The history of previous myocardial infarction, stroke, and cancer was ascertained using a questionnaire and was verified with information from general practitioners or hospital discharge information. Information on the history of diabetes mellitus was obtained from a standardized questionnaire.

Ascertainment of follow-up events

Municipal registries provided information on vital status and were checked at 5-y intervals. Two men were lost to follow-up during the study. Their time to follow-up was censored after their last physical exam. Information on the cause of death during 15 y of follow-up was obtained from hospital discharge data, cancer registries, and/or general practitioners. One clinical epidemiologist ascertained the final causes of death. The underlying causes of death were coded according to the Ninth Revision of the International Classification of Diseases (ICD-9). CVD cover ICD-9 codes 390–459. This included ischemic heart disease (ICD-9 codes 410–414) and stroke (codes 430–438) as main causes and also other diseases of the circulatory system. The first, 2nd, and 3rd cause of death was used to identify mortality from CVD. Information on the cause of death was lacking for 2 men and their follow-up was censored after they had died.

Data analysis

Relative risks (RR) and 95% CI were calculated for a 1-SD increase of antioxidants from diet only by using Cox regression models. To check the assumption on linearity, we also performed analyses for tertiles of antioxidant intake. Because this yielded similar findings, we report only RR based upon continuous analysis. In time-dependent analysis, we analyzed the cumulative mean intake of antioxidants using methods for repeated measurements (33). Specifically, mortality between 1985 and 1990 was related to the intake reported in 1985; mortality between 1990 and 1995 was related to the mean intake of 1985 and 1990 and mortality from the period 1995 to 2000 was related to the mean intake of 1985, 1990, and 1995. The intake of antioxidants and other dietary variables was adjusted for total energy intake by using the residual method (34).

We also investigated the total intake of vitamin C and alpha-tocopherol from diet and vitamin supplements. Because the information on dosage of vitamin supplements was not collected, we performed analyses in tertiles of intake, in which users of vitamin C were forced into the highest tertile of vitamin C, users of vitamin E in the highest tertile of alpha-tocopherol, and users of multivitamin supplements in the highest tertile of vitamin C and alpha-tocopherol. In these analyses, the lowest tertile served as the reference category.

Finally, we assessed whether a diet rich in 3 major antioxidants was related to CVD mortality by constructing a dietary antioxidant score, i.e. the sum of carotenoids, the sum of

alpha-tocopherol and gamma-tocopherol, and vitamin C. First, tertiles of intake (1 to 3) were computed for each of the 3 antioxidants. These 3 tertile variables were then summed, yielding a score that ranged from 3 (indicating a diet poor in antioxidants) to 9 (indicating a diet rich in antioxidants). Tertiles of the antioxidant score were computed and entered in the Cox regression models, in which the lowest tertile served as the reference. At baseline, the antioxidant score in the lowest tertile ranged from 3 to 5, the middle tertile consisted of men with a score of 6, and the score in the highest tertile ranged from 7 to 9.

All analyses were adjusted for age and total energy intake. We then adjusted additionally for current and former cigarette smoking. This model was extended with nondietary covariates, including BMI (kg/m^2), physical activity (min/wk), alcohol consumption (yes or no), diet prescription (yes or no), vitamin supplement use (yes or no), socioeconomic status (high vs. low), and the use of antihypertensive medication (yes or no), aspirin (yes or no), and anticoagulants (yes or no). The final model included additional adjustments for the intake of dietary fiber, folate, saturated fat, polyunsaturated fat, and *trans* fatty acids. Physical activity and nutrient intakes were entered in the models as cumulative means. Effect modification by smoking, alcohol consumption, physical activity, BMI, and socioeconomic status was evaluated by including product terms into the multivariable model B and were considered significant at $P < .05$. All analyses were performed with SAS version 9.1 (SAS Institute) and reported P-values are 2-sided.

Results

Characteristics of the study population

Characteristics of the study population in 1985, 1990, and 1995 are shown in **Table 1**. At baseline, the mean age of the men was ~72 y. During the study, the proportion of smokers decreased from one-third at baseline to one-fifth 10 y later. The use of vitamin supplements was ~15% throughout the study. The mean intake of most nutrients remained stable throughout the study, although the intake of lycopene increased and the intake of *trans* fatty acids decreased substantially during 10 y.

At baseline, vegetables contributed 82% to the intake of beta-carotene, with carrots (40%) and leafy vegetables (33%) as major sources. Carrots provided 94% of the amount of alpha-carotene. Main sources of vitamin E were margarines (43% contribution to the intake of alpha-tocopherol, 36% to gamma-tocopherol). For vitamin C, major sources were fruits (44%) and vegetables (23%).

Intakes of several dietary antioxidants were associated ($P < .05$) with each other. Examples are alpha-carotene and beta-carotene (Spearman $r = 0.74$), beta-cryptoxanthin and

zeaxanthin (r = 0.65), alpha-tocopherol and gamma-tocopherol (r = 0.56), and vitamin C with beta-cryptoxanthin (r = 0.64) and zeaxanthin (r = 0.60).

Table 1: Selected characteristics of the study population in 1985, 1990, and 1995

	Study year		
Characteristic	**1985**	**1990**	**1995**
Number of men participating	559	375	202
Demographic and risk factor data		Mean ± SD	
Age, y	71.8 ± 5.2	75.7 ± 4.5	80.1 ± 4.3
Body mass index, kg/m^2	25.6 ± 3.1	25.4 ± 3.2	25.1 ± 3.4
Current smoking, %	32.7	24.3	18.8
Physical activity, min/wk, median [IQR]	465 [225, 870]	480 [210, 750]	300 [110, 630]
Current alcohol consumption, %	432 (77)	284 (75)	159 (79)
Diet prescription, %	16.3	14.4	14.4
Use of vitamin C supplements, %	6.1	9.3	8.4
Use of multivitamin supplements, %	7.3	5.9	6.9
Aspirin, %	12.2	12.0	10.4
Antihypertensive medication, %	10.6	12.3	12.4
Anticoagulant medication, %	8.1	9.9	5.0
Dietary intake*			
Total energy, kJ/d	9572 ± 2096	8925 ± 1969	9001 ± 1904
Saturated fatty acids, g/d	44.3 ± 8.6	37.0 ± 8.5	39.1 ± 8.0
Trans fatty acids, g/d	11.2 ± 5.5	6.9 ± 3.4	4.5 ± 1.5
Monounsaturated fatty acids, g/d	27.5 ± 6.1	27.1 ± 4.7	27.8 ± 5.7
Polyunsaturated fatty acids, g/d	17.4 ± 7.0	16.4 ± 7.6	16.6 ± 7.2
Fiber, g/d	24.8 ± 6.1	23.4 ± 6.3	23.5 ± 5.5
Folate, µg/d	187 ± 54	183 ± 57	189 ± 47
Vitamin E, mg α-TE/d	12.5 ± 5.1	13.5 ± 6.2	13.4 ± 5.2
Alpha-tocopherol, mg/d	9.1 ± 4.6	9.1 ± 4.9	7.5 ± 3.5
Gamma-tocopherol, mg/d	9.3 ± 6.2	7.7 ± 6.0	7.9 ± 5.8
Vitamin C, mg/d	88 ± 38	94 ± 47	96 ± 45
Beta-carotene, µg/d	2766 ± 1474	2179 ± 1360	2487 ± 1408
Alpha-carotene, µg/d, median [IQR]	428 [136, 767]	231 [86, 622]	414 [148, 778]
Beta-cryptoxanthin, µg/d	47 ± 75	64 ± 86	109 ± 173
Lutein, µg/d	2477 ± 1490	1836 ± 1114	1853 ± 1107
Zeaxanthin, µg/d	109 ± 43	99 ± 49	99 ± 47
Lycopene, µg/d, median [IQR]	104 [39, 305]	166 [50, 579]	194 [59, 639]

Values are presented as mean ± SD, n (%), or median [IQR].
* Shown are dietary intakes; contributions from vitamin supplements were not included.

Dietary antioxidants and CVD mortality

After 15 y of follow-up, comprising 5744 person-years, 383 men had died (68.5%). CVD were the cause of death in 197 men. Major cardiovascular causes of death were ischemic heart disease (n = 89) and stroke (n = 52). In time-dependent analysis and after adjustment for age and total energy intake, a-carotene and beta-cryptoxanthin were significantly related to a lower CVD mortality (**Table 2**). After further adjustment for smoking, BMI, physical activity, alcohol use,

Table 2: Relative risks* of 15-y cardiovascular mortality by intake of dietary antioxidants in the Zutphen Elderly Study 1985-2000

Antioxidant	Mean ± SD daily intake at baseline (1985)†	Adjusted for age and energy intake‡		Adjusted for age, energy and smoking§		Multivariable- adjusted model A¶		Multivariable- adjusted model B#	
		RR	95% CI	RR	95% CI	RR	95% CI	RR	95% CI
Total carotenoids	6372 ± 3201 µg	0.90	0.76-1.05	0.92	0.79-1.08	0.90	0.77-1.06	0.82	0.69-0.98
Alpha-carotene	541 ± 586 µg	0.81	0.67-0.99	0.84	0.69-1.02	0.81	0.66-0.98	0.81	0.66-0.99
Beta-carotene	2766 ± 1474 µg	0.89	0.75-1.05	0.91	0.77-1.08	0.88	0.74-1.05	0.80	0.66-0.97
Luteine	2477 ± 1490 µg	1.08	0.93-1.25	1.08	0.93-1.24	1.06	0.92-1.22	0.95	0.81-1.12
Lycopene	442 ± 1112 µg	0.85	0.71-1.02	0.87	0.73-1.04	0.88	0.73-1.05	0.91	0.76-1.08
Beta-cryptoxanthin	47 ± 75 µg	0.84	0.71-0.99	0.85	0.72-1.00	0.85	0.72-1.01	0.86	0.72-1.03
Zeaxanthin	109 ± 43 µg	0.94	0.81-1.09	0.95	0.82-1.10	0.92	0.79-1.07	0.88	0.70-1.10
Vitamin C	88 ± 38 mg	0.89	0.76-1.04	0.92	0.78-1.07	0.91	0.78-1.07	1.02	0.85-1.23
Vitamin E**	12.5 ± 11.9 mg alpha-TE	0.93	0.79-1.10	0.93	0.79-1.10	0.92	0.78-1.09	0.92	0.76-1.12
Alpha-tocopherol	9.1 ± 4.6 mg	0.98	0.85-1.14	0.99	0.85-1.14	1.00	0.86-1.16	0.96	0.82-1.12
Gamma-tocopherol	9.3 ± 6.2 mg	1.04	0.90-1.20	1.02	0.88-1.18	1.01	0.87-1.18	0.94	0.79-1.12

* Shown are relative risks and 95%CI for a 1-SD increase in intake of dietary antioxidants; the intake from vitamin supplements was not included.

† Shown are mean intakes ± SD from diet; contributions by vitamin supplements were not included.

‡ Adjusted for age (continuous), and energy intake (continuous).

§ Adjusted for age (continuous), energy intake (continuous), and smoking (indicator variables of current and former smoking).

¶ Adjusted for age (continuous), energy intake (continuous), smoking (indicator variables of current and former smoking), body mass index (continuous), physical activity (continuous), alcohol consumption (yes or no), socioeconomic status (high versus medium and low), use of multivitamin supplements (yes or no), use of vitamin C supplements (yes or no), use of aspirin (yes or no), use of antihypertensive drugs (yes or no), use of anticoagulants (yes or no).

As Model A, with additional adjustment for diet prescription, intake of fiber, β-carotene, vitamin C, α-tocopherol, folate, saturated fatty acids, *trans* fatty acids, polyunsaturated fatty acids. Intake of all carotenoids was not adjusted for other carotenoids and no adjustment was made for the antioxidant under investigation.

** Alpha-tocopherol Equivalents (alpha-TEs) as calculated by alpha-tocopherol + beta-tocopherol × 0.40 + gamma-tocopherol × 0.10 + delta-tocopherol × 0.01.

socioeconomic status, the use of medications, vitamin supplements, and dietary factors, a 1-SD increase in intake of alpha- and beta-carotene was associated with ~20% lower RR of death from CVD. An association of similar strength was observed for the consumption of carrots, the major source of alpha- and beta-carotene, in relation to 15-y CVD mortality (multivariate-adjusted RR = 0.83; 95% CI = 0.68-1.00). Intakes of carotenoids other than alpha- and beta-carotene were not significantly associated with CVD mortality, nor were intakes of vitamin C and vitamin E analyzed as alpha-TE, alpha-tocopherol, or gamma-tocopherol.

To assess whether the relations of alpha- and beta-carotene with CVD mortality differed by levels of established CVD risk factors, we performed stratified analyses. We did not find significantly different associations for smokers and nonsmokers, for users and nonusers of alcoholic beverages, for those in the highest or lowest 2 tertiles of minutes of physical activity, for men with BMI < 25 and ≥25 kg/m^2, or between levels of socioeconomic status (P > .05 for all).

To avoid bias from change in diet and lifestyle due to nonfatal cardiovascular events, analyses were performed in which we stopped updating the information on diet and physical activity if subjects reported a nonfatal myocardial infarction or stroke (whichever came first) during the follow-up of the study, comprising 136 men. This, however, did not change our findings. We also analyzed the associations in nonusers of vitamin supplements (n = 504) and found virtually the same results. We finally performed analyses without possible subclinical CVD cases at baseline by excluding men who died within the first 2 y of follow-up (n = 15). This strengthened the multivariate-adjusted RR for a 1-SD increase to 0.72 (95% CI = 0.57-0.90) for alpha-carotene and 0.74 (0.60-0.91) for beta-carotene.

Table 3: Relative risks* of 15-y cardiovascular mortality by intake of antioxidant vitamins C and E from diet and supplements in the Zutphen Elderly Study 1985-2000

	Tertiles of total intake			P for linear trend
Antioxidant vitamin	**Lowest**	**Middle**	**Highest**	
Vitamin C				
Adjusted for age and energy	1.00	0.83 (0.58-1.20)	0.67 (0.46-0.98)	.03
Adjusted for age, energy, and smoking	1.00	0.84 (0.58-1.20)	0.70 (0.48-1.02)	.06
Multivariate-adjusted model A†	1.00	0.83 (0.57-1.20)	0.70 (0.48-1.03)	.07
Multivariate-adjusted model B‡	1.00	0.95 (0.64-1.42)	0.86 (0.57-1.30)	.45
Vitamin E				
Adjusted for age and energy	1.00	0.75 (0.52-1.08)	0.65 (0.44-0.94)	.03
Adjusted for age, energy, and smoking	1.00	0.75 (0.52-1.08)	0.65 (0.45-0.95)	.03
Multivariate-adjusted model A†	1.00	0.76 (0.52-1.10)	0.67 (0.46-0.98)	.05
Multivariate-adjusted model B‡	1.00	0.82 (0.56-1.21)	0.73 (0.49-1.08)	.12

* Shown are relative risks (95% CIs) for cardiovascular mortality by tertiles of total antioxidant intake. Users of vitamin supplements were forced into the highest tertile; for details see text.

† Adjusted for age (cont.), energy (cont.), body mass index (cont.), physical activity (cont.), alcohol consumption (yes or no), socioeconomic status (high versus medium and low), use of aspirin (yes or no), use of antihypertensive drugs (yes or no), use of anticoagulants (yes or no).

‡ As Model A, with additional adjustment for diet prescription, intake of fiber, beta-carotene, folate, saturated fatty acids, *trans* fatty acids, polyunsaturated fatty acids. The intake of vitamin C was adjusted for the intake vitamin E, and vice versa.

Total intake of vitamins C and E, and CVD mortality

The total intake of vitamin C and vitamin E, i.e. from diet and vitamin supplements together, was studied in tertiles. After adjustment for age, energy intake, and lifestyle not including dietary factors (Model A), total vitamin E intake was significantly and total vitamin C intake borderline significantly associated with CVD mortality, indicating ~30% lower CVD mortality risk in the highest tertile compared with the lowest (**Table 3**). Further adjustment for dietary factors (Model B), especially *trans* fatty acids, attenuated these relations to yield nonsignificant findings (P for trend > .10 for both).

Dietary antioxidant score and CVD mortality

A diet high in 3 major antioxidants, i.e. carotenoids, vitamin E, and vitamin C, was related to a lower CVD mortality, although this finding was borderline (P for trend = .05) at the customary level of significance (**Table 4**). To investigate the importance of alpha- and beta-carotene in this antioxidant score, we reassessed the score in which alpha- and beta-carotene were not included. This alternative score was composed of the sum of carotenoids other than alpha- and beta-carotene, vitamin C, and the sum of alpha- and gamma-tocopherol. RR (95% CI) according to the multivariable-adjusted model B for increasing tertiles of this antioxidant score were 1.00, 1.02 (0.71-1.47), and 0.80 (0.55-1.16) (P for trend = .34).

Discussion

In this prospective cohort study in Dutch elderly men, dietary alpha-carotene and beta-carotene were inversely related to CVD mortality. Carrots were the primary source of both alpha- and beta-carotene and their consumption was also associated with a lower risk of CVD death. In contrast, the intake of 4 other carotenoids and that of vitamin E and vitamin C were not associated with mortality from CVD.

Table 4: Relative risks* of 15-y cardiovascular mortality by dietary antioxidant score in the Zutphen Elderly Study 1985-2000

	Tertiles of total intake			P for linear trend
Model	**Lowest**	**Middle**	**Highest**	
Adjusted for age and energy	1.00	0.94 (0.66-1.34)	0.74 (0.53-1.03)	.04
Adjusted for age, energy, and smoking	1.00	0.96 (0.68-1.37)	0.78 (0.56-1.08)	.08
Multivariate-adjusted model A†	1.00	0.94 (0.66-1.34)	0.75 (0.54-1.05)	.06
Multivariate-adjusted model B‡	1.00	0.87 (0.60-1.25)	0.73 (0.60-1.05)	.05

* Shown are relative risks (95% CIs) by tertiles of dietary antioxidant score, contributions by vitamin supplements were not included (for details see text).
† Adjusted for age (cont.), energy (cont.), body mass index (cont.), physical activity (cont.), alcohol consumption (yes or no), socioeconomic status (high versus medium and low), use of multivitamin supplements (yes or no), use of vitamin C supplements (yes or no), use of aspirin (yes or no), use of antihypertensive drugs (yes or no), use of anticoagulants (yes or no).
‡ As Model A, with additional adjustment for diet prescription, intake of fiber, folate, saturated fatty acids, *trans* fatty acids, polyunsaturated fatty acids.

The strengths of this study include its prospective design, the nearly complete mortality follow-up, and the relatively high CVD mortality rate. Also, the use of cumulative means of repeated measurements of diet and physical activity better reflect long-term exposure and reduce within-person variation (33). This study also has limitations. First, the credibility of observational studies is often criticized because of the potential of residual confounding due to measurement error and unmeasured variables (35). In this study, men with higher intakes of alpha- and beta-carotene were less likely to smoke, more often used multivitamin supplements, and had a higher socioeconomic status. Although the relation of alpha- and beta-carotene with CVD mortality did not differ between levels of these variables, we cannot exclude the possibility of residual confounding by these or other factors. Second, as many dietary carotenoids, especially alpha- and beta-carotene, were correlated with each other, their independent association with CVD mortality was difficult to establish. Third, the sample size of the study population limited the power to detect small effects of antioxidants and their dietary sources. Finally, information about the dose and frequency of vitamin supplement use was not collected in this study. Although we forced users of vitamin supplements in the highest tertile of antioxidant intake, this may have resulted in some misclassification.

Several prospective cohort studies on dietary carotenoids showed an inverse relation between the intake of beta-carotene or total carotenoids and the risk of coronary heart disease (22,36), although sometimes only in subgroups (15). This is in line with the inverse relation between the intake of beta-carotene and CVD mortality in the current study. The Nurses' Health Study also confirmed the inverse relation for alpha-carotene (22).

Findings from observational studies that addressed relations between circulating carotenoids and intermediaries for CVD and clinical CVD are inconsistent. Subjects with high blood concentrations of a- or b-carotene had less atherosclerosis compared with subjects with low concentrations (37,38), although this finding is not consistent in all studies (39,40). Yet, circulating levels of either alpha- and beta-carotene, or both, are inversely associated with future CVD in some (24,41) but not all studies (13,42).

In this study, we constructed an antioxidant score combining 6 carotenoids, 2 tocopherols, and vitamin C, in which each of these 3 antioxidants was given the same weight. Such an approach may be preferable to studying 1 antioxidant at a time, as single antioxidants are less likely to lower CVD risk (19–21), whereas a diet rich in multiple antioxidants may do so. However, the weak inverse association between the antioxidant score and CVD mortality in this study was driven by alpha- and beta-carotene and did not clearly support the hypothesis that a diet rich in multiple antioxidants lowers CVD risk. To our knowledge, only 1 other study has applied a score in which dietary antioxidants were included (43). In that study population, which consisted of male smokers, an oxidative balance score based on the dietary intake of beta-carotene, vitamin C, and iron was not related to CVD mortality (43).

Alpha and beta-carotene are considered to be important antioxidants. It has been hypothesized that lipoprotein oxidation induced by reactive nitrogen species within the arterial

wall preferably depletes these carotenes compared with other fat-soluble antioxidants (44). When the diet no longer provides sufficient amounts of these carotenes, their concentrations in LDL and plasma will decrease and lipoproteins will become more prone to oxidation. However, although it has been shown that in vitro lipoprotein oxidation induced by reactive nitrogen species depletes a- and b-carotene (44), it is yet not clear whether reactive nitrogen species are an important cause of in vivo lipoprotein oxidation. Also, recently it was shown that circulating carotenoids, including alpha- and beta-carotene, are inversely associated with inflammation and oxidative stress and positively associated with markers of endothelial function (14), which suggests that they may influence CVD risk through different pathways. Alternatively, it may also be that other nutrients and dietary bioactive compounds, correlated with dietary alpha- and beta-carotene, may account for the observed inverse association of these 2 carotenoids with cardiovascular mortality.

Research on vitamin E has been mainly focused on alpha-tocopherol, whereas scientific interest in gamma-tocopherol was raised only recently (45). Gamma-tocopherol constitutes ~70% of the vitamin E in the typical American diet (46) and 50% in the diet consumed by the population in this study. Our findings do not support a role of vitamin E in lowering mortality from CVD. Observational studies on vitamin E and CVD have yielded conflicting results, whereas large-scale trials with vitamin E largely produced null results (19). The most conspicuous study is the Alpha-Tocopherol Beta-Carotene trial, in which 50 mg/d synthetic vitamin E did not affect primary and secondary prevention of CVD in male Finnish smokers (47,48), but baseline serum levels of alpha-tocopherol were, paradoxically, inversely related to CVD mortality (49).

Despite the possibility that vitamin C may exert anti-inflammatory effects (11) and may improve endothelial function (12), dietary vitamin C intake was not related with CVD mortality in this study or in large prospective cohort studies on dietary vitamin C intake and cardiovascular events (15,50–53). Although in a recent meta-analysis of large prospective cohort studies vitamin C from foods and supplements was related to a lower risk of coronary heart disease (54), the few trials that have investigated the efficacy of vitamin C supplementation on mortality do not support a role for this antioxidant in reducing overall mortality (55).

In summary, intakes of alpha- and beta-carotene, especially from carrots, were inversely related to a CVD death in elderly men. More observational studies on the intake of individual carotenoids and their specific sources, as well as foods correlated with their intake, in relation to CVD are warranted. Our findings on dietary vitamin C and alpha- and gamma-tocopherol do not support a role for these antioxidants in lowering CVD mortality.

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5

Plasma Carotene and Alpha-Tocopherol in Relation to 10-Year All-Cause and Cause-Specific Mortality in European Elderly: the Survey in Europe on Nutrition and the Elderly, a Concerted Action (SENECA)

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Background: Only a few observational studies have related plasma carotene and alpha-tocopherol to mortality in elderly subjects.

Objective: The objective was to study the association of plasma carotene (alpha- and beta-carotene) and alpha-tocopherol with all-cause and cause-specific mortality in elderly subjects who participated in a European prospective study.

Design: Plasma concentrations of carotene and alpha-tocopherol were measured in 1168 elderly men and women. After a follow-up period of 10 y, 388 persons had died. The association between plasma antioxidants and mortality was analyzed by using Cox proportional hazard models. To put our results in context, we performed a meta-analysis of 5 studies on plasma antioxidants and all-cause mortality in elderly populations.

Results: Plasma carotene concentrations were associated with a lower mortality risk [adjusted rate ratio (RR) for an increment of 0.39 μmol/L: 0.79; 95% confidence interval (CI): 0.70, 0.89]. This lower mortality risk was observed for both cancer (RR: 0.59; 95% CI: 0.44, 0.79) and cardiovascular disease (RR: 0.83; 95% CI: 0.70, 1.00). The lower risk in cardiovascular death risk was confined to those with a body mass index (in kg/m^2) <25 (RR: 0.67; 95% CI: 0.49, 0.94). Plasma concentrations of alpha-tocopherol were not associated with all-cause or cause-specific mortality. The results for both plasma antioxidants and all-cause mortality were confirmed by the meta-analysis.

Conclusions: This prospective study suggests that high plasma concentrations of carotene are associated both with a lower mortality from all causes and with cancer in the elderly. For cardiovascular mortality, the inverse association was confined to elderly with body mass indexes <25.

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Introduction

Aging is associated with higher levels of oxidative stress (1), which are likely to play an important role in the development of cardiovascular disease (CVD) (2) and cancer (3). Oxidative stress is lower among subjects with a high antioxidant status (4). Therefore, elderly subjects with a high antioxidant status may have a lower risk of these chronic diseases.

Carotene and vitamin E are 2 fat-soluble antioxidants that have received much scientific attention during the past decade. Observational studies relating plasma or serum concentrations of beta-carotene to CVD and cancer and relating vitamin E to CVD are mainly conducted in middle-aged populations. A high status of beta-carotene was related to a lower risk of CVD in most (5–8) but not all (9) observational studies. A few studies of vitamin E status found an inverse association with CVD (10,11), but most others did not (5,7,11,12,13). A high beta-carotene status was strongly related to a low risk of cancer, especially lung cancer (14).

Studies of beta-carotene and vitamin E status in elderly populations are limited to a few observational studies that related plasma concentrations of these antioxidants to mortality (15–19). Most of these studies focused on all-cause mortality and had a small sample size. Although several studies found an inverse association of serum beta-carotene or total carotenoid concentrations with all-cause (15,18,19) and cardiovascular mortality (15,18), this association was in most cases not significant. One study did not find a relation between plasma beta-carotene and all-cause mortality (17). Plasma alpha-tocopherol was not associated with either all-cause (15–18) or cardiovascular mortality (15,16,18) in elderly subjects. Only one study investigated the relation of plasma carotenoids and vitamin E to cancer mortality in an elderly population, and it found no association (15).

Measures of overweight and obesity, such as body mass index, are consistently found to be inversely associated with levels of plasma beta-carotene (20–22), whereas their association with plasma alpha-tocopherol is less clear (20,22,23). Obesity has been shown to be associated with systemic low-grade inflammation (24) and oxidative stress (25). Taken together, these observations could indicate that plasma beta-carotene in particular is depleted because of oxidative stress in obese subjects.

We have studied whether plasma concentrations of carotene and alpha-tocopherol were associated with all-cause and cause-specific mortality in apparently healthy elderly subjects who participated in a prospective European study. We examined whether these associations were modified by body mass. Finally, to put our results in context, we performed a meta-analysis of observational studies of both plasma antioxidants in relation to all-cause mortality in elderly populations.

Subjects and methods

Study population

The Survey in Europe on Nutrition and the Elderly, a Concerted Action (SENECA) is a prospective study investigating whether diet and lifestyle influence the health of elderly people in various European countries (26). Subjects were excluded if they were psycho-geriatric patients living in nursing homes, did not speak the country's language fluently, or were not able to answer questions independently.

Between December 1988 and March 1989, baseline measurements took place in a random age-gender stratified sample of European elderly aged 70-75 y (27). Nine study centers, in which 2038 subjects were examined, collected blood samples for antioxidant measurements and provided information about vital status and causes of death. Participation rates varied between 34% and 62% (27). Plasma antioxidants were actually measured in 1682 subjects. We excluded 102 participants with missing information on important variables and another 392 with a history of myocardial infarction, stroke, diabetes, or cancer. In addition, 16 participants were excluded because information on follow-up time was lacking, and 4 participants were excluded because their plasma antioxidant concentrations exceeded the mean by >9 times the SD. This study is therefore based on 1168 European elderly.

All participants gave written informed consent. Approval of the study was obtained from the participating SENECA centers.

Antioxidant and lipid measurements

Blood samples were obtained by venipuncture after an overnight fast. Plasma and sera were stored at –80 ºC before transport to the Division of Human Nutrition at Wageningen University for measurement of serum total and HDL cholesterol. For plasma antioxidant measurements, plasma samples were then send to the laboratories of Hoffmann-La Roche Ltd (Basel, Switzerland). Plasma concentrations of carotene and alpha-tocopherol were determined by HPLC methods (28,29). For the present study the sum of alpha- and beta-carotene was reported by the laboratory. CVs for the day-to-day variance of duplicate assays of one sample were 3.0% for carotene and 1.8% for alpha-tocopherol. Total and HDL cholesterol and triacylglycerols were measured in sera by using enzymatic calorimetric methods in the standardized lipid laboratory of Wageningen University (30).

Lifestyle assessment and health status

Baseline information on smoking, years spent in full-time education, the use of vitamin supplements, alcohol consumption, and the prevalence of diabetes, myocardial infarction, stroke, and cancer was obtained by using questionnaires (31). Alcohol consumption was categorized as use and nonuse. Household, sports, and leisure time physical activities were estimated by using a

validated questionnaire (32). Physical activity was classified in the total study population by sex-specific tertiles.

Body weight was measured to the nearest 0.5 kg on a calibrated scale. Subjects were weighed in the morning after breakfast and after emptying the bladder; they were wearing light underclothing. Height was measured to the nearest 0.1 cm while subjects were standing erect and wearing no shoes. Body mass index (BMI) was calculated by dividing body weight (in kg) by the square of height (m^2).

Case ascertainment

Information on vital status was collected in 1999–2000. One experienced clinical epidemiologist coded the causes of death by using the ninth revision of the World Health Organization International Classification of Diseases (ICD9) (33). CVD is covered by ICD9 codes 390–459, and cancer is covered by codes 140–209. The cause of death was unknown for 104 subjects. For the analysis of cause-specific mortality, the follow-up time of these subjects was censored. Only 4 subjects were lost to follow-up; their follow-up time was censored after 5 y. After 10 y of follow-up, 388 had died–38% of CVD and 25% from cancer.

Statistical analysis

Descriptive analyses were performed with stratification for cohort and tertiles of plasma carotene and alpha-tocopherol. Because the distribution of plasma carotene was skewed to the right, values were log transformed and, therefore, geometric means and geometric SDs are presented.

Cox proportional hazards models were used to obtain rate ratios (RR) and 95% CIs for the association between a 1-SD increment in plasma antioxidant concentration and mortality. Although women had a higher average concentration of both plasma carotene and alpha-tocopherol, all analyses were done for men and women combined because the associations with mortality did not differ between men and women. Analyses were adjusted for age (continuous) and sex (Model 1); in additional multivariate analyses, they were also adjusted for other covariates [Model 2: BMI (continuous), serum total cholesterol (continuous), serum HDL cholesterol (continuous), current smoking (yes or no), alcohol consumption (yes or no), physical activity (low versus medium and high tertiles), and SENECA center (indicator variables)]. All analyses were further adjusted for either plasma carotene or plasma alpha-tocopherol (Model 3).

Plasma carotene and alpha-tocopherol were measured 5 y later in a subsample of 644 participants. Using Pearson's coefficient for correlation between the measurements at baseline and 5 y later, we adjusted our risk estimates for measurement error and within-person variance (regression dilution bias) (34).

To examine the roles of body mass, smoking, and sex, stratified analyses were performed. Possible interactions of plasma concentrations of antioxidants with body mass or smoking for mortality were studied by entering product terms in the Cox regression models.

We performed a meta-analysis of observational studies of the association of plasma (beta-)carotene or alpha-tocopherol with all-cause mortality in subjects aged ≥60 y, including the current study. We performed a systematic, computerized literature search by using a combination of the Medical Subject Heading terms "aged," "beta-carotene," "carotenoids," "vitamin E," "antioxidants," and "mortality." References lists of selected articles were inspected. In addition to 3 studies which measured plasma beta-carotene (17–19), we included one study that measured plasma carotenoids (15). Reported adjusted RRs were used to calculate new mortality rates in the lowest and highest category of plasma antioxidant. On the basis of these event rates, RRs were calculated in which the lowest plasma category was taken as the reference. We used a random-effects model to combine the results of all studies (35). Heterogeneity was assessed by inconsistency squared (I^2), which describes the percentage of total variation between studies that is due to heterogeneity rather than to chance; higher values indicated greater heterogeneity (36).

All data were analyzed by using SAS software (version 9.1; SAS Institute, Cary NC, USA). Statistical tests were 2-sided, and P values < .05 were considered significant.

Results

Plasma carotene varied from 0.28 μmol/L in Belgian subjects to 0.69 μmol/L in Swiss subjects. Plasma alpha-tocopherol ranged from 25.3 μmol/L in the Belgians to 35.6 μmol/L in the Swiss. Selected characteristics of the participants from the SENECA centers are shown in **Table 1**. Plasma concentrations of carotene and alpha-tocopherol were positively associated with serum total and serum HDL cholesterol and inversely associated with smoking (**Table 2** and **Table 3**). Plasma carotene was inversely related to serum triacylglycerols and physical activity, whereas plasma alpha-tocopherol was positively associated with serum triacylglycerols and years spent in full-time education.

After multivariate adjustment, subjects with BMI s ≥ 25 had lower mean plasma carotene concentrations compared than did subjects with BMIs < 25 (0.47 μmol/L and 0.60 μmol/L, respectively; P < .0001). Plasma alpha-tocopherol was not associated with BMI. Women had higher concentrations of both plasma alpha-tocopherol and plasma carotene than did men (P < .0001 for both after multivariate adjustment).

Plasma antioxidants and mortality

As shown in **Table 4**, plasma carotene concentrations were inversely associated with mortality due to all causes, CVD, and cancer. The strongest inverse association was observed for mortality due to cancer. Additional adjustment for plasma alpha-tocopherol yielded similar results.

Excluding subjects with subclinical CVD and cancer by omitting the first 2 y of follow-up resulted in similar associations. The inverse association with cancer mortality became stronger (RR for an increment of 0.39 μmol/L plasma carotene: 0.50; 95% CI: 0.35, 0.72),

Table 1: Baseline characteristics of apparently healthy participants (n=1168) by SENECA study center

Characteristic	Hamme, Belgium (n=137)	Roskilde, Denmark (n=122)	Haguenau, France (n=144)	Romans, France (n=107)	Padua, Italy (n=156)	Culemborg, Netherlands (n=149)	Villa Franca, Portugal (n=112)	Betanzos, Spain (n=118)	Yverdon, Switzerland (n=123)
Demographics and lifestyle									
% Men	57	49	53	50	49	45	52	47	46
Age (years)	73.0 (1.8)	72.9 (3.1)	72.9 (1.7)	73.1 (1.8)	73.5 (1.7)	72.8 (1.7)	72.4 (1.6)	72.7 (1.9)	72.9 (1.7)
Body Mass Index (kg/m^2)	26.3 (3.9)	25.4 (4.2)	27.2 (4.2)	26.0 (3.8)	25.5 (4.0)	27.0 (3.8)	26.9 (4.1)	27.9 (3.9)	26.6 (4.1)
Education (years)	9.0 (2.4)	10.3 (3.4)	9.4 (2.7)	8.2 (3.0)	8.9 (4.7)	8.6 (2.8)	2.6 (2.7)	6.7 (3.7)	9.4 (2.2)
% Smoker	27	43	17	7	23	23	8	19	14
% Alcohol use	62	81	85	79	84	65	45	52	72
% Low physical activity	41	33	39	59	24	29	21	29	24
% Vitamin supplement use	3	59	0	0	15	23	6	0	9
Plasma antioxidants and serum lipoproteins									
Plasma carotene (µmol/L) *	0.28 (0.21)	0.50 (0.35)	0.49 (0.37)	0.67 (0.47)	0.67 (0.49)	0.50 (0.26)	0.59 (0.49)	0.43 (0.32)	0.69 (0.44)
Plasma alpha-tocopherol (µmol/L)	25.3 (8.3)	30.4 (8.1)	31.7 (8.7)	30.8 (8.2)	32.9 (8.5)	29.9 (7.2)	28.9 (8.5)	30.8 (8.6)	35.6 (8.1)
Plasma alpha-tocopherol (µmol/L) †	24.6 (0.5)	30.0 (0.6)	32.7 (0.5)	30.6 (0.6)	33.4 (0.5)	29.8 (0.5)	29.9 (0.6)	31.5 (0.6)	33.7 (0.6)
Serum total cholesterol (mmol/L)	6.5 (1.3)	6.5 (1.3)	6.1 (1.1)	6.4 (1.1)	6.3 (1.0)	6.4 (1.1)	6.2 (1.2)	6.2 (1.1)	6.8 (1.1)
Serum HDL cholesterol (mmol/L)	1.30 (0.35)	1.43 (0.40)	1.36 (0.37)	1.42 (0.38)	1.53 (0.41)	1.31 (0.35)	1.23 (0.34)	1.36 (0.38)	1.39 (0.38)
Serum triacylglycerols (mmol/L)	1.4 (0.7)	1.3 (0.8)	1.4 (0.8)	1.4 (0.6)	1.5 (0.7)	1.4 (0.7)	1.3 (0.7)	1.4 (1.3)	1.4 (0.8)

Values are means (SD) unless otherwise indicated.

*Geometric mean (geometric SD).

† Mean plasma alpha-tocopherol (SE), adjusted for total serum cholesterol.

Table 2: Baseline characteristics of the study population (n=1,168) by tertiles of plasma carotene

Characteristic	Tertiles of plasma carotene			P value*
	Lowest tertile (n=385)	Medium tertile (n=390)	Highest tertile (n=393)	
Plasma carotene, µmol/L†	0.22 (0.12)	0.54 (0.09)	1.12 (0.39)	
Plasma carotene, µmol/L‡	0.23 (0.22-0.24)	0.54 (0.52-0.56)	1.10 (1.06-1.14)	
Demographics and lifestyle				
Age, y	73.0 (2.3)	73.0 (1.7)	72.8 (1.8)	.32
% Men	70	47	34	<.0001
Body Mass Index, kg/m^2	27.3 (4.0)	26.8 (4.2)	25.6 (3.8)	<.0001
Education, y	8.0 (3.7)	8.2 (3.5)	8.5 (4.2)	.18
% Smoker	34	18	11	<.0001
% Alcohol use	75	70	65	.02
% Low physical activity	36	36	26	.004
% Vitamin supplement use	12	11	16	.11
Plasma tocopherol and serum lipoproteins				
Plasma alpha-tocopherol, µmol/L	26.5 (8.2)	31.5 (7.9)	34.0 (8.1)	<.0001
Plasma alpha-tocopherol, µmol/L§	28.1 (0.35)	31.4 (0.34)	32.6 (0.34)	<.0001
Serum total cholesterol, mmol/L	6.0 (1.1)	6.4 (1.1)	6.7 (1.2)	<.0001
Serum HDL cholesterol, mmol/L	1.29 (0.37)	1.35 (0.37)	1.48 (0.38)	<.0001
Serum triacylglycerols, mmol/L	1.5 (1.0)	1.4 (0.7)	1.3 (0.6)	.002

Values are means (SD) unless otherwise indicated.
* P value based on analysis of (co)variance or Chi-square test.
† Geometric mean (geometric SD).
‡ Geometric mean (95% CI), adjusted for gender.
§ Mean plasma alpha-tocopherol (SE), adjusted for total serum cholesterol.

though the association with cardiovascular mortality was slightly attenuated (RR: 0.89; 95% CI: 0.74, 1.07).

The inverse association between plasma carotene and risk of death due to CVD was confined to subjects with a low body mass. Persons with BMIs < 25 had RRs of 0.67 (95% CI: 0.49, 0.94), whereas those with a BMIs ≥ 25 had RRs of 0.97 (95% CI: 0.79, 1.20) (P for interaction = .033; based on model 3 without adjustment for BMI). The association between plasma carotene and CVD death did not differ between men and women (P for interaction = .65), and nor did between smokers and non-smokers (P for interaction = .63).

After adjustment for age, sex, and other potential confounders, plasma alpha-tocopherol was not significantly associated with mortality due to all causes, CVD, or cancer. These results did not differ significantly by strata of BMI, smoking, or sex (data not shown).

With the use of Pearson's correlation coefficients between baseline and follow-up measurements of plasma carotene (r = 0.73) and alpha-tocopherol (r = 0.68; P < .0001 for both), we adjusted for measurement error and within-person variation of both plasma antioxidants. For associations between plasma carotene and mortality, this resulted in age- and sex-adjusted RRs that were 7– 16% lower than those obtained by using only baseline information of plasma carotene.

Table 3: Baseline characteristics of the study population (n=1168) by tertiles of plasma alpha-tocopherol

Characteristic	Tertiles of plasma alpha-tocopherol			P value*
	Lowest tertile (n=371)	Medium tertile (n=399)	Highest tertile (n=398)	
Plasma alpha-tocopherol, µmol/L	21.8 (4.4)	29.9 (2.0)	39.9 (6.0)	
Plasma alpha-tocopherol, µmol/L†	21.9 (21.4-22.3)	29.9 (24.4-30.3)	39.8 (39.4-40.3)	
Demographics and lifestyle				
Age, y	72.9 (1.8)	73.0 (2.2)	73.0 (1.8)	.63
% Men	65	50	24	<.0001
Body Mass Index, kg/m^2	26.3 (4.1)	26.6 (4.1)	26.7 (4.0)	.37
Education, y	7.9 (3.9)	8.3 (3.9)	8.6 (3.5)	.03
% Smoker	27	21	15	<.0001
% Alcohol use	70	71	68	.64
% Low physical activity	37	32	30	.09
% Vitamin supplement use	10	13	16	.07
Plasma carotene and serum lipoproteins				
Plasma carotene, µmol/L‡	0.35 (0.27)	0.55 (0.37)	0.70 (0.45)	<.0001
Serum total cholesterol, mmol/L	5.6 (1.0)	6.3 (0.8)	7.2 (1.0)	<.0001
Serum HDL cholesterol, mmol/L	1.3 (0.3)	1.4 (0.4)	1.4 (0.4)	<.0001
Serum triacylglycerols, mmol/L	1.2 (0.8)	1.3 (0.6)	1.7 (0.9)	<.0001

Values are means (SD) unless otherwise indicated.
* P value based on analysis of (co)variance or Chi-square test.
† Mean plasma alpha-tocopherol (95%CI), adjusted for gender.
‡ Geometric mean (geometric SD).

Meta-analysis on plasma antioxidants and all-cause mortality

We identified 5 observational studies, including the current study, that reported associations of plasma (beta)-carotene, plasma vitamin E, or both with all-cause mortality in elderly populations. For the current study, multivariate adjusted RRs for the highest versus the lowest antioxidant quartile were used. After combining all studies, elderly subjects with high concentrations of plasma carotene had a RR of 0.72 (95% CI: 0.59, 0.87; **Figure 1**) compared with those with low plasma carotene concentrations, whereas high plasma vitamin E concentrations were not associated with all-cause mortality (RR: 1.07; 95% CI 0.94, 1.22; **Figure 2**). Heterogeneity between studies was moderate, varying from 36% for studies of vitamin E status to 50% for those of carotene status.

Discussion

In this prospective study conducted in European elderly subjects, plasma carotene concentrations were associated with lower mortality risks due to all causes, cancer and CVD. The inverse relationship with CVD mortality was, however, confined to subjects with a BMI < 25. Plasma concentrations of alpha-tocopherol were not associated with both all-cause or cause-specific mortality.

Table 4: Relative risks and 95%CIs for the association of plasma antioxidants with all-causes and cause specific mortality among participants of the SENECA study (n=1,168)

Causes of mortality	Number of deaths (%)	RR (95%CI)	
		Plasma carotene*	Plasma alpha-tocopherol†
All-causes	388 (33.2)		
Crude model		0.67 (0.60-0.75)	0.81 (0.73-0.90)
Model 1 ‡		0.76 (0.68-0.85)	0.90 (0.81-0.99)
Model 2 §		0.79 (0.70-0.89)	0.96 (0.84-1.10)
Model 3‖		0.79 (0.70-0.89)	1.01 (0.88-1.15)
Cardiovascular diseases	148 (12.7)		
Crude model		0.74 (0.63-0.87)	0.81 (0.69-0.95)
Model 1 ‡		0.83 (0.70-0.98)	0.89 (0.75-1.05)
Model 2 §		0.82 (0.68-0.98)	0.83 (0.67-1.03)
Model 3‖		0.83 (0.70-1.00)	0.87 (0.70-1.07)
Cancer	96 (8.2)		
Crude model		0.52 (0.39-0.67)	0.82 (0.67-1.00)
Model 1 ‡		0.62 (0.47-0.80)	0.94 (0.76-1.15)
Model 2 §		0.60 (0.45-0.80)	1.01 (0.77-1.32)
Model 3‖		0.59 (0.44-0.79)	1.11 (0.85-1.44)
Other causes	53 (4.5)		
Crude model		0.59 (0.43-0.82)	0.85 (0.64-1.11)
Model 1 ‡		0.67 (0.48-0.93)	0.94 (0.71-1.24)
Model 2 §		0.71 (0.50-1.01)	1.01 (0.72-1.42)
Model 3‖		0.70 (0.49-1.01)	1.07 (0.77-1.49)
Unknown causes	104 (8.9)		
Crude model		0.72 (0.59-0.88)	0.76 (0.62-0.92)
Model 1 ‡		0.79 (0.65-0.97)	0.82 (0.67-1.00)
Model 2 §		0.94 (0.75-1.17)	0.97 (0.75-1.27)
Model 3‖		0.94 (0.75-1.17)	0.99 (0.76-1.29)

Data are relative risks for a 1-standard deviation change of *0.39 μmol/L plasma carotene and †8.5 μmol/L plasma alpha-tocopherol.
‡Adjusted for age (continuous) and gender.
§As model 1, but with additional adjustment for body mass index (continuous), serum total cholesterol (continuous), serum HDL cholesterol (continuous), current smoking (yes/no), alcohol consumption (yes/no), physical activity (lowest versus the medium and highest tertile), and SENECA center (indicator variables).
‖As model 2 but with additional adjustment for either plasma alpha-tocopherol or plasma carotene (both continuous).

The strengths of our study are the performance of all assays of plasma antioxidant concentrations in one laboratory, the wide range in plasma concentrations of carotene and alpha-tocopherol, the longitudinal study design, and the standardized and validated methods to assess lifestyle factors and anthropometric variables (27). The limitations of this study also must be considered. First, although vital status follow-up was nearly complete, there were 104 deceased subjects for whom the cause of death could not be ascertained, which limited our power to study cause-specific associations. Second, baseline plasma values of carotene and alpha-tocopherol are assumed to be stable in persons during the years of follow-up, but these concentrations could change over time. However, concentrations of both plasma antioxidants at baseline correlated strongly with those 5 y later.

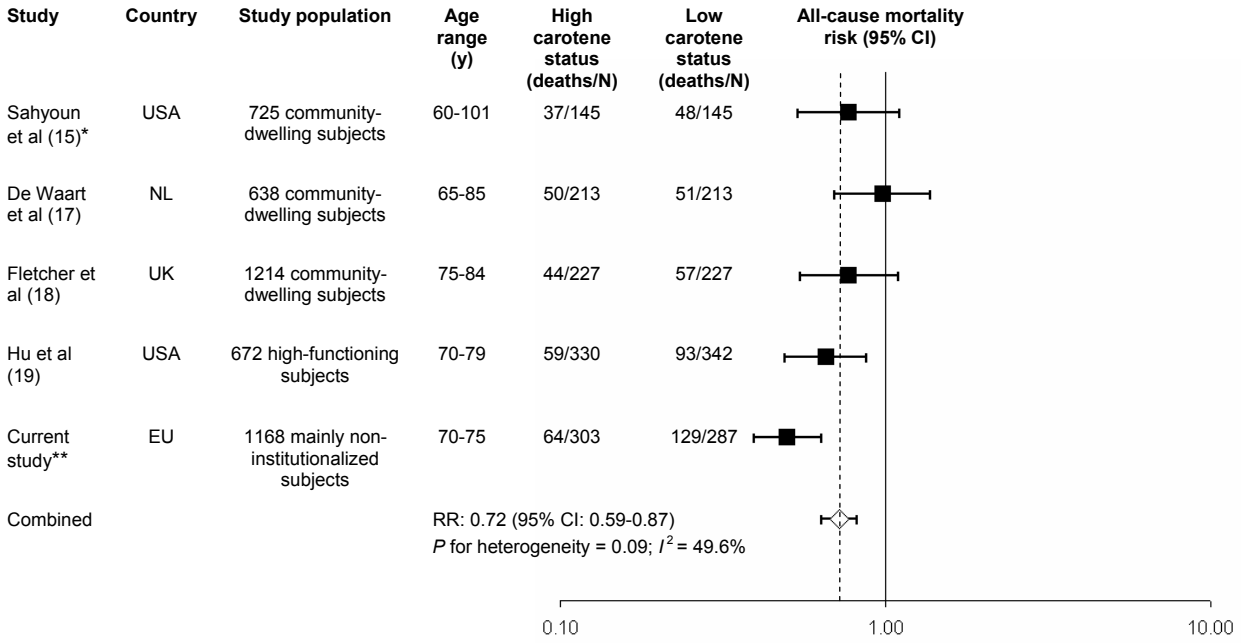

Figure 1: Meta-analysis of the association of high and low carotene status with all-cause mortality in elderly subjects. USA, United States of America; NL, Netherlands; UK, United Kingdom; EU, Europe; I^2, inconsistency squared. *This study investigated plasma carotenoids. **This study investigated plasma alpha- and beta-carotene. The *P* value represents the result of the chi-square test for heterogeneity.

In the current study, plasma carotene was strongly inversely associated with mortality from all causes, whereas no association was observed between plasma alpha-tocopherol and all-cause mortality. Previous observational studies in elderly subjects related plasma carotene, carotenoids, or vitamin E to all-cause mortality (15–19), but the sample size of most of these studies was relatively small. Therefore, we performed a meta-analysis, including the current study, with all-cause mortality as the endpoint. Plasma carotene was strongly associated with a lower risk of all-cause mortality, whereas plasma alpha-tocopherol was not associated with all-cause mortality.

We observed an inverse association of plasma carotene with cancer and cardiovascular mortality. Together with results from other observational studies, these findings are in sharp contrast with those from large-scale supplementation trials (37–40), which makes it very unlikely that pharmacological doses of beta-carotene are effective in preventing CVD and caner. It was argued that the inverse associations between antioxidants and chronic disease in observational studies could be confounded by smoking or by socioeconomic status (41). We adjusted our analyses for current smoking. An alternative adjustment for smoking, the use of information of former smoking, did not result in different risk estimates. In our study, the number of years spent in full-time education was not significantly related to plasma carotene, and it did not confound the relation between plasma carotene and all-cause mortality. However, residual confounding by smoking, socioeconomic status, or other factors can not be excluded.

Plasma carotene was inversely associated with cancer mortality in the current study. This relation became stronger after excluding subclinical cancer cases and adjustment for errors in

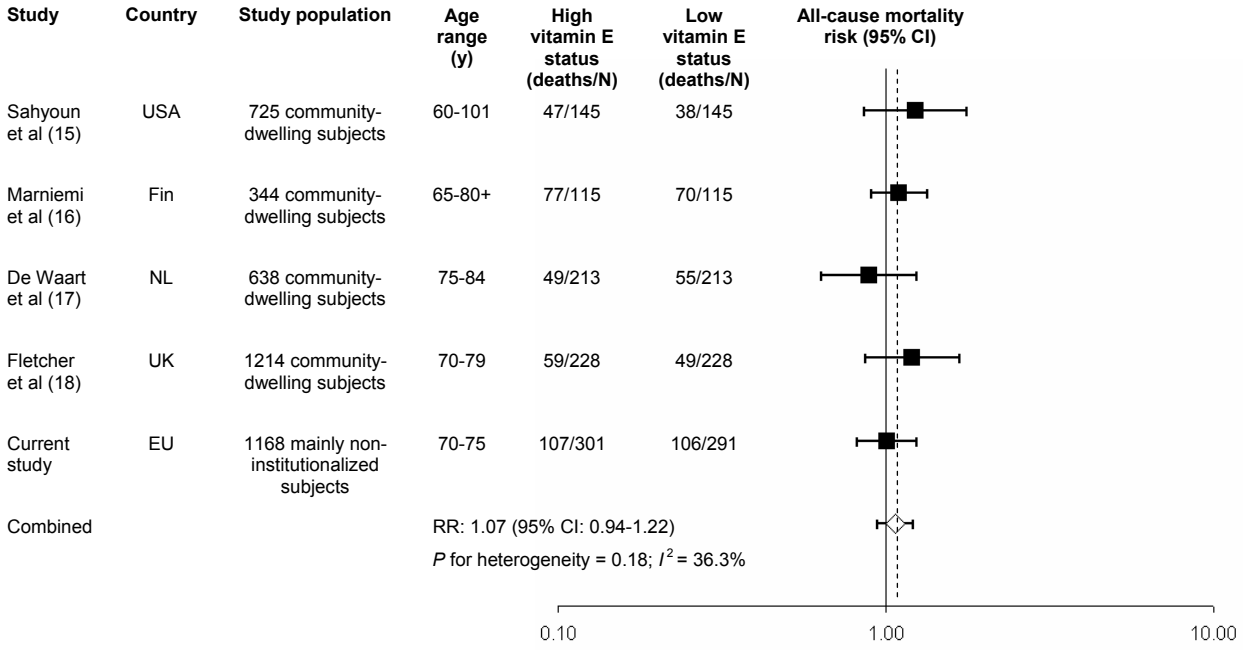

Figure 2: Meta-analysis of the association of high and low vitamin E status with all-cause mortality in elderly subjects. USA, United States of America; Fin, Finland; NL, Netherlands; UK, United Kingdom; EU, Europe; I^2, inconsistency squared. The *P* value represents the result of the chi-square test for heterogeneity.

measurement of and biological fluctuation in plasma carotene. Because large-scale trials did not show that pharmalogical doses of beta-carotene prevent cancer, the question remains whether lower doses of beta-carotene would reduce the incidence of cancer in susceptible populations. An alternative explanation is that other factors related to plasma carotene are responsible for the inverse associations found in observational studies (42). Plasma carotene may also be a marker of a concerted effect of multiple other nutrients in fruit and vegetables.

In the current study, the inverse association between plasma carotene and CVD mortality was confined to subjects with BMIs < 25. A possible explanation for this difference in association between plasma carotene and CVD mortality by BMI could be the relationship with inflammation. It has been shown that BMI is positively associated to C-reactive protein (24), an indicator of inflammation. Our data suggest that, in persons with BMIs < 25, who are likely to have a low level of inflammation, plasma beta-carotene is inversely related to CVD mortality. However, in overweight and obese subjects, who are characterized by a high level of inflammation, this relation was not observed. These results therefore suggest that, in the presence of a low inflammation burden, carotene may be protective against CVD.

Results of observational studies on plasma vitamin E in relation to CVD mortality in elderly subjects are inconsistent. In British elderly subjects, a nonsignificantly lower risk of CVD death was observed for subjects in the 2 upper quintiles of plasma alpha-tocopherol (18), whereas in elderly living in the United States, plasma alpha-tocopherol was positively associated with heart disease mortality (15). In the current study, plasma alpha-tocopherol was not related to CVD mortality or to all-cause mortality. This observation was confirmed by our meta-

analysis as well as by a meta-analysis of trials, in which vitamin E supplementation did not reduce all-cause mortality and high doses of vitamin E were even found to increase the risk of all-cause mortality (43).

In conclusion, in the current study, conducted in European elderly, plasma concentrations of carotene were associated with a lower risk of death due to all causes and that due to cancer. Subjects with a high plasma carotene concentration and a BMI < 25 had also a lower risk of CVD death. The relationship between plasma carotene, BMI, and CVD merits further study.

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Cocoa Intake, Blood Pressure, and Cardiovascular Mortality: the Zutphen Elderly Study

6

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Background: Small, short-term, intervention studies indicate that cocoa-containing foods improve endothelial function and reduce blood pressure. We studied whether habitual cocoa intake was cross-sectionally related to blood pressure and prospectively related with cardiovascular mortality.

Methods: Data used were of 470 elderly men participating in the Zutphen Elderly Study and free of chronic diseases at baseline. Blood pressure was measured at baseline and 5 years later, and causes of death were ascertained during 15 years of follow-up. Habitual food consumption was assessed by the cross-check dietary history method in 1985, 1990, and 1995. Cocoa intake was estimated from the consumption of cocoa-containing foods.

Results: One third of the men did not use cocoa at baseline. The median cocoa intake among users was 2.11 g/d. After adjustment, the mean systolic blood pressure in the highest tertile of cocoa intake was 3.7 mm Hg lower (95% confidence interval [CI], –7.1 to –0.3 mm Hg; P = .03 for trend) and the mean diastolic blood pressure was 2.1 mm Hg lower (95% CI, –4.0 to –0.2 mm Hg; P = .03 for trend) compared with the lowest tertile. During follow-up, 314 men died, 152 of cardiovascular diseases. Compared with the lowest tertile of cocoa intake, the adjusted relative risk for men in the highest tertile was 0.50 (95% CI, 0.32-0.78; P = .004 for trend) for cardiovascular mortality and 0.53 (95% CI, 0.39-0.72; P < .001) for all-cause mortality.

Conclusion: In a cohort of elderly men, cocoa intake is inversely associated with blood pressure and 15-year cardiovascular and all-cause mortality.

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Introduction

Cocoa has a rich history, covering a period of more than 2600 years (1). In ancient history, numerous positive properties to human health have been ascribed to cocoa and chocolate. Already in the 18th century, cocoa was believed to strengthen the heart and reduce angina pectoris (2), but these benefits were not based on scientific evidence. After the finding that cocoa and chocolate contain phenolic compounds (3), scientific interest was triggered. Cocoa is a rich source of flavan-3-ols, also known as flavanols or catechins, a subclass of flavonoids. The flavan-3-ols in cocoa are present as monomers (4), and as oligomers and polymers, better known as procyanidins (5).

In the past few years, the results of several small randomized trials were published in which the effects of cocoa-containing foods were studied on intermediate end points of cardiovascular diseases (CVDs). Consumption of chocolate or cocoa drinks rich in flavan-3-ols lowered blood pressure (6-8) and improved endothelial function (8-11) and insulin sensitivity (7,8). Although these results are promising, most studies (6-10) used chocolate or cocoa that contained much higher amounts of flavan-3-ols than commercially available products. Furthermore, the question remains whether the observed effects are long-standing or transitory, and whether they extend to clinical CVDs.

To our knowledge, observational studies examining the association of cocoa intake with blood pressure or CVD have not been published. We, therefore, have estimated the intake of cocoa from the habitual consumption of cocoa-containing foods and evaluated whether cocoa intake was inversely related to blood pressure and cardiovascular mortality in elderly men living in Zutphen, the Netherlands.

Methods

Study population

The Zutphen Elderly Study is the continuation of the Zutphen Study, the Dutch contribution to the Seven Countries Study (12). Baseline examinations took place between March 9 and June 25, 1985, and repeated examinations took place in the same period in 1990 and 1995. At baseline, 367 of the 555 men in the original cohort who were still alive participated. In addition, a random sample of 711 other men of the same age and also living in Zutphen was selected. In total, 1266 men aged 65 to 84 years were invited, of whom 939 (74.2%) participated in the study. In 876 men, dietary intake was estimated. Information on risk factors and chronic disease prevalence was available for 790 men. Men with a history of CVDs, diabetes mellitus, or cancer at baseline (n = 266) or those who were taking antihypertensive medication (n = 54) were excluded from the analysis. Therefore, our study population consisted of 470 men.

Assessment of diet and cocoa intake

The habitual dietary intake of the subjects in the month preceding the interview was estimated in 1985, 1990, and 1995 by experienced dieticians using a cross-check dietary history method adapted to the Dutch situation (13). All subjects were interviewed at home for about 1 hour in the presence of the person who usually prepared the meals. The habitual consumption of foods during the whole week was determined, and verified with the quantities of foods bought per week. Also, information on the type of diet prescribed by a physician was collected.

The intake of calories (energy) and nutrients in 1985, 1990, and 1995 was calculated using the Netherlands' food composition table from 1987/1988, 1989/1990, and 1996, respectively. The most recent version of this table (14) was used to calculate the intake of magnesium, vitamin E, beta carotene, and folate in all examination years, because only this version contains information on these nutrients. Earlier collected information on *trans* fatty acids in foods consumed in the Zutphen Elderly Study was used to calculate the intake of these fatty acids (15).

We identified 24 cocoa-containing foods that were reported by the Zutphen elderly subjects. For each examination year, the consumption of these foods was multiplied with their individual cocoa content, which was derived from the Conversion Model for Consumer Foods to Primary Agricultural Products (16) or from food labels. The intake of cocoa from individual foods was summed to yield actual cocoa in grams per day for each subject.

The reproducibility of the dietary history method was tested 3 and 12 months after the start of the study in a sample of the present study (13). For the consumption of sugar confectionary, which is the most important source of cocoa in the present study, the 3- and 12-month reproducibility was $r = 0.72$ and $r = 0.76$, respectively (13). Baseline cocoa intake correlated with the cocoa intake that was estimated 5 and 10 years later (Spearman $r = 0.45$ and $r = 0.43$, respectively; $P < .001$ for both). The reproducibility of total calorie intake was about $r = 0.80$ after 3 and 12 months (13).

Collection of risk factor and morbidity data

Systolic and diastolic blood pressure (Korotkoff phase V) were measured twice at the end of the physical examination by 1 of 5 physicians using a random-zero sphygmomanometer (Hawksley & Sons Ltd, West Sussex, United Kingdom) while participants were in the supine position. The mean values of the 2 blood pressure measurements are presented herein. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters.

Total and high-density lipoprotein cholesterol levels were measured in nonfasting serum samples using standard validated methods (17). Remaining serum samples were stored at –20°C. Serum homocysteine levels were determined in stored samples 10 years after the examination (18).

The history of myocardial infarction, angina pectoris, and intermittent claudication was ascertained using the Dutch translation of the questionnaire formulated by Rose and Blackburn

(19). For history of heart failure, diabetes mellitus, and cancer, a standardized questionnaire was developed and the physicians' conclusion was used in the present analyses. Physical activity was estimated using a validated questionnaire originally designed for retired men (20). This questionnaire asks about the frequency and duration of different activities, such as walking, cycling, and gardening. The time spent on each activity was summed to yield minutes of physical activity. Information on cigarette smoking, socioeconomic status, and the use of antihypertensive drugs, aspirin, and anticoagulants was also collected by questionnaire.

Ascertainment of follow-up events

Municipal registries provided information on vital status and were checked at 5-year intervals. Two men were lost to follow-up during the study and were censored after their last physical examination. Information on the cause of death was obtained from hospital discharge data and/or general practitioners, and up to 1990 also from Statistics Netherlands, Voorburg. The final causes of death were ascertained by one clinical epidemiologist and coded according to the *International Classification of Diseases, Ninth Revision* (ICD-9). Cardiovascular diseases refer to ICD-9 codes 390 to 459. Because the underlying cause of death in elderly people is often difficult to ascertain, we included CVDs coded as primary (n = 118), secondary (n = 30), and tertiary (n = 4) cause of death in our analysis. Information on the cause of death was lacking for 1 man, and his follow-up was censored at the date of death.

Statistical analysis

Cocoa intake was related to blood pressure cross-sectionally, combining data from 470 men at baseline and data from 324 men who were repeatedly examined in 1990, composing 794 total observations. A random intercept model (SAS Proc Mixed; SAS Institute Inc, Cary, NC, USA) was used to calculate adjusted means and 95% confidence intervals (CIs) of blood pressure for each tertile of cocoa intake. In addition to an age-adjusted model, we used 3 multiple regression models to adjust for other factors that are associated with cocoa intake or are well-known determinants of blood pressure. Dietary covariates were adjusted for total calorie intake according to the residual method (21).

Relative risks (RRs) and 95% CIs for the association between cocoa intake and cardiovascular and all-cause mortality were estimated using Cox proportional hazards models in which the middle and the highest tertiles of cocoa intake were compared with the lowest tertile. In the time-dependent analyses, mortality between 1985 and 1990 was related to cocoa intake estimated in 1985; mortality between 1990 and 1995 was related to the mean cocoa intake in 1985 and 1990; and mortality between 1995 and 2000 was related to the mean cocoa intake in 1985, 1990, and 1995 (22). Besides an age-adjusted model, we used a multivariate-adjusted model that included lifestyle factors related to CVD risk and BMI as an indicator of calorie balance (model A). This model was extended with diet prescription, total calorie intake, and calorie-adjusted consumption of foods (model B). In model C, we also adjusted for dietary

intake using calorie-adjusted intake of nutrients instead of foods. Adjustment for continuous distributed covariates was done time dependently; for discrete variables, we used baseline data. Stratified analyses were performed for major cardiovascular risk factors. All statistical tests were 2-sided.

Results

Cocoa intake and baseline characteristics

In 1985, one third of the study population did not use cocoa. The median intake in the middle tertile was 0.92 g/d; in the highest tertile, this was 4.18 g/d. All cocoa-containing foods that were consumed at baseline are listed in **Table 1**. Plain chocolate and chocolate bars contributed two thirds to the total intake of cocoa.

Table 1: Cocoa containing food items consumed by elderly men in Zutphen at baseline*

Cocoa containing food	Cocoa content (g/100g)	Contribution to total cocoa intake (%)
Chocolate confectionary		
Plain chocolate, dark	43.0	28.4
Plain chocolate, milk	30.0	21.8
Chocolate bar with nuts	22.5	3.3
Chocolate candy bar†	15.0	9.5
Chocolates (bonbons)	18.0	2.3
M&Ms, chocolate	28.5	<0.1
M&Ms, peanuts	16.5	0.4
Chocolate cookies	9.0	1.1
Cocoa sandwich filling		
Chocolate spread	13.5	0.6
Chocolate nut spread	6.0	0.6
Chocolate confetti, dark	38.7	10.9
Chocolate confetti, milk	27.0	4.3
Cocoa desserts		
Chocolate custard	8.3	4.4
Chocolate pudding	2.5	0.6
Chocolate pudding with sauce	2.1	0.1
Chocolate mousse	22.6	0.1
Drinks		
Cocoa drink, full fat	1.5	2.4
Cocoa drink, skimmed	1.5	1.9
Miscellaneous		
Cocoa powder	100.0	5.9
Cocoa powder sweetened	25.0	0.3
Nutritional supplement‡	42.0	1.2

*Data are given for men free of chronic diseases and not using antihypertensive medication at baseline. †Bounty, Mars, Milky Way (Mars Inc, McLean, Va, USA), Nuts (Nestlé, Vevey, Switzerland).
‡Ovomaltine (cocoa flavor) (Novartis, Basal, Switzerland).

Men who consumed cocoa-containing foods used more low- and medium-fat dairy foods, sugar confectionary foods, and cookies and savory foods, and were more likely to consume alcohol and nuts and seeds (**Table 2**). Cocoa intake was inversely associated with the consumption of meat and coffee. Furthermore, cocoa intake was positively related with calorie intake, and with intake of calcium (P = .03) and magnesium (P = .04). The median cocoa intake among users was 2.11 g/d in 1985, 2.30 g/d in 1990, and 2.36 g/d in 1995.

Cocoa intake and blood pressure

After adjustment for potential confounders, cocoa intake was inversely associated with systolic and diastolic blood pressure (**Table 3**). The mean systolic blood pressure was 3.7 mm Hg lower (95% CI, –7.1 to –0.3 mm Hg; P = .03) in the highest tertile of cocoa intake compared with the lowest tertile after multivariate adjustment, including consumption of foods (model B). This difference was –3.1 mm Hg (95% CI, –6.5 to 0.2 mm Hg; P = .07) after adjustment according to model C, which included intake of nutrients rather than foods. The mean diastolic blood pressure was 2.1 mm Hg lower in the highest tertile of cocoa intake compared with the lowest tertile (95% CI, –4.0 to –0.2 mm Hg; P = .03; model B). Adjustment for covariates in model C yielded similar estimates.

Additional multivariate-adjusted analysis showed that neither systolic nor diastolic blood pressure was related to the consumption of sugar confectionary (P = .84 and P = .16 for linear trend, respectively), and to the consumption of cookies and savory foods (P = .31 and P = .20 for linear trend, respectively). After multivariate adjustment, the mean systolic blood pressure did not differ between consumers and nonconsumers of nuts and seeds (P = .44), whereas the diastolic blood pressure was higher among consumers (P = .02).

Cocoa intake and mortality

During the 4908 person-years of follow-up between 1985 and 2000, 314 (66.8%) of the 470 men died. Cardiovascular diseases were the cause of death for 152 men. The results of the time-dependent Cox proportional hazards regression model showed that cocoa intake was inversely related to cardiovascular mortality (**Table 4**). After adjustment for age, BMI, lifestyle factors, drug use, and food and calorie intake (model B), the RR for cardiovascular mortality for men in the highest tertile of cocoa intake was 0.50.

Adjustment for intake of nutrients (model C) instead of foods resulted in similar risk estimates. Analyzing baseline cocoa intake rather than cumulative average cocoa intake in time-dependent analysis yielded similar results (multivariate-adjusted RR of cardiovascular death for the highest vs the lowest tertile, 0.51; 95% CI, 0.33-0.78; P = .006 for linear trend).

The consumption of sugar confectionary was not significantly associated with cardiovascular mortality (P = .54 for linear trend), nor was the consumption of cookies and savory foods (P = .45 for linear trend), nor the consumption of nuts and seeds (P = .12).

Table 2: Selected characteristics of elderly men in 1985 in Zutphen, free of chronic diseases and not using antihypertensive drugs, by tertiles of cocoa intake

Characteristic	Tertiles of cocoa intake			P value*
	Lowest tertile (n=165)	Medium tertile (n=149)	Highest tertile (n=156)	
Cocoa intake, g/day†	0 (0-0)	0.92 (0.60-1.45)	4.18 (2.90-6.10)	
Demographics. lifestyle, risk factors, and drug use				
Age, y	72.1 (5.6)	72.0 (5.4)	71.3 (4.4)	.38
High socioeconomic status, %	33.3	30.2	31.4	.83
Positive family CVD history, %	17.0	19.5	17.9	.85
Leisure time physical activity, h/wk†	10.5 (4.5, 20.5)	9.8 (4.5, 18.9)	10.7 (6.3, 20.4)	.40
Current cigarette smoking, %	35.8	31.5	33.3	.73
Alcohol consumer, %	70.9	77.2	83.3	.03
Prescribed diet, %	15.2	12.8	9.0	.24
Use of vitamin supplements, %	15.2	12.8	14.1	.83
Body mass index, kg/m^2	25.9 (3.2)	25.4 (3.0)	25.3 (2.7)	.24
Resting heart rate, beats/min‡	70.7 (10.8)	70.9 (11.9)	70.8 (10.6)	.97
Serum total cholesterol, mmol/L	6.1 (1.1)	6.0 (1.2)	6.1 (1.1)	.53
Serum HDL cholesterol, mmol/L	1.2 (0.3)	1.1 (0.3)	1.1 (0.3)	.46
Serum homocysteine, µmol/L	14.8 (8.1)	14.8 (5.9)	14.8 (8.1)	.96
Aspirin use, %	13.9	12.1	7.7	.20
Anticoagulant use, %	15.2	10.7	10.9	.39
Food intake, g/day				
Bread and cereal products	165 (72)	162 (61)	158 (63)	.66
Potatoes	182 (96)	165 (84)	181 (88)	.17
Vegetables	181 (77)	173 (65)	169 (61)	.27
Fruits†	157 [100, 243]	189 [116, 271]	167 [112, 263]	.13
Low- and medium-fat dairy†	250 [124, 461]	321 [165, 493]	330 [212, 490]	.03
Meat	122 (50)	113 (40)	109 (37)	.02
Fish	18 (21)	20 (23)	19 (24)	.82
Butter and hard margarine	49 (37)	55 (39)	54 (35)	.35
Vegetable oil and soft margarine†	5 [0, 22]	2 [0, 23]	3 [0, 21]	.26
Sugar confectionary other than chocolate	47 (37)	53 (34)	61 (38)	<.001
Cookies and savory products	31 (29)	40 (29)	50 (34)	.003
Coffee†	428 [288, 624]	396 [226, 496]	400 [284, 517]	.05
Tea†	372 [201, 590]	392 [180, 600]	400 [223, 610]	.60
% nuts and seeds consumers	32.1	40.3	55.1	<.001
Energy intake, kJ§	8795 (2207)	9345 (1914)	10011 (2149)	<.001

Data are given as mean (SD), unless otherwise indicated. Abbreviations: CVD, cardiovascular diseases; HDL, high-density lipoprotein.

*Based on analysis of variance, Kruskal-Wallis, or Chi-square test.

† Median [interquartile range].

‡ Based on 461 observations because of missing data.

§Energy contributed by alcohol is not included.

To explore whether blood pressure contributed to the lower cardiovascular mortality risk observed in cocoa users, we also adjusted for baseline blood pressure in model B. Neither systolic nor diastolic blood pressure, however, affected our risk estimates.

Table 3: Systolic and diastolic blood pressure according to cocoa intake among elderly men in Zutphen, free of chronic diseases and not using antihypertensive drugs, by tertiles of cocoa intake

	Tertiles of cocoa intake			
Blood pressure	**Lowest (<0.36 g/d)**	**Middle (0.36-2.30 g/d)**	**Highest (>2.30 g/d)**	**P for trend**
Systolic				
Crude	149.7 (147.3-152.2)	148.8 (146.5-151.1)	147.0 (144.6-149.5)	.08
Age-adjusted	149.6 (147.2-152.0)	148.8 (146.4-151.1)	147.0 (144.6-149.5)	.09
Model*				
A	149.9 (147.4-152.4)	148.9 (146.6-151.2)	146.9 (144.4-149.4)	.07
B	150.2 (147.7-152.8)	149.0 (146.7-151.3)	146.5 (144.0-149.1)	.03
C	150.0 (147.5-152.6)	148.8 (146.5-151.2)	146.9 (144.4-149.4)	.06
Diastolic				
Crude	84.4 (82.9-85.8)	83.6 (82.3-85.0)	82.2 (80.8-83.6)	.02
Age-adjusted	84.5 (83.1-85.9)	83.7 (82.3-85.0)	82.2 (80.8-83.6)	.01
Model*				
A	84.2 (82.8-85.6)	83.8 (82.5-85.1)	82.5 (81.1-83.8)	.05
B	84.4 (83.0-85.8)	83.8 (82.5-85.1)	82.3 (80.9-83.7)	.03
C	84.3 (82.9-85.7)	83.8 (82.5-83.7)	82.3 (80.9-83.7)	.03

Data are given as mean (95% confidence interval) blood pressure values, in mm Hg.
* Model A was adjusted for age (continuous), body mass index (continuous), alcohol intake (yes or no), physical activity (continuous), cigarette smoking (yes or no), diet prescription (yes or no), aspirin use (yes or no), anticoagulant use (yes or no), and the physician who measured blood pressure (categorical); B, variables given for A and further adjusted for the consumption of vegetables, fruit, meat, low- and medium fat dairy, nuts and seeds, sugar confectionary other than chocolate, cookie and savory foods, coffee, and energy intake (all continuous); and C, those variables given for A and further adjusted for intake of potassium, sodium, calcium, magnesium, and total energy intake (all continuous).

Cocoa intake was also inversely related to all-cause mortality in time-dependent analysis (Table 4). Similar results were obtained when baseline cocoa intake was related to 15-year all-cause mortality (multivariate-adjusted RR for the highest vs the lowest tertile, 0.59; 95% CI, 0.44-0.79; P = .003 for trend).

The association between cocoa intake and cardiovascular mortality did not differ significantly between strata of BMI, cigarette smoking, physical activity, calorie intake, alcohol consumption, or socioeconomic status (P > .30 for all) (**Table 5**).

The consumption of sugar confectionary was not significantly associated with cardiovascular mortality (P = .54 for linear trend), nor was the consumption of cookies and savory foods (P = .45 for linear trend), nor the consumption of nuts and seeds (P = .12).

To explore whether blood pressure contributed to the lower cardiovascular mortality risk observed in cocoa users, we also adjusted for baseline blood pressure in model B. Neither systolic nor diastolic blood pressure, however, affected our risk estimates.

Discussion

In the present study, usual daily cocoa intake was inversely related to blood pressure in cross-sectional analysis. In prospective analysis, usual cocoa intake was associated with a 45% to 50% lower risk of cardiovascular and all-cause death.

Table 4: Relative risks for the association between cocoa intake and 15-year mortality among elderly men in Zutphen, free of chronic diseases and not using antihypertensive drugs at baseline

	Tertiles of cocoa intake			P for trend
Mortality	**Lowest (<0.36 g/d)**	**Middle (0.36-2.30 g/d)**	**Highest (>2.30 g/d)**	
Number of men	161	147	162	
Person-years	1481	1573	1854	
CVD mortality				
Cases, n (%)	58 (36.0)	50 (34.0)	44 (27.2)	
Mortality rate*	39.2	31.8	23.7	
RR (95% CI)				
Age-adjusted	1.00	0.79 (0.54-1.15)	0.58 (0.39-0.86)	.008
Model†				
A	1.00	0.84 (0.57-1.24)	0.67 (0.45-1.01)	.05
B	1.00	0.70 (0.47-1.05)	0.50 (0.32-0.78)	.004
C	1.00	0.79 (0.53-1.19)	0.50 (0.32-0.78)	.004
All-cause mortality				
Cases, n (%)	122 (75.8)	100 (68.0)	92 (56.8)	
Mortality rate*	82.4	63.6	49.6	
RR (95% CI)				
Age-adjusted	1.00	0.76 (0.58-0.99)	0.57 (0.43-0.75)	<.001
Model†				
A	1.00	0.81 (0.62-1.05)	0.65 (0.49-0.86)	<.001
B	1.00	0.73 (0.55-0.97)	0.53 (0.39-0.72)	<.001
C	1.00	0.79 (0.60-1.05)	0.52 (0.38-0.71)	<.001

Abbreviations: CI, confidence interval; CVD, cardiovascular diseases; RR, relative risk.
* Mortality rate is given per 1000 person-years.
†Model A was adjusted for age (continuous), body mass index (continuous), alcohol intake (yes or no), physical activity (continuous), cigarette smoking (yes or no), diet prescription (yes or no), aspirin use (yes or no), anticoagulant use (yes or no); B, those variables given for A and further adjusted for the consumption of vegetables, fruit, meat, low- and medium fat dairy, nuts and seeds, sugar confectionary other than chocolate, cookie and savory foods, coffee, and energy intake (all continuous); and C, those variables given in model A and further adjusted for intake of potassium, sodium, calcium, magnesium, and total energy intake (all continuous).

To our knowledge, the present study is the first epidemiological study reporting inverse relationships of cocoa intake with blood pressure and with cardiovascular and all-cause mortality. In the Harvard Alumni Study (23), consumers of candy had a lower risk of all-cause mortality compared with subjects who almost never consumed candy. However, the investigators were not able to differentiate between consumption of chocolate and sugar candy. In the Nurses' Health Study, the frequency of consumption of chocolate bars and chocolate pieces was not associated with a lower risk of coronary heart disease after 14 years of follow-up (24). Because cocoa is not only present in chocolate, this association may have been underestimated in this study.

A major concern in observational studies is the possibility of residual confounding. In our study, cocoa users consumed less meat and coffee; consumed more dairy, sugar confectionary, cookies, and savory foods; and were more likely to use alcoholic drinks and nuts and seeds. Consequently, cocoa intake was positively associated with calorie intake. However, we did not observe a positive association of cocoa intake with BMI or physical activity. Because

Table 5: Relative risks for the association between cocoa intake and 15-year cardiovascular mortality among elderly men in Zutphen, free of chronic diseases and not using antihypertensive drugs at baseline, stratified by major risk factors*

	Tertiles of cocoa intake			
Variable	**Lowest (<0.36 g/d)**	**Middle (0.36-2.30 g/d)**	**Highest (>2.30 g/d)**	**P for trend**
Body mass index				
≥25	1.00	0.90 (0.53-1.51)	0.55 (0.31-0.98)	.03
<25	1.00	0.63 (0.31-1.25)	0.40 (0.20-0.79)	.01
Cigarette smoking				
Yes	1.00	0.59 (0.29-1.22)	0.42 (0.18-0.95)	.06
No	1.00	0.76 (0.45-1.30)	0.52 (0.30-0.92)	.03
Physical activity				
High	1.00	1.31 (0.41-4.20)	0.40 (0.11-1.54)	.07
Low	1.00	0.67 (0.42-1.05)	0.50 (0.31-0.81)	.009
Energy intake				
High	1.00	0.54 (0.28-1.06)	0.48 (0.26-0.87)	.04
Low	1.00	0.95 (0.56-1.63)	0.54 (0.28-1.05)	.06
Alcohol consumption				
Yes	1.00	0.65 (0.41-1.02)	0.51 (0.32-0.84)	.02
No	1.00	1.29 (0.45-3.74)	0.18 (0.04-0.73)	.01
Socioeconomic status				
High	1.00	0.59 (0.23-1.48)	0.42 (0.16-1.13)	.12
Low	1.00	0.75 (0.46-1.21)	0.46 (0.27-0.78)	.004

Abbreviations: CI, confidence interval; CVD, cardiovascular diseases; RR, relative risk.
* Adjusted for age (continuous), body mass index (continuous), alcohol intake (yes or no), physical activity (continuous), cigarette smoking (yes or no), diet prescription (yes or no), aspirin use (yes or no), anticoagulant use (yes or no), consumption of vegetables, fruit, meat, low- and medium fat dairy, nuts and seeds, sugar confectionary other than chocolate, cookie and savory foods, coffee, and energy intake, and total energy intake (all continuous).

BMI was measured accurately, we cannot rule out that residual confounding by physical activity, and by dietary factors, may partly explain our results.

Chocolate confectionary contributed about two thirds to the total intake of cocoa in our study. We considered the possibility of reverse causation, i.e., that healthy subjects consume more chocolate confectionary than those who are not healthy. However, we limited our study to subjects without chronic diseases and to those not using antihypertensive drugs at baseline. Also, the association between cocoa intake and cardiovascular mortality did not differ between subjects with a high and low level of physical activity, which can also be considered as a marker of general health. Finally, the consumption of sugar confectionary other than chocolate, cookies and savory foods, and nuts and seeds, which were all strongly related to cocoa intake, was not associated with cardiovascular mortality, suggesting a specific effect of cocoa on cardiovascular mortality.

A few small short-term intervention studies have evaluated the effect of dark chocolate consumption on blood pressure. In hypertensive and normotensive persons, daily consumption of 100 g of dark chocolate for 2 weeks reduced the average systolic blood pressure between 4.1 and 11.9 mm Hg and the average diastolic blood pressure between 1.8 and 8.5 mm Hg (6,8). However, daily consumption of 46 g of dark chocolate did not affect blood pressure after 2

weeks in healthy subjects (10). In summary, these studies suggest that large amounts of dark chocolate lower blood pressure, whereas a smaller amount appears to have no effect.

The present study indicates that men with a usual daily cocoa intake of about 4.2 g, which is equal to 10 g of dark chocolate per day, had a lower systolic and diastolic blood pressure compared with men with a low cocoa intake. Although this amount is one tenth of the dose that is used in most intervention studies, it suggests that long-term daily intake of a small amount of cocoa may lower blood pressure.

Mechanistic studies suggest that the flavan-3-ols in cocoa-containing foods are likely to be responsible for the reduction in blood pressure and the improvement of endothelial function. Consumption of flavan-3-ol–rich chocolate or cocoa is shown to increase arterial (9,11) and peripheral vasodilation (10), whereas this effect is less (10) or absent (9,11) after consumption of flavan-3-ol–low chocolate or cocoa. An increased activity of nitric oxide is likely to play a major role in this process (9,10). This is supported by the observation that polymeric procyanidins increased endothelial nitric oxide synthase activity in cultured endothelial cells (25). Although there is little doubt that flavan-3-ols have a beneficial effect on endothelial function and blood pressure, other bioactive substances in cocoa, including theobromine (26), may also contribute to its effects.

The lower cardiovascular mortality risk associated with cocoa intake could not be attributed to the lower blood pressure observed with cocoa use. An explanation may be that blood pressure itself was not a risk factor for cardiovascular and all-cause mortality in the Zutphen Elderly Study (27). Our findings, therefore, suggest that the lower cardiovascular mortality risk related with cocoa intake is mediated by mechanisms other than lowering blood pressure. The improvement of endothelial function by flavan-3-ols in cocoa may be a plausible candidate (8–11). Dark chocolate also exhibits several metabolic effects (7,8). Daily 100 g of dark chocolate for 2 weeks reduced fasting insulin and glucose levels and decreased glucose and insulin responses after an oral glucose load (7,8). Cocoa-containing foods and flavan-3-ols may also reduce cardiovascular risk by the inhibition of platelet function (28–30) and low-density lipoprotein oxidation (31–33), the modulation of cytokine production (24–36), and the beneficial effect on serum cholesterol levels (8,33,37).

To explore whether possible misclassification of cardiovascular mortality occurred in our study, we also studied the association of cocoa intake with all-cause mortality. We observed a similar risk for all-cause mortality as for cardiovascular mortality. This may implicate that cocoa intake is also associated with noncardiovascular mortality. Because cocoa is a rich source of antioxidants, it may also be related to other diseases that are linked to oxidative stress (e.g., pulmonary diseases, including chronic obstructive pulmonary disease (38), and certain types of cancer) (39). However, this merits further investigation.

In conclusion, to our knowledge, this is the first observational study that found that habitual cocoa intake was inversely associated with blood pressure in cross-sectional analysis

and with cardiovascular and all-cause mortality in prospective analysis. Before drawing conclusions, confirmation by other observational and experimental studies is needed.

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Serum Glutathione Peroxidase 3 Activity in Relation to Cardiovascular Mortality: the Minnesota Heart Survey

7

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Background: Oxidative stress has been proposed as an initiating factor in the development of atherosclerosis. If true, then glutathione peroxidase-3 (GPx-3) activity may be inversely related to cardiovascular disease, but prospective studies have not been reported.

Methods and Results: In a case-control study nested within the Minnesota Heart Survey, we assayed serum GPx-3 activity in 130 cardiovascular deaths and 240 controls identified after 5 to 12 years of follow-up. Participants were predominantly of white race and aged 26 to 85 years. Logistic regression models were used to calculate odds ratios adjusted for age, sex, baseline year, body mass index, smoking, alcohol intake, physical activity, serum total and high-density lipoprotein (HDL) cholesterol, systolic blood pressure, serum non-fasting glucose, and serum activity of gamma glutamyltransferase. Odds ratios [95% confidence intervals (CIs)] for cardiovascular mortality for increasing quartiles of serum GPx-3 activity were 1.00, 0.90 (0.48 to 1.68), 0.77 (0.40 to 1.49), and 0.42 (0.21 to 0.86) (P for trend = .02). This inverse association was confined to those with below median serum HDL cholesterol concentration (P for interaction = .006), with odds ratios (95% CIs) for increasing quartiles of 1.00, 0.82 (0.36 to 1.88), 0.60 (0.23 to 1.59), and 0.17 (0.06 to 0.47) (P for trend = .001). GPx-3 was not related in subjects with above median HDL cholesterol (P for trend = .82).

Conclusions: A high serum GPx-3 activity in those with a low serum HDL cholesterol concentration may contribute to reduced cardiovascular mortality.

Submitted for publication.

Introduction

Glutathione peroxidases (GPx) form a superfamily that consists of different isoforms which vary in cellular location. GPx-1 is solely found in the cytosol, whereas GPx-3 is present mainly in circulating high-density lipoprotein particles (1,2). This enzyme's main biological function is to catalyze the reactions of glutathione to reduce lipid hydroperoxides to their corresponding alcohols and to reduce hydrogen peroxide to water (2), thereby contributing to overall reduction of oxidative stress.

The activity of GPx-1 in erythrocytes predicted incident cardiovascular death, infarction, or stroke in a 6.5 year followup study of angiographically studied patients (3). Also, a low activity of erythrocyte GPx-1 has been associated with an increased risk of cardiovascular events in patients with coronary heart disease (4). Although a recent case-control study found that several single nucleotide polymorphisms in the GPx-3 gene promoter region were associated with early strokes (5), no prospective studies on GPx-3 activity in serum and cardiovascular diseases have been published.

A nested case control design within the Minnesota Heart Survey presented an opportunity for a pilot prospective assessment of the concept that serum GPx-3 activity is inversely related to cardiovascular disease. Consistent with its presumed role in reducing oxidative stress, we hypothesized that a higher activity of GPx-3 would be associated with a lower cardiovascular mortality. An inverse relation was anticipated to be stronger in those with a low HDL cholesterol concentration, as these subjects are likely to need additional antioxidant activity to protect LDL from oxidation.

Methods

Minnesota Heart Survey and selection of cases and controls

Methods of the Minnesota Heart Survey (MHS) have been published previously (6-8). Briefly, the MHS, initiated in 1980, is an ongoing population-based surveillance on trends of risk factors for coronary heart disease in residents of the 7 county Minneapolis-St. Paul metropolitan area. A two-stage self-weighting cluster design was used selecting households at random. Within each household, one individual was randomly selected to participate in the survey except for surveys 1980-1981, 1995-1997, and 2000-2002 when all age-eligible household members were invited to participate (9). Mortality follow-up was accomplished by matching with Minnesota death certificates on or before December 31, 2002. Written consent was obtained from all study participants. Consent and data collection procedures for each survey were approved by the University of Minnesota Research Subjects' Protection Programs Institutional Review Board.

Given 5-12 years of followup for mortality, these data presented an opportunity to test the concept that GPx-3 activity can protect against cardiovascular disease. Nevertheless, the

study was regarded as a pilot because MHS was not designed to study this question and there were several known disadvantages. Specifically, participants were not fasting, clinic blood handling was differential between the two surveys, and there were an unknown number of missing blood samples.

For the current analysis, we used data and stored blood samples from the surveys conducted in 1990-1992 and 1995-1997. We identified 173 cardiovascular disease deaths (International Classification of Diseases ninth revision codes 390-459 or tenth revision codes I00-I99) and 2 age and sex matched controls per case (n = 346). Serum was available for 137 of the participants who had died and for 250 of the controls. Three of the controls had died during follow-up from non-cardiovascular disease. The subjects ranged in age from 26 to 85 years. Absence of blood sample occurred more often in the 1995-1997 survey: 96 cases and 198 controls were seen in 1990-1992, while 41 cases and 51 controls were seen in 1995-1997. Complete covariate data were available in 130 cases 95 in 1990 and 35 in 1995) and 240 controls (197 in 1990 and 43 in 1995). Findings were barely altered if the 16 missing covariate values were imputed from the participant's sex, age, body mass index (BMI), and diabetes status. Nevertheless, we report on those with complete data because 12 of the missing values were in high-density lipoprotein (HDL) cholesterol, and this variable played a central role in these analyses.

Clinical assessment

Height was measured in stocking feet with a wooden triangle and a rigid ruler attached to a wall. Weight was measured without coat and shoes with a beam balance; the balance was calibrated daily with a 22.7-kg (50-lb) weight. BMI was calculated as weight (kg) divided by the square of height (m^2).

Systolic and fifth phase diastolic blood pressure were measured with a random zero sphygmomanometer (Hawksley, West Sussex, United Kingdom) throughout the study by trained technicians according to a standard procedure (9). The average of two blood pressure measurements taken 1 minute apart is presented.

Information on age, sex, leisure-time physical activity, smoking, and the consumption of alcoholic beverages was obtained by interviewer administered questionnaires. For leisure-time physical activity, four questions were asked about the intensity, duration, and frequency of exercise sessions and a physical activity score (in MET-hr/day) was derived (10).

Laboratory assays in serum

Non-fasting blood was drawn according to standardized protocols that differed in one respect (typical duration of refrigeration in clinic at 4ºC) between the 1990 and 1995 surveys. After clotting, serum was isolated and refrigerated up to several days in the 1990 survey but generally within 24 hours in the 1995 survey. Upon receipt in the laboratory, all serum samples were stored at –70ºC, and serum was used for all subsequent laboratory analyses.

Total cholesterol was assayed with an AutoAnalyzer II (Technicon Corporation) with a nonenzymatic method between 1990 and 1992 and with an enzymatic method thereafter. HDL cholesterol was measured with an enzymatic method after precipitation of non-HDL cholesterol with heparin and Mn2+ (1990-1992) (11) or magnesium dextran sulfate (1995-1997) (12). Glucose, urea nitrogen, aspartate aminotransferase, alanine aminotransferase and gamma glutamyltransferase were measured using a Vitros 950 multi-channel analyzer (Ortho Clinical Diagnostics, Raritan, NJ) using their standard thin-layer reflectance spectrophotometric methods. Average analytical CV's for these assays at the high end of the normal reference range were, <3% for all analytes.

GPx-3 activity in all samples was determined in 2006 by a modified kinetic assay as previously described (1). Briefly, the serum samples, glutathione and glutathione reductase were aliquoted in quadruplicate into a 96 well microtitre plate, with a phosphate buffer. The plate was warmed to 37ºC and nicotinamide adenine dinucleotide phosphate (NADPH) was added and the plate was shaken. The decrease in absorbance at 340 nm was determined in a microtitre plate reader (FLUOstar, BMG, Offenberg, Germany). The coefficient of variation was 5% within plate.

The GPx-3 activity was generally lower in samples collected in 1990 than in those collected in 1995. During characterization of the GPx-3 assay (work associated with, but not reported in Chen et al (1)), we observed a loss of serum GPx-3 activity in samples stored for 48 hours at 4ºC compared to aliquots of the same samples that had quickly been frozen at -70ºC. This difference presumably occurred because of the longer pre-freezing refrigeration time in 1990. To rule out a difference between plates as the source of the difference in GPx-3 activity between surveys, we investigated whether 1990 and 1995 samples run on the same plate had widely differential values. Because the samples were run in the order received, there was only one such plate, for which GPx-3 mean activity (SD) was 0.36 (0.13) U/ml in thirteen 1990 samples versus 0.76 (0.07) U/ml in eight 1995 samples (P for difference <.0001).

Data analysis

Case-control differences were similar within participants in the 1990 survey as within participants in the 1995 survey and the primary findings pool data from the two surveys. However, given GPx-3 differences and case/control differences in blood sample availability between years, we also present survey specific data to demonstrate this consistency. In the pooled analyses, we adjusted for baseline year and used baseline year-specific quartiles based on the distribution in controls in assessing the association between GPx-3 activity and cardiovascular mortality. Relative risks for cardiovascular mortality were studied by calculating odds ratios (ORs) and 95% confidence intervals (CIs) in logistic regression models. The lowest quartile was the reference.

All analyses were adjusted for age, sex, and baseline year (model 1). In multivariable analysis, we first included BMI, cigarette smoking, physical activity, and the intake of alcohol

(model 2). This model was then extended with serum total and HDL cholesterol, systolic blood pressure, non-fasting serum glucose, and serum gamma glutamyltransferase (model 3). Trends across quartiles of GPx-3 activity were assessed by modeling one variable for quartiles of GPx-3 activity as a continuous variable.

Stratified analyses for HDL cholesterol were conducted to assess whether the relation between serum GPx-3 and cardiovascular mortality differed in those below vs. above sex-specific median values for HDL cholesterol. The sex-specific median values for HDL cholesterol were similar for 1990-1992 and 1995-1997. A product term of GPx-3 (quartiles) and HDL cholesterol (continuous) was entered into the multivariable model to assess the statistical significance of this interaction. All analyses were conducted with SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Baseline characteristics

Of the 130 cardiovascular mortality cases included in the analyses, 66 (51%) had died of coronary heart disease, 25 (19%) of atherosclerotic stroke, 22 (17%) of other atherosclerotic cardiovascular diseases and 17 (13%) of non-atherosclerotic cardiovascular diseases. 95% of cases and 97% of controls were of white race. Overall, cases were more likely to smoke, had higher average systolic blood pressure, non-fasting serum glucose and serum gamma glutamyltransferase, and lower average serum HDL cholesterol than did controls (**Table 1**). Furthermore, cases were more likely to use medication for hypertension and hyperlipidemia, and to self-report having diabetes. These differences were mutually independent, apart from higher prevalent diabetes in the cases, which lost statistical significance after regression adjustment for other risk factors for cardiovascular diseases. The GPx-3 activity was lower in cases than controls, but varied between surveys, with mean (SD) activity for samples collected in 1990 0.32 (0.12) U/ml, while those collected in 1995 had mean 0.51 (0.19) U/ml. Case-control difference were generally similar between surveys, although, like GPx-3, aspartate aminotransferase and alanine aminotransferase activities were higher in both cases and controls in 1995 than in 1990, while gamma glutamyltranferase activity and serum lipid, glucose, and urea nitrogen concentrations were comparable between the two surveys (data not shown). Among controls, GPx-3 activity was unrelated or inconsistently related to the covariates studied (**Table 2**) and there were no notable differences in GPx-3 associations with covariates between the 1990 and 1995 surveys (data not shown).

Table 1: Baseline (1990-1992 or 1995-1997) characteristics of the total study population by case-control status, the Minnesota Heart Survey

Characteristic	Cases (n=130)	Controls (n=240)	P*
Male sex, %†	57.7	56.7	.85
Age, y†	69.2 (11.9)	67.9 (12.2)	.33
BMI, kg/m2	27.8 (5.1)	27.3 (4.3)	.16
Current smoker, %	20.8	8.3	<.001
Alcohol, drinks per week	5.6 (12.8)	3.4 (6.8)	.07
Physical activity, MET-hr/day	8.5 (12.7)	7.8 (10.7)	.60
Serum cholesterol, mg/dl			
Total	220 (43)	216 (38)	.43
High-density lipoprotein (HDL)	41 (14)	45 (15)	.03
Lipid-lowering medication, %	13.8	6.7	.02
Hypercholesterolemia, %‡	41.5	32.1	.06
Blood pressure, mm Hg			
Systolic	135.9 (18.5)	130.1 (18.9)	.005
Diastolic	75.7 (12.7)	74.6 (10.6)	.37
Antihypertensive use, %	40.8	25.4	.002
Hypertension, %§	66.9	43.8	<.001
Self-reported diabetes, %	19.2	7.5	<.001
Aspirin use, %	39.2	35.0	.52
Enyme activities in serum			
Glutathione peroxidase-3, U/mL‖	0.34 (0.01)	0.37 (0.01)	.02
Gamma glutamyltransferase, U/L	43.3 (69.7)	25.9 (18.3)	.006
Serum glucose, mg/dL¶	130 (62)	109 (36)	<.001
Urea nitrogen, mg/dL	19.7 (9.2)	18.1 (4.9)	.07

Data are given as mean (SD), unless otherwise indicated.
*Based on 2-sample t-test, Mann-Whitney U test, or Chi-square test.
†Matching factor.
‡Defined as a serum total cholesterol >200 mg/dl.
§Defined as a blood pressure of ≥140/90 mm Hg or the use of antihypertensive medication.
‖Given are mean (standard error) glutathione peroxidase-3 activities adjusted for baseline year in covariance analysis.
¶Glucose concentrations were measured in non-fasting sera.

Serum activity of GPx-3 and cardiovascular mortality

Serum GPx-3 activity was inversely and dose-dependently related to cardiovascular mortality after controlling for the matching factors age and sex, and baseline year (**Table 3**). Additional adjustment for conventional cardiovascular risk factors and the activity of gamma glutamyltransferase had little effect on the strength of the association. The regression coefficient predicting the case-control logit per standard deviation of GPx-3 was -0.48 (P = .02) in 1990 and was -0.1 (P = .70) in 1995.

The association between GPx-3 and cardiovascular mortality was restricted to subjects with below sex-specific median concentration of serum HDL cholesterol (cutpoints were 38 mg/dl in men and 48 mg/dl in women), whereas GPx-3 was not significantly related in those with higher HDL cholesterol concentration (P for interaction, .006). For those below median HDL cholesterol, the multivariable adjusted regression coefficient predicting the case-control

Table 2: Cross-sectional relation between glutathione peroxidase 3 activity and characteristics at baseline: the Minnesota Heart Survey*

Characteristic	Baseline serum glutathione peroxidase 3 activity†				P trend‡
	Q1	Q2	Q3	Q4	
Number of controls	59	60	61	60	
Male sex, %	49.2	61.7	55.7	60.0	.81
Age, y	68.5 (11.1)	68.1 (12.8)	68.4 ±13.5)	66.6 (11.3)	.52
BMI, kg/m^2	27.8 (4.8)	26.9 (3.4)	26.8 (4.6)	27.7 (4.4)	.46
Current smoker, %	8.5	11.7	6.6	8.3	.86
Alcohol, drinks per week	3.1 (5.6)	4.7 (9.4)	3.2 (5.1)	2.5 (6.1)	.33
Physical activity, MET-hr/day	8.2 (11.4)	6.8 (9.5)	7.0 (9.8)	9.1 (12.2)	.63
Serum cholesterol, mg/dL					
Total	219 (39)	213 (43)	219 (34)	214 (36)	.73
High-density lipoprotein	42 (15)	45 (14)	50 (14)	43 (14)	.03
Lipid-lowering medication, %	5.1	11.7	0.0	10.0	.04
Hypercholesterolemia, %‖	33.9	35.0	31.1	28.3	.89
Blood pressure, mm Hg					
Systolic	127.8 (18.4)	128.2 (16.9)	130.6 (19.4)	133.9 (20.7)	.26
Diastolic	73.5 (11.3)	74.0 (9.6)	75.4 (11.6)	75.3 (10.0)	.71
Antihypertensive use, %	27.1	20.0	24.6	30.0	.64
Hypertension, %§	39.0	33.3	50.8	51.7	.11
Self-reported diabetes, %	8.5	6.7	4.9	10.0	.74
Aspirin use, %	42.4	23.3	36.1	33.3	.17
Gamma glutamyltransferase, U/L	25.7 (19.8)	23.6 (14.0)	26.2 (17.2)	28.1 (21.6)	.61
Serum glucose, mg/dL¶	108 (26)	105 (24)	110 (30)	115 (55)	.47
Urea nitrogen, mg/dL	19.6 (6.8)	17.8 (3.8)	17.7 (4.4)	17.4 (3.9)	.05

Data are given as mean (SD), unless otherwise indicated.
†Shown are year-specific (1990-1992 and 1995-1997) quartiles of glutathione peroxidase 3 activity, based on the distribution in controls. Ranges of glutathione peroxidase 3 activity in U/ml for increasing quartiles were 0.09-0.23, 0.24-0.30, 0.31-0.42, and 0.43-0.76 in 1990-1992, and 0.17-0.35, 0.36-0.47, 0.48-0.56, and 0.57-1.03 in 1995-1997.
‡Based on modeling quartiles of glutathione peroxidase 1 activity as a continuous variable.
§Defined as a blood pressure of ≥140/90 mm Hg or the use of antihypertensive medication.
‖Defined as a serum total cholesterol >200 mg/dL.
¶Glucose concentrations were measured in non-fasting sera.

logit per standard deviation of GPx-3 was -0.67 (P = .01) in 1990 and was -0.62 (P = .10) in 1995, compared to values for those at or above median HDL cholesterol, namely -0.08 in 1990 and 0.43 in 1995, neither significantly different from zero. We also displayed the association of GPx-3 activity and HDL cholesterol with cardiovascular mortality with the joint lowest GPx-3 quartile / below median HDL cholesterol as the single reference category. Compared to this reference, the multivariable-adjusted risk of cardiovascular mortality decreased across increasing quartiles of GPx-3 in those with below median HDL cholesterol concentration (**Figure**). In contrast, in those at or above the median HDL cholesterol concentration, the cardiovascular mortality risk across quartiles of GPx-3 was nearly constant and similar to the risk seen in those with a low HDL cholesterol concentration and high GPx-3 activity.

Table 3: Odds ratios and 95% confidence intervals (CIs) of cardiovascular mortality by serum glutathione peroxidase-3 activity: the Minnesota Heart Survey

	Quartiles of baseline serum glutathione peroxidase-3 activity*				Regression coefficient (SE)‡	P
	Q1	Q2	Q3	Q4		
All subjects						
Number of cases/controls	43/59	35/60	32/61	20/60		
Odds ratio (95% CI)						
Model A§	1.00	0.79 (0.44-1.40)	0.69 (0.38-1.25)	0.44 (0.23-0.83)	-0.30 (0.14)	.03
Model B‖	1.00	0.79 (0.43-1.43)	0.70 (0.38-1.30)	0.44 (0.23-0.86)	-0.28 (0.14)	.05
Model C¶	1.00	0.90 (0.48-1.68)	0.77 (0.40-1.49)	0.42 (0.21-0.86)	-0.30 (0.15)	.04
Low HDL cholesterol**						
Number of cases/controls	35/35	20/26	15/20	9/33		
Odds ratio (95% CI)						
Model A§	1.00	0.74 (0.35-1.57)	0.72 (0.32-1.65)	0.26 (0.11-0.64)	-0.52 (0.19)	.005
Model B‖	1.00	0.78 (0.36-1.71)	0.71 (0.29-1.69)	0.23 (0.09-0.58)	-0.57 (0.20)	.004
Model C¶	1.00	0.82 (0.36-1.88)	0.60 (0.23-1.59)	0.17 (0.06-0.47)	-0.68 (0.22)	.002
High HDL cholesterol**						
Number of cases/controls	8/24	15/34	17/41	11/27		
Odds ratio (95% CI)						
Model A§	1.00	1.28 (0.46-3.58)	1.15 (0.42-3.14)	1.16 (0.39-3.42)	0.09 (0.22)	.67
Model B‖	1.00	1.33 (0.44-4.02)	1.34 (0.45-4.00)	1.29 (0.39-4.26)	0.15 (0.23)	.53
Model C¶	1.00	1.38 (0.44-4.35)	1.34 (0.43-4.16)	1.22 (0.35-4.27)	0.12 (0.24)	.62

*Shown are year-specific (1990-1992 and 1995-1997) quartiles of glutathione peroxidase 3 activity, based on the distribution in controls. Ranges of glutathione peroxidase 3 activity in U/ml for increasing quartiles were 0.09-0.23, 0.24-0.30, 0.31-0.42, and 0.43-0.76 in 1990-1992, and 0.17-0.35, 0.36-0.47, 0.48-0.56, and 0.57-1.03 in 1995-1997.

‡Shown are regression coefficients (standard errors) predicting the case-control logit per standard-deviation (0.16 U/ml) increase of glutathione peroxidase 3 activity.

§Adjusted for matching factors age (continuous) and sex, and baseline year.

‖Adjusted as in model 1, but with additional adjustment for body mass index (continuous), current cigarette smoking, alcohol use (dummy variables in drinks per week: 1-6, 7-14, >14), and physical activity (continuous).

¶Adjusted as in model 2, but with additional adjustment for total cholesterol (continuous), high-density lipoprotein (HDL) cholesterol (continuous), systolic blood pressure (continuous), non-fasting glucose (continuous), and gamma glutamyltransferase (continuous). In stratified analysis, adjustment for HDL cholesterol was omitted.

** Low high-density lipoprotein (HDL) cholesterol was defined as lower than the median serum value of 38 mg/dl for men and 48 mg/dl for women, high serum HDL as greater or equal than 38 mg/dl in men and greater or equal than 48 mg/dl in women.

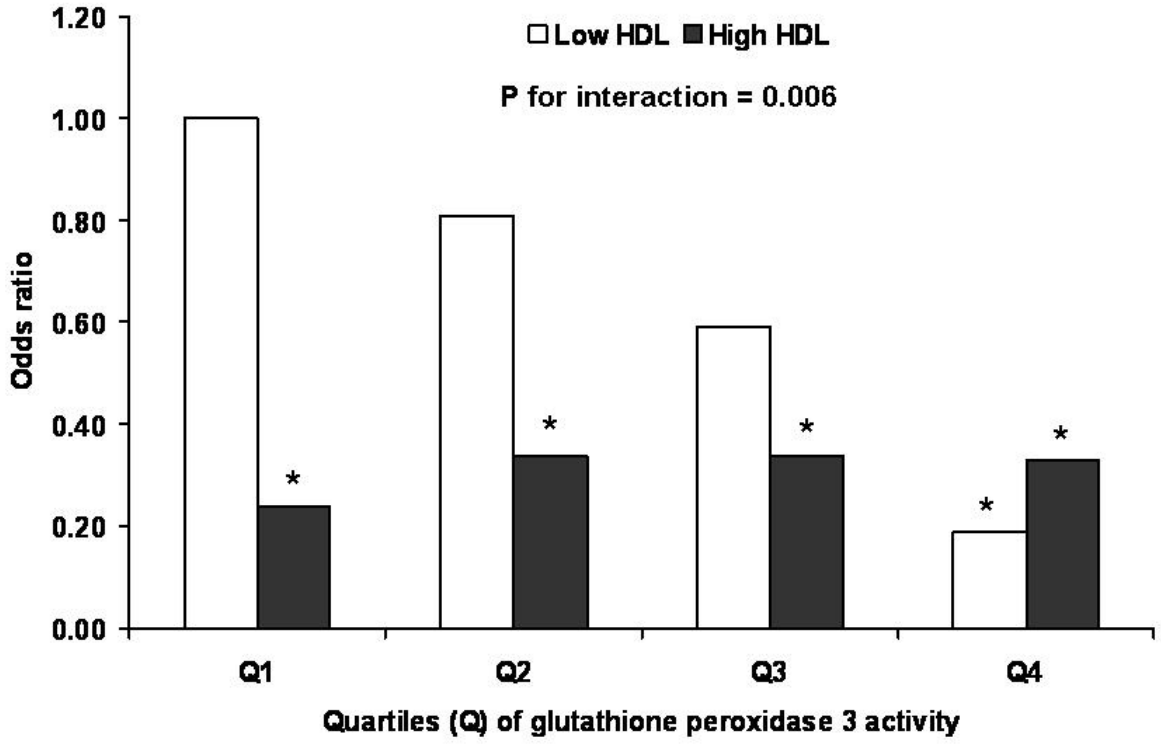

Figure: Odds ratios for cardiovascular mortality for joint levels of glutathione peroxidase 3 activity and high-density lipoprotein (HDL) cholesterol, adjusted for age, sex, baseline year, body mass index (continuous), current cigarette smoking, alcohol use (dummy variables in drinks per week: 1-6, 7-14, >14), and physical activity (continuous), total cholesterol (continuous), high-density lipoprotein (HDL) cholesterol (continuous), systolic blood pressure (continuous), non-fasting glucose (continuous), and gamma glutamyltransferase (continuous). Glutathione peroxidase 3 activities were year-specific quartiles, based on the distribution among controls. For HDL cholesterol, sex-specific median values were used as cutpoints; the median value was 38 mg/dl in men and 48 mg/dl in women. Those in the lowest quartile of glutathione peroxidase-3 activity and with below median HDL cholesterol served as the reference. The interaction tested was between quartiles of glutathione peroxidase-3 activity (as a continuous variable coded 1 to 4) and HDL cholesterol (continuous). * denotes $P < 0.05$ as compared to the reference.

After excluding cases that had died of non-atherosclerotic cardiovascular diseases (n=13), the relation in those with below median HDL cholesterol concentration remained essentially similar, with multivariable-adjusted odds ratios (95% CIs) for increasing quartiles of GPx-3 activity of 1.00, 0.83 (0.35 to 1.95), 0.58 (0.21 to 1.62), and 0.20 (0.07 to 0.57) (P for trend = .003). Additional adjustment for the use of aspirin or medication for hyperlipidemia or hypertension, or the prevalence of diabetes mellitus did not influence the estimates. The relation between GPx-3 activity and cardiovascular mortality did not differ significantly between smokers and non-smokers (P for interaction = .52), or alcohol intake (P for interaction = .08) or age (P for interaction = .14).

Discussion

In the current nested case-control study, the activity of GPx-3 in serum was inversely associated with cardiovascular mortality in a linear fashion, especially in subjects with low serum HDL cholesterol concentration. This relation was independent of conventional risk factors of cardiovascular diseases and gamma glutamyltransferase activity.

It is widely accepted that oxidized plasma lipids initiate and promote cardiovascular disease (13). GPx-3, which is the form found in HDL particles, is one enzyme that catalyzes the reduction of plasma lipid hydroperoxides. Although no population based studies have been published on the role of GPx-3 in cardiovascular disease, a few basic studies suggest that it may have a protective role (14,15). One study found that homocysteine decreased the concentration of nitric oxide through inhibition of GPx-1 (14), which was supported by an in vitro observation that homocysteine inhibited GPx-3 activity (1). Porter et al. (16) and Dogru-Abbasoglu et al. (17) in small cross-sectional studies have observed that patients with cardiovascular diseases have lower plasma, platelet, and erythrocyte (16) and plasma (17) GPx-3 activities than do normals. GPx-1 activity was inversely related to incident atherosclerotic events in the ATHEROGENE study in CAD patients with coronary artery disease (3,4). The ATHEROGENE study also found that the prediction of new events from homocysteine was stronger in those with low GPx-1 than in those with higher values (18). Similarly, Voetsch et al. (5) reported that the presence of several genetic variants in the promoter region of the GPx-3 gene were associated with an increased risk of premature arterial ischemic strokes. Finally, a case-control study (19) found that serum glutathione concentrations in the adolescent sons of recent myocardial infarction victims were significantly lower than those of sons of age-matched controls. Our finding of an inverse association between the activity of GPx-3 and cardiovascular mortality is consistent with these observations.

In the current study, the inverse relation was confined to those with low HDL cholesterol concentration. This may suggest that GPx-3 is an important antioxidant enzyme in persons with low HDL cholesterol concentration. In the Veterans Affairs High-Density Lipoprotein Cholesterol Intervention trial (VA-HIT) (20), patients with coronary heart disease and low HDL cholesterol concentrations were treated with the fibric acid derivative gemfibrozil. Although gemfibrozil significantly increased the HDL cholesterol concentrations and reduced coronary heart disease as compared to placebo, the increase in HDL cholesterol could only explain 23% of the reduction in coronary heart disease (21). This suggests that mechanisms other than HDL cholesterol are involved in the reduction of coronary heart disease, perhaps an increase in GPx-3 activity (2). This hypothesis is supported by the finding that treatment of dyslipidemia with fenofibrate for 120 days was accompanied by an increase of the activity of GPx in erythrocytes by 80% (22). Another study of fenofibrate (23) failed to observe such an increase, but red cells do not form new protein once in circulation, and the treatment duration of 30 days was insufficient for much red cell turn over.

This study has several limitations, mostly arising from the fact that the MHS was not designed to study the question posed here. Many scheduled blood samples were not collected, especially in 1995, and for unknown reasons this differentially affected those selected as controls in 1995. The number of cardiovascular deaths was relatively small, resulting in a sample size limitation, which limited the power to study the associations of GPx-3 activity in more detail. There was a systematic difference in GPx-3 activity for samples collected in 1990 versus 1995, probably accounted for by differential prefreezing refrigeration time. It is unlikely that any deterioration in samples would have affected cases and controls differentially, but the 1995 survey GPx-3 activity values are likely to be more representative of general population levels than are those reported here from the 1990 survey. Because we also noted systematically lower activity values for samples collected in 1990 compared to those collected in 1995 for the serum activity of aspartate aminotransferase and alanine aminotransferase, but not for gamma glutamyltranferase or for serum levels of lipids, glucose, and urea nitrogen, we therefore could not rule out some deterioration during the 5 years longer storage time at –70ºC as contributory, although such deterioration seems unlikely at this storage temperature. We removed any effect of this difference in GPx-3 by verifying that the case-control difference was similar between baseline years, computing year-specific quartiles for the activity of GPx-3, and adjusting all analyses for baseline year. Despite all these problems, this MHS nested case-control study provided an important opportunity for prospective study of GPx-3 and cardiovascular disease incidence.

In conclusion, our nested case control study indicates that a high activity of GPx-3 is related to a lower risk of cardiovascular mortality, particularly in persons with low concentration of HDL cholesterol. A high serum GPx-3 activity in those with low serum HDL cholesterol concentration may contribute to reduced cardiovascular mortality.

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General Discussion: The Antioxidant Paradox, Oxidative Stress, and Cardiovascular Diseases

8

The research described in this thesis aimed to elucidate the role that antioxidants and oxidative stress play in the development of cardiovascular diseases from an epidemiological perspective. Specifically, the objectives were to confirm previous observational studies on beta-carotene and vitamin E in relation to CVD, and to evaluate emerging antioxidants and indicators of oxidative stress in relation to CVD. The main findings were a three-fold lower 40-year mortality from coronary heart disease (CHD) in the Cretan cohort (Greece) compared with the Zutphen cohort (The Netherlands; Chapter 3) of the Seven Countries Study. This lower CHD mortality was paralleled by a higher intake of carotenoids from fruits and vegetables, particularly tomotoes, and a higher intake of alpha-tocopherol from olive oil. Also, the Cretan men had lower intakes of saturated and *trans* fatty acids than did the Zupthen men. These differences in intake were confimed at the age of 80 years and older, when the Cretan cohort had higher plasma concentrations of major dietary antioxidants such as lycopene and alpha-tocopherol, lower levels of iron status, lower levels of oxidative stress and inflammation, and a more favorable plasma fatty acid composition (Chapter 2). Earlier prospective cohort studies that found inverse associations between antioxidants and risk of cardiovascular diseases (CVD) were confirmed for dietary and blood concentrations of (beta-)carotene, but not for vitamin E (Chapters 4 and 5). Novel findings were the inverse association between the intake of cocoa, a rich source of flavanol antioxidants, with blood pressure and 15-year CVD and all-cause mortality (Chapter 6), and the inverse relation of serum glutathione peroxidase-3, an antioxidant enzyme, with CVD mortality, particularly in persons with low HDL cholesterol concentrations (Chapter 7). This chapter puts these findings into perspective and gives suggestions for future research.

The antioxidant paradox: no clear-cut answer yet

The antioxidant paradox refers to the sharp contrast that exists in evidence concerning the cardiovascular benefits of antioxidants (1), primarily vitamin E and beta-carotene, obtained from experimental animal studies and human observational studies (generally suggesting beneficial effects) on the one hand, and large-scale randomized trials (finding no or even harmful effects) on the other hand. There is yet no clear-cut answer for this apparent paradox, although a plethora of possible explanations exists.

In retrospect, the first large prospective cohort studies for vitamin E showed consistently strong associations (2-5) which were considered too large to be explained by confounding (relative risks for CHD were between 0.4-0.6 in (3-5)). However, the results of later published studies on vitamin E were less consistent (6), and two prospective cohort studies described in this thesis did not find an association of either vitamin E intake or vitamin E concentrations in blood with CVD mortality.

An all-embracing explanation for the first positive cohort studies is lacking. One of the reasons may be that subjects with a high intake of vitamin E, in particular vitamin E supplement users, differ in a multitude of aspects from those with a low intake. By definition, observational studies do not randomize participants with respect to exposure as is done in clinical trials. Although differences between subjects in observational studies are usually accounted for by statistical adjustments, it is sometimes not certain whether these adjustments are sufficient (7). Residual confounding arises when a factor related to both exposure and disease, while not involved in the causal pathway, is not at all or (due to measurement error or improper categorization) poorly accounted for. This is a major threat to the validity of observational studies.

Multiple factors have been suggested to be inadequately accounted for in observational studies on antioxidants and CVD. First, multiple social and behavioral factors acting across the course of life have been proposed (8). In the British Women's Heart and Health Study, Lawlor et al. (8) showed that adulthood plasma concentrations of vitamin C, and to a lesser extent also vitamin E, are inversely related to multiple measures of socioeconomic status from childhood through adulthood. They propose that observational studies should account for a wide range of socioeconomic and behavioral factors and demonstrated in the same population that plasma vitamin C is not longer associated with coronary heart disease after adjustment for these factors (9). However, this approach has been criticized by others (10,11), due to a lack of biological and quantitative plausibility and because many of the proposed measures, such as adulthood leg-length as marker for early life exposure, have multifactorial causes (12).

A second possible factor that has been proposed to induce residual confounding is (low-grade) systemic inflammation. Because inflammation itself has consistently been shown a risk factor for coronary heart disease (13), and plasma levels of antioxidants have been inversely related to measures of inflammation (14-17), it may be that plasma antioxidants are simply a

surrogate for inflammation. Alternatively, it may also be possible that inflammation lies within the biological pathway in which antioxidants lower CVD risk. This is supported by the observation that supplementation with antioxidants reduces measures of inflammation in various populations (18-21). If antioxidants truly lower inflammation, then observational studies on antioxidants and CVD can evaluate the contribution by the reduction in inflammation by adjusting for it in the statistical analysis. However, the observational studies on antioxidants and CVD risk that accounted for inflammation did not show substantial attenuation of the risk estimates (16,22,23), which suggests that the inverse associations between antioxidants and CVD are explained by other factors than inflammation.

A third factor, smoking, was initially proposed as confounder in the relation between beta-carotene and lung cancer (24). However, it may also be relevant in the relation between antioxidants and CVD as smoking is a strong CVD risk factor and has been related to lower blood concentrations of antioxidants (17,25). These lower concentrations are at least partly due to smoking-induced destruction of antioxidants (26-28). Low blood concentrations of antioxidants may therefore simply reflect the risk of cigarette smoking. However, without a few exceptions (4,29), the inverse relation between antioxidants and CVD has been shown, also in this thesis, in both smokers and non-smokers (4,5,30). This makes it unlikely that smoking is a major confounding factor. It can, however, not be excluded that residual confounding by several factors together partly explain the inverse association between antioxidants and CVD.

Perhaps the most likely explanation is that other dietary antioxidants and compounds with bioactive potential are responsible for the apparent protective effects of single antioxidants in observational studies. For ages, the human body has been exposed to a wide array of antioxidants and bioactive compounds from plant foods, among which tocopherols, tocotrienols, carotenoids, and polyphenols. The Zutphen Elderly Study, in which dietary intake was repeatedly assessed by means of the dietary history method and intake of antioxidants were estimated by using a detailed food composition table, confirms earlier studies that found an inverse association of CVD mortality with alpha- and beta-carotene in the diet (Chapter 3). This inverse relation was also confimed with concentrations of carotene in blood in SENECA (Chapter 4). An explanation for these findings is that carotene (31), like vitamin C (32), is a marker for fruit and vegetable consumption and, thus, for their total antioxidant and bioactive potential. Taking a single antioxidant in the form of a supplement in dosages beyond dietary intake may disrupt the delicate biochemical balance between all these compounds (33,34). This may explain the increased risk of CVD and mortality that is related with the use of antioxidant supplements (35-38), as it is thought that high dosages of antioxidants exhibit pro-oxidant rather than antioxidant effects (39).

Apart from observational studies, the large-scale trials on antioxidants and CVD have been criticized too (1,40-43), including [A] the choice to test a single antioxidant instead of multiple, [B] in the *synthetic* form rather than the *natural* one with higher activity (vitamin E), [C] in a dose that was either too low or too high, [D] for a period that was too short, [E] in

patients with established CVD or high risk groups, but without any proven oxidative stress, [F] and lack of monitoring therapy compliance. Concerning the dose, it might be that antioxidants within the normal dietary range offer protection, whereas amounts beyond that (i.e., from supplement use) increase CVD risk (44). Although the total evidence, i.e. from all studies together, may suggest such a curvilinear association, this has not been shown within a single population.

Finally, the negative results from the trials might also suggest that the oxidation modification hypothesis is incorrect (45). However, the lack of benefit shown by the large-scale trials does not necessarily disprove the oxidation hypothesis. Rather, it shows that the type and dosage of antioxidants tested in the trials lack benefit. As long as the precise underlying mechanisms of *in vivo* lipoprotein oxidation are unknown, it remains uncertain which antioxidants, if any, at what dosages, would be most effective (40).

In summary, despite many possible explanations, the antioxidant paradox remains unsolved. The most likely explanation is that single antioxidants as studied in observational studies, were not only reflecting the actual intake of the antioxidant itself, but rather of multiple antioxidants and bioactive compounds.

Cocoa, a food component recently discovered to be rich in antioxidants

Although cocoa has only recently been proposed as a healthy food component, the interest in the beneficial health effects of cocoa is not new. Already three centuries ago, it was thought that cocoa relieved angina pectoris (46). The empirical research on cocoa and cardiovascular health, however, started only a decade ago, after the finding that chocolate is a rich source of antioxidants, in particular flavanols (47,48). The first observational data in humans came from the island-dwelling Kuna Indians in Panama. While living off Panama's coast, they consume large amounts of cocoa drink and rarely develop hypertension, whereas this apparent protection by cocoa is lost upon migration to Panama city (49).

An important biological mode of action by which cocoa is thought to exert its beneficial effects, is by improving endothelial function. The vasodilator nitric oxide (NO) plays an essential role in maintaining endothelial function. Endothelial dysfunction is recognized as an early event in the development of atherosclerosis (50) and occurs when the bioavailability of NO decreases. Consumption of cocoa and chocolate rich in flavanols improves vasodilation (51-54) likely by improving NO bioavailability (54,55). Apart from improving endothelial function, other possible mechanisms of action include inhibition of platelet function (56,57), increase in HDL cholesterol concentrations (58), modulation of cytokine production (59), improvement of glycemic control (60,61), and inhibition of LDL oxidation (62).

One of the possible effects of improving endothelial function is a lower blood pressure. Previous experimental studies have tested the blood pressure lowering potential of dark chocolate (53,60,61,63) or cocoa drink (61). In a meta-analysis (64), 100 grams of dark chocolate per day for at least 7 days was shown to lower systolic blood pressure by 4.7 (95% CI, -7.6 to -1.8) mm Hg and diastolic blood pressure by 2.8 (95% CI, -4.8 to -0.8) mm Hg. The findings of the Zutphen Elderly Study indicate that blood pressure can be lowered with already 4 grams of cocoa per day, comparable to about 10 grams of regular dark chocolate (Chapter 6). Indeed, a more recently published intervention study found that 6.3 grams of dark chocolate per day for 18 weeks lowered systolic blood pressure with 2.9 mm Hg ($P < .001$) and diastolic blood pressure with 1.9 mm Hg ($P < .001$), as compared to an isocaloric amount of white chocolate (65).

Apart from the inverse association between habitual cocoa intake and blood pressure, the Zutphen Elderly Study also indicates that a daily consumption of 4 grams of cocoa lowers CVD mortality by 50% as compared to those not consuming cocoa. Up to present, only two other observational studies have published results on cocoa and incident CVD. Chocolate consumption was prospectively related to a lower CVD mortality in the Iowa Women's Health Study (66) and to a lower odds of non-fatal myocardial infarction in an Italian case-control study (67).

Taken together, evidence is accumulating that cocoa and its flavanols improve endothelial function and lower blood pressure by increasing the bioavailability of NO. Whether this translates to a reduced CVD incidence is tempting, but so far there is insufficient evidence in this emerging area of research.

Oxidative stress

Oxidative stress can be defined as a higher rate of oxidant production compared to the rate of their inhibition and degradation. This definition makes oxidative stress a theoretical model and more conceptual than operational in biomedical research (68). There is still much to learn about the oxidative mechanisms *in vivo*. Together with the lack of validated indicators of oxidative stress and the difficulty in distinguishing oxidative stress as a cause or consequence of disease, this makes many scientists skeptical about the role of oxidative stress in disease etiology.

Oxidative stress in humans can be monitored by [A] measuring products of oxidative damage, [B] by challenging the potential of individuals or their tissue (usually blood) to withstand *in vivo* or *in vitro* oxidation, and by [C] measuring activities of antioxidant enzymes. This thesis describes only two of a wide range of measureable products of oxidative processes, i.e. concentrations of serum hydroperoxides and malondialdehyde (MDA). In addition, activities of two enzymes involved in the metabolism of glutathione were described, i.e. gamma-glutamyltransferase, of which high activities have been related to an increased CVD risk, and

glutathione-peroxidase 3, of which high activities have been related to a lower CVD risk. There is, however, little agreement about which is the best indicator of oxidative stress.

Ideally, a biomarker of oxidative stress should be predictive for and reflect biological events related to the pathogenesis of CVD; should be stable over a period of time in individuals; should provide identical results when assayed in the same laboratory as well as in different laboratories; and should be stable (at least in its ranking) during specimen collection, freezing storage, processing and assaying (69). Additional criteria apply for the use in large-scale observational studies: the biomarker should also be measurable by an assay that performs well in the lower concentration range; the measurement should be feasible on a large scale (reasonable costs and high throughput), and utilize a minimum of specimen.

For most biomarkers, however, there is currently no sufficient information available necessary to evaluate these criteria (70). Various products of low-density lipoprotein (LDL) oxidation can be measured in blood, but many lack precision (71), and a reduction in LDL oxidation by vitamin E supplementation does not substantially lower the progression of atherosclerotis (72). MDA is an extensively studied product of polyunsaturated fatty acid peroxidation and its concentration was nonsignificantly lower among elderly men in Crete than in Zutphen (Chapter 3). Although there has been much concern about the sensitivity and specificity (73,74), MDA has also been found an stable and effective marker of smoking related oxidative stress (75). Serum hydroperoxides and activity of gamma-glutamyltransferase, were much lower in Crete compared to Zutphen, though need to be related to clinical CVD end points in future studies.

The biomarker of oxidative stress that meet most criteria for use in large-scale observational studies at present are the isoprostanes, which have become the gold standard for *in vivo* oxidative stress, at least with respect to lipid peroxidation (76). Isoprotanes are stable, specific, and unique end products of arachidonic acid oxidation (77). Although isoprostanes may be a valid biomarker of lipid peroxidation, measures of lipid peroxidation alone do not represent overall oxidative stress, due to a lack of correlation with oxidative stress measures in other molecules, including DNA and protein (78). Therefore, it is recommended to measure multiple biomarkers of oxidative stress and also concurrently measure markers of inflammation, as oxidative stress and inflammation often parallel each other (70).

Future epidemiological research

Antioxidants and oxidative stress in relation to CVD form a controversial topic that has been extensively studied in the past two decades. Hopeful signs come from studies on foods rich in flavonoids and their effects on endothelial function and blood pressure. On the other hand, extensive research on beta-carotene and vitamin E has led to the antioxidant paradox.

Given the evidence that a diet rich in plant foods, including fruit and vegetables, lowers CVD risk, and that beta-carotene and vitamin E alone are not responsible for this effect, suggests

that multiple antioxidants and other compounds with bioactive potential may synergistically offer cardiovascular protection. Therefore, it is worthwhile to study multiple antioxidants simultaneously. Since 1941 it has been suggested that vitamin C recycles vitamin E (79,80). Yet, the first direct evidence that vitamin C and vitamin E work together *in vivo* has only recently been published (81). More of these studies are needed to indentify which antioxidants are working in concert. Up to present, nutritional epidemiology has heavily relied on studying one nutrient at a time, by using the so called 'single-nutrient approach.' Although this approach is powerful and the first to recommend for not commonly studied components in the diet, there is a need for additional study designs that address multiple dietary compounds (nutrients or foods) simultaneously. The research in this thesis continues to suggest an inverse association between beta-carotene and CVD, and also that alpha-carotene intake is related to a lower CVD risk. However, beta-carotene itself is not likely to be a causal factor, and the finding for alpha-carotene merits further study. It may be that beta-carotene, and perhaps also alpha-carotene, are just markers of a healthy diet. One relatively new approach in nutritional epidemiology is studying patterns of dietary intake by using different analytical techniques (82-85). By using these designs, patterns of dietary antioxidants can be studied.

But perhaps we should go one step further backwards. It has even been suggested that studying nutrients is too simplistic and that foods rather than nutrients should be the unit of nutritional research (86). By estimating the intake of, for example, beta-carotene from fruit and vegetables, researchers will lose information about the food sources of beta-carotene and its interactions with other bioactive compounds. During the past few years, results on food and food pattern analyses have become a topic of interest in observational studies (87-89). However, there is a lack of randomized trials on the effects foods on CVD. Compared to randomized intervention trials testing the efficacy of beta-carotene, trials on foods, such as fruit and vegetables, are only sparingly published (90-93) and need to be encouraged. Specifically, trials with an increased intake of plant foods rich in carotene or flavanols and other flavonoids need to be conducted. Studying hard end points, such as incident CVD, might be difficult as long-term compliance to a high fruit and vegetable diet is difficult. Therefore, surrogate end points for CVD, including blood pressure, inflammation, blood lipids, atherosclerotic progression, and oxidative stress should be used.

In order to measure the efficacy of (foods rich in) antioxidants in reducing oxidative stress and inhibiting lipoprotein oxidation, observational and intervention studies should use valid biomarkers. Measuring the ability of an antioxidant to lower the *ex vivo* lipoprotein oxidation is widely used, but may not be satisfactory enough. Thus, there is a need for new biomarkers. Apart from isoprostanes, the serum concentration of hydroperoxides may be worthwhile to consider. Currently, it is recommended to measure multiple measures of oxidative stress (70), together with measures of inflammation (94). Also, the use of information on antioxidant capacity of foods (95) or redox-active compounds in the diet (96) may be useful in observational epidemiology. The incorporation of relevant biomarkers and nutrigenomics (97)

may enable researchers to investigate molecular interactions, influences of genetic polymorphism, new valid intermediate endpoints, and differentiate persons who are more susceptible for oxidative stress from those who are not. In the future, the use of genetic epidemiology may be helpful in underpinning the causal inference of antioxidants in observational studies. For example, by using the variance in functional genes, researchers may differentiate subjects with a high and low antioxidant status or oxidative stress, independent from behavioral, social, and environmental factors (98).

The research on the cardiovascular health effects of cocoa has only just begun. Although evidence is accumulating that cocoa may improve CVD risk factors and lower CVD risk itself, much research still needs to be done before any recommendations can be made (99). At present, only two prospective cohort studies have been published on cocoa and incident CVD, with promising results (Chapter 6 and (66)). More of such studies on both intermediate and clinical end points are needed to confirm these findings. A biomarker of cocoa or flavanol intake would be very useful to estimate the intake accurately. Intervention studies need to increase their duration and use lower, more realistic amounts of chocolate or cocoa; use an appropriate placebo for chocolate that, apart from having no flavanols, is difficult to distinguish from the experimental flavanol-rich chocolate; properly assess and publish the content of flavanols as well as other nutrients and bioactive components; and, attempt to publish null or negative results to prevent publication bias. Also, although nearly all scientific interest is going out to flavanols (100), cocoa also contains other compounds with bioactive potential, including flavonols (101), another flavonoid subclass, and methylxanthines such as theobromine (102). Finally, the dominance of industry-funded human intervention studies on the health effects of cocoa is worrisome. Of 28 identified studies (99), 19 had industrial involvement by either sponsorship or authorship, whereas 9 can be classified as industry-independent. Though the interest of the food industry in this new area of research is understandable, studies without involvement of the industry, funded by governmental and other non-profit organizations, should be encouraged.

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Summary

Cardiovascular diseases (CVD) remain the number one cause of death worldwide. During the past 20 y, there has been a great interest in the role of antioxidants in the prevention of CVD. Antioxidants are proposed to lower CVD risk by reducing oxidative stress in general and the oxidation of low-density lipoproteins (LDL) in particular. In this thesis, the role of antioxidants and oxidative stress in the development of CVD was investigated from an epidemiological perspective. The aim was to confirm previous observational studies on antioxidants and CVD risk and to investigate the association of emerging antioxidants and oxidative stress with CVD. These objectives were evaluated in cross-cultural and prospective cohort studies.

In **Chapters 2** and **3**, the Cretan (Greece) and Zutphen (The Netherlands) cohorts of the Seven Countries Study were compared cross-culturally with respect to coronary heart disease (CHD) mortality, diet, lifestyle, and biomarkers of nutrient intake. At baseline in 1960, 676 men in Crete and 878 men in Zutphen (age, 40-59 y), were examined. After 40 y of follow-up, the median survival time for all-cause mortality was 32 y in Crete (95% confidence interval [CI]: 31 to 33 y) compared to 26 y in Zutphen (95% CI: 24 to 27 y; P for difference <0.0001). The CHD mortality rate was 3 times lower in Crete. Compared to Zutphen, the Cretan diet was higher consumption of fruit, tomatoes, and olive oil, amongst others, and a lower intake of meat, dairy, poultry, solid fats and sugar products. Consequently, the intake of carotenoids, alpha-tocopherol, and vitamin C was higher, and that of saturated and *trans* fatty acids lower in Crete.

At the age of ~80 y and older, the survivors of the Cretan and Zutphen cohorts were compared with respect to their antioxidant status, iron status, and level of oxidative stress. This analysis included 105 men in Crete and 139 men in Zutphen (age range, 79-98 y). Two previously proposed indicators of oxidative stress, i.e. serum hydroperoxides and the activity of gamma-glutamyltransferase, were on average lower in Cretan men than men in Zutphen ($P < .01$ for both). The most pronounced difference in iron status was a twofold lower mean serum ferritin concentration in men in Crete ($P < .0001$), a marker of the amount of stored iron. Finally, Cretan men had consistently higher plasma concentrations of major antioxidants, including a higher mean concentration of beta-carotene ($P = .001$) and even a nearly fourfold higher mean concentration of lycopene ($P < .0001$), a carotenoid exclusively found in tomatoes. Given the cross-cultural design and the old age of the survivors, it is difficult to judge the strength the relation between these factors and CHD risk.

The Zutphen cohort was used to investigate whether dietary antioxidants were related to CVD mortality (**Chapter 4**). In this study, about 500 men (age, 65-84 y) participated since 1985 and were followed for 15 years. Of six dietary carotenoids, only alpha- and beta-carotene were inversely related to CVD mortality [relative risk (RR) for 1 standard deviation increase in intake of both carotenoids: ~0.80; P for both <.05]. Carrots were the dietary source of these carotenes,

and its consumption was also inversely related to CVD mortality (0.83; 95% CI: 0.68–1.00). Vitamin E from dietary sources was not related to CVD mortality, nor was dietary vitamin C. Also, the total intake of vitamin E and vitamin C as estimated from dietary intake and the use of vitamin supplements were not associated with CVD mortality. Finally, multiple dietary antioxidants were investigated by constructing an 'antioxidant score.' This score was weakly inversely related to CVD mortality, and this association was driven by alpha- and beta-carotene.

In the 'Survey in Europe on Nutrition and the Elderly: a Concerted Action' (SENECA), the association between serum concentrations of carotene and alpha-tocopherol with mortality was evaluated (**Chapter 5**). In this study, 1168 men and women (age, 70-75 y) from eight European countries were examined and followed for 10 y. Plasma concentrations of carotene were inversely related to mortality [RR for 1 standard deviation increase in plasma concentration: 0.83 (95% CI: 0.70-1.00) for CVD mortality and 0.79 (95% CI: 0.70-0.89) for all-cause mortality]. Plasma concentrations of vitamin E were not related to CVD and all-cause mortality. For both antioxidants, the findings on all-cause mortality were confirmed by a meta-analysis of 5 observational studies, including SENECA.

Data from the Zutphen Elderly Study was also used to address a food component naturally rich in certain flavonoids, which became more recently a topic of intensive scientific interest: cocoa (**Chapter 6**). The intake of cocoa from cocoa-containing foods was cross-sectionally related to blood pressure, and prospectively to 15-y CVD mortality. As compared to men who did not use cocoa, the intake of 4 g of cocoa per day was related to a 3.7 (95% CI: –7.1 to –0.3) mm Hg lower mean systolic blood pressure and a 2.1 (95% CI: –4.0 to –0.2) mm Hg lower mean diastolic blood pressure (for both P trend < .05). The relative risk of CVD mortality was also 50% lower in these men (RR: 0.50; 95% CI: 0.32-0.78; P trend = .004).

In the Minnesota Heart Survey, a nested case-control study was conducted on the association between CVD mortality and the serum activity of glutathione peroxidase 3 (GPx-3), a scarcely studied antioxidant enzyme but considered important in lowering oxidative stress (**Chapter 7**). This analysis included data of 130 CVD deaths and 240 control subjects (age, 26-85 y), from who blood was earlier collected, either in 1990-1992 or 1994-1997. Persons in the highest quartile of serum activity of GPx-3 had on odds ratio of CVD mortality of 0.42 (95% CI: 0.21-0.86; P trend = .02). This inverse association was only evident in persons with below median concentrations of HDL cholesterol (odds ratio: 0.17; 95% CI: 0.06-0.47; P trend = .001); no association was observed in those with median or higher concentrations of HDL cholesterol (P interaction = .006). These findings may indicate that having a low concentration of HDL cholesterol may not increase the risk of CVD mortality as long as the activity of GPx-3 is high. However, due to small numbers and a systematic difference in GPx-3 activity between samples from the two used baseline periods, these findings need to be confirmed in other studies.

The results described in this thesis were put into perspective in **Chapter 8**. There is a sharp contrast between the favorable results on beta-carotene and vitamin E from observational studies and the null-findings or even harmful effects of these antioxidants found in large-scale randomized trials (the 'antioxidant paradox'). The research described in this thesis continous to suggest an inverse relation between beta-carotene and CVD mortality, but supports no favorable or harmfull role of vitamin E. Altough the interpretation of the findings on beta-carotene remains ambiguous, it may be that beta-carotene reflects a diet rich in multiple bioactive compounds, including alpha-carotene. The inverse relation of cocoa with blood pressure and CVD described in this thesis are supported by short-term intervention studies on intermediate risk factors for CVD. However, the research on cocoa and its flavanols has only just started and it is too early for drawing firm conclusions.

Oxidative stress is a term that is more conceptual than operational. Its evaluation as a CVD risk factor is hampered by the current lack of validated biomarkers. Criteria for an ideal biomarker of oxidative stress are described. Until such biomarkers are not available, it is recommended to measure multiple indicators of oxidative stress, together with indicators of inflammation.

Future epidemiological research should evaluate multiple antioxidants simultaneously, for example by applying new analytical methods to study food patterns. Also, more intervention studies on foods rich in antioxidants such as carotene and flavanols are needed. Such studies should use established intermediate CVD end points. Intervention studies on the cardiovascular health effects of cocoa should increase in duration, use more realistic amounts of cocoa and a suitable placebo, and should publish the content of flavanols and other nutrients. The few published prospective cohort studies on cocoa or chocolate and CVD need to be confirmed by others. A biomarker of flavanol or cocoa intake would be very useful in this respect.

Samenvatting

Hart- en vaatziekten (HVZ) vormen wereldwijd nog steeds de belangrijkste doodsoorzaak. In de afgelopen 20 jaar was er grote belangstelling voor de rol van antioxidanten in de preventie van HVZ. Antioxidanten worden verondersteld het risico op HVZ te verkleinen via het verlagen van oxidatieve stress, in het bijzonder de oxidatie van het *low-density lipoprotein* (LDL) cholesterol. In dit proefschrift is de rol van antioxidanten en oxidatieve stress op het ontstaan van HVZ onderzocht vanuit epidemiologisch perspectief. Het doel was om eerder gepubliceerde observationele onderzoeken over antioxidanten in de voeding en HVZ te bevestigen en de relatie van nieuwe antioxidanten en indicatoren van oxidatieve stress met HVZ te bestuderen. Hiervoor werden cross-culturele en prospectieve cohortstudies gebruikt.

In **Hoofdstuk 2** en **3** zijn twee cohorten van de Zeven Landen Studie, namelijk het cohort op Kreta (Griekenland) en het cohort in Zutphen (Nederland) met elkaar vergeleken wat betreft sterfte aan coronaire hartziekte (CHZ), voeding, leefstijl en biomarkers voor nutriëntinname. Tijdens het eerste onderzoek in 1960 werden hiervoor 676 mannen in Kreta en 878 mannen in Zutphen (leeftijd 40-59 jaar) onderzocht. Na 40 jaar follow-up was de mediane overlevingsduur voor totale sterfte 32 jaar in Kreta (95% betrouwbaarheidsinterval [BI]: 31 tot 33 jaar), vergeleken met 26 jaar in Zutphen (95% BI: 24 tot 27 jaar; $P < .0001$). De sterfte aan CHZ was 3 keer lager in Kreta. Vergeleken met Zutphen bevatte het Kretenzer voedingspatroon onder andere meer fruit, tomaten en olijfolie, en minder vlees, zuivel, gevogelte, zogenaamde 'harde' vetten en suikerprodukten. Daarom was de inname van carotenoiden, alfa-tocoferol en vitamine C hoger, en dat van verzadigde en transvetzuren lager in Kreta.

Op de leeftijd van ~80 jaar en ouder werden de overlevenden van het Kreta- en Zutphencohort vergeleken wat betreft hun antioxidantstatus, ijzerstatus, en mate van oxidatieve stress. Dit onderzoek had betrekking op 105 mannen in Kreta en 139 mannen in Zutphen (leeftijdsrange 79-98 jaar). Twee veronderstelde indicatoren voor oxidatieve stress in serum, te weten de concentratie hydroperoxides en de activiteit van gamma-glutamyltransferase, waren gemiddeld lager in de Kretenzer mannen ($P < .01$ voor beide). Het meest opvallende verschil in ijzerstatus was een twee keer zo laag gemiddeld serumferritinegehalte in de Kretenzer mannen ($P < .0001$). Tenslotte hadden zij ook hogere plasmaconcentraties van antioxidanten in de voeding, waaronder een hoger gemiddelde concentratie van beta-caroteen ($P = .0001$) en een bijna vier keer zo hoog gemiddelde concentratie van lycopeen ($P < .0001$), een carotenoid dat alleen in tomaten voorkomt. Gezien het cross-culturele design van dit onderzoek en de hoge leeftijd van de overlevenden is de sterkte van de relatie tussen deze factoren en het CHZ-risico moeilijk vast te stellen.

Het cohort in Zutphen werd gebruikt om de relatie tussen antioxidanten in de voeding gerelateerd en sterfte aan HVZ te onderzoeken (**Hoofdstuk 4**). Hiervoor werden gegevens

gebruikt van ongeveer 500 mannen (leeftijd 60-80 jaar) die vanaf 1985 werden onderzocht. Van in totaal zes verschillende carotenoiden in de voeding waren alleen alfa- en beta-caroteen invers gerelateerd aan sterfte door HVZ [relatief risico (RR) voor een toename van 1 standaarddeviatie in inname voor beide carotenoiden: ~0,80; P voor beide <.05] . Wortels waren de belangrijkste bron van deze carotenoiden in de voeding en de consumptie ervan was ook invers gerelateerd aan sterfte door HVZ (RR: 0,83; 95% CI: 0,68–1,00). Vitamine E in de voeding was niet gerelateerd aan sterfte door HVZ, evenals vitamine C. Ook de gecombineerde inname van deze antioxidant-vitamines in de voeding en voedingssupplementen was niet gerelateerd aan sterfte door HVZ. Tenslotte werden verschillende antioxidanten samengevat in een 'antioxidantscore.' Deze score was zwak invers gerelateerd aan HVZ-mortaliteit, wat verklaard kon worden door de belangrijke bijdrage van alfa- en beta-caroteen aan deze score.

In de 'Survey in Europe on Nutrition and the Elderly: a Concerted Action' (SENECA) werden de relaties tussen serumconcentraties van carotene en alfa-tocoferol met sterfte bestudeerd (**Hoofdstuk 5**). In dit onderzoek werden 1168 mannen en vrouwen (leeftijd 70-75 jaar) uit acht verschillende Europese landen onderzocht en gevolgd voor 10 jaar. Plasmaconcentraties van caroteen waren invers gerelateerd aan sterfte door HVZ [RR voor een toename van 1 standaarddeviatie in plasmaconcentratie: 0,83; 95% betrouwbaarheidsinterval (BI): 0,70-1,00] en aan totale mortaliteit (RR: 0,79; 95% BI: 0,70-0,89). Plasmaconcentraties van vitamine E waren niet gerelateerd aan HVZ- en totale sterfte. Voor beide antioxidanten werden de bevindingen voor totale sterfte bevestigd in een meta-analyse van 5 observationele onderzoeken, waaronder SENECA.

Gegevens van de Zutphen Studie werden ook gebruikt voor een onderzoek naar een voedingsingrediënt dat van nature rijk is aan bepaalde flavonoiden en recentelijk in wetenschappelijke belangstelling is komen te staan: cacao (**Hoofdstuk 6**). De inname van cacao uit voedingsmiddelen werd cross-sectioneel onderzocht in relatie tot bloeddruk en prospectief in relatie tot 15-jaars sterfte aan HVZ. Ten opzichte van mannen die geen cacao gebruikten, was de inname van 4 g cacao per dag geassocieerd met een 3,7 (95% BI: –7,1 to –0,3) mm Hg lager gemiddelde systolische bloeddruk en een 2,1 (95% BI: –4,0 to –0,2) mm Hg lagere diastolische bloeddruk (P trend voor beide < .05). Het relatief risico voor sterfte aan HVZ bij mannen die dezelfde hoeveelheid cacao gebruikten was de helft van het risico voor mannen die geen cacao aten (RR: 0,50; 95% BI: 0,32-0,78; P voor trend = .004).

In de Minnesota Heart Survey werd een genest patiënt-controleonderzoek uitgevoerd naar de relatie tussen de serumactiviteit van glutathioneperoxidase 3 (GPx-3), een weinig onderzocht maar belangrijk verondersteld antioxidantenzym, en sterfte ten gevolge van HVZ (**Hoofdstuk 7**). Dit onderzoek had betrekking op gegevens van 130 sterfgevallen van HVZ en 240 controlepersonen (leeftijd 26-85 jaar), van wie op een eerder tijdstip bloed was afgenomen,

ofwel in de periode 1990-1992, ofwel tussen 1994-1997. De odds ratio voor sterfte aan HVZ voor personen in het hoogste kwartiel van GPx-3-activiteit bedroeg 0,42 (95% BI: 0,21-0,86; P trend = .02). Deze inverse associatie werd alleen waargenomen voor personen met een HDL-concentratie beneden de mediane waarde (RR: 0,17; 95% BI: 0,06-0,47; P trend = .001); er werd geen associatie gevonden voor degenen met HDL-concentraties gelijk of hoger dan de mediaan (P interactie = .006). Deze bevindingen suggereren dat een lage HDL-concentratie niet gepaard hoeft te gaan met een verhoogd risico op sterfte aan HVZ zolang de activiteit van GPx-3 hoog is. Echter, door de kleine omvang van het onderzoek en een systematisch verschil in GPx-3-activiteit tussen samples van de twee verschillende baselineperioden, is bevestiging van deze resultaten in andere onderzoeken noodzakelijk.

De resultaten die beschreven zijn in dit proefschrift werden in perspectief geplaatst in **Hoofdstuk 8**. Er is een groot verschil tussen de gunstige resultaten van beta-caroteen en vitamin E uit observationeel onderzoek en de nulbevindingen of zelfs schadelijke effecten van deze antioxidanten uit grootschalige gerandomiseerde interventiestudies (de ‘antioxidantparadox’). Het onderzoek dat in dit proefschrift beschreven is, blijft duiden op een inverse relatie tussen beta-caroteen en sterfte aan HVZ, maar ondersteunt geen gunstige of schadelijke rol voor vitamin E. Hoewel de resultaten van beta-caroteen niet eenduidig geïnterpreteerd kunnen worden, is het mogelijk dat beta-caroteen een voedingspatroon weerspiegelt dat rijk is aan meerdere bioactieve componenten, waaronder alfa-caroteen. De in dit proefschrift beschreven inverse associatie van cocoa met bloeddruk en sterfte aan HVZ wordt ondersteunt door kortdurende interventiestudies met intermediaire eindpunten voor HVZ. Het onderzoek naar cacao en flavanolen is echter pas van start gegaan en het is nog te vroeg om definitieve conclusies te trekken.

Oxidatieve stress is een term die meer conceptueel dan operationeel is. Onderzoek naar oxidatieve stress als risicofactor voor HVZ wordt bemoeilijkt door het ontbreken van gevalideerde biomarkers. Criteria voor een ideale biomarkers worden beschreven. Zonder dergelijke biomarkers verdient het de aanbeveling om meerdere indicatoren van oxidatieve stress tezamen met indicatoren van ontstekingprocessen te onderzoeken.

Toekomstig epidemiologisch onderzoek dient meerdere antioxidanten tegelijkertijd te bestuderen. Dit kan bijvoorbeeld door het toepassen nieuw ontwikkelde analysemethoden die het mogelijk maken voedingspatronen te bestuderen. Tevens zijn meer interventiestudies noodzakelijk met voedingsmiddelen die rijk aan antioxidanten zijn zoals caroteen en flavanolen. Dergelijke studies dienen onderbouwde risicofactoren voor HVZ als eindpunt te gebruiken. Interventieonderzoek naar de effecten van cocoa op risicofactoren voor HVZ moet zich richten op de lange-termijn effecten van realistische hoeveelheden cocoa, een geschikte placebo gebruiken, en gehaltes van flavanolen en andere nutriënten in de publicatie vermelden. Meer prospectief cohortonderzoek naar de relatie tussen de inname van cocoa en HVZ is noodzakelijk.

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Brian

About the author

Gijsbertus Marinus (Brian) Buijsse was born on April the 25th, 1975, in Alkmaar (The Netherlands). After having completed his studies on cooking and food technology, he started the BSc program Nutrition & Dietetics at the Hogeschool van Amsterdam. As a part of that study, he contributed to the conduct of a clinical trial on the effects of the hormone leptin in humans at Maastricht University. After having received his BSc degree in spring 1999, he continued his studies at Wageningen University, where he obtained his MSc degree in Nutrition & Health with a major in epidemiology. Thereafter he was appointed as a PhD fellow to the Division of Human Nutrition of Wageningen University. During this period, he performed the research described in this thesis primarily at the National Institute for Public Health and the Environment (RIVM) in Bilthoven. In the final stage, he visited the Division of Epidemiology and Community Health of the University of Minnesota (Minneapolis, USA). Currently, he is working as a postdoctoral fellow at the Department of Epidemiology of the German Institute of Human Nutrition (DIfE) Potsdam-Rehbrücke in Nuthetal (Germany).

Publications

Original research papers

- Buijsse B, Feskens EJ, Kwape L, Kok FJ, Kromhout D. Both alpha- and beta-carotene, but not tocopherols and vitamin C, are inversely related to 15-year cardiovascular mortality in Dutch elderly men. J Nutr. 2008;138:344-50.
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- Buijsse B, Boer JMA, Streppel MT, Ocké MC, Savelkoul M, Wendel-Vos W, Verhagen H. Epidemiologische aspecten van ischemische hartziekten in relatie tot voeding. Informatorium voor Voeding en Dietetiëk, Dieetleer 3A, december 2007. Bohn Stafleu en Loghum, Houten. [in Dutch]
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- Holtzman J, Buijsse B, Steffen LM, Lee D-H, Erickson RR, Eckfeldt JH, Luepker RV, Jacobs DR. The inverse correlation between serum glutathione peroxidase 3 activity and cardiovascular disease mortality in the Minnesota Heart Survey. Circulation. 2008;117:E248.
- Buijsse B, Feskens EJM, Kok FJ, Kromhout D. Cocoa intake in relation to blood pressure and cardiovascular mortality in elderly men. Circulation. 2006;113:E372.
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- Buijsse B, Feskens EJM, Streppel MT, Kok FJ, Kromhout D. Chocolate consumption and blood pressure in elderly men: the Zutphen Elderly Study. Eur Heart J. 2004;25:235.

Educational programme

Discipline specific activities

Courses

- Regression analysis, Erasmus Summer Programme, Rotterdam (NL), 2002
- Survival analysis, Erasmus Summer Programma, Rotterdam (NL), 2002
- Vascular biology, CARIM/VU, Arnhem (NL), 2002
- Epidemiologic data analysis by Dr K. Rothman, Bilthoven (NL), 2002
- Nutritional and lifestyle epidemiology, Wageningen (NL), 2005
- Topics in meta-analysis, Erasmus Summer Programme, Rotterdam (NL), 2005
- Methods in public health research, Erasmus Summer Programme, Rotterdam (NL), 2005
- Functional Foods, ABS Graduate School, Helsinki (Finland), 2006

Meetings

- Annual meetings NWO Nutrition, Arnhem (NL), 2002, 2003, and 2004
- Annual symposiums of the Netherlands Epidemiology Society (WEON), 2002 (Nijmegen, NL) and 2003 (Rotterdam, NL)
- The coronary heart disease epidemic can be tamed by diet and lifestyle: 40-year results of the Seven Countries Study, Zutphen (NL), 2002
- Health Benefits of the Mediterranean Diet, Crete (Greece), 2003
- Traditional Mediterranean Diet: Past, Present, and Future, Athens (Greece), 2004
- Annual conference of the European Society of Cardiology, Munich (Germany), 2004
- 4th European Congress on Nutrition and Health in the Elderly, Toulouse (France), 2004
- Annual Conference on Cardiovascular Disease Epidemiology and Prevention, American Heart Association, Phoenix, Arizona (USA), 2005

General courses

- PhD Week, VLAG, Bilthoven (NL), 2002
- Written English, WUR/CENTA, Wageningen (NL), 2003
- Scientific English, VU/RIVM, Bilthoven (NL), 2004

Optional courses and activities

- Preparation PhD research proposal, Wageningen (NL), 2002
- PhD Tour Australia, Wageningen University (Australia), 2003
- Mini-symposium Nutrition and Antisocial Behaviour among Young Adult Prisoners, Division of Human Nutrition, Wageningen (NL), 2004
- Journal Club, Wageningen University, Wageningen (NL), 2002-2005
- Review Club, RIVM, Bilthoven (NL), 2002-2005
- Strategy Day, RIVM, Bilthoven (NL), 2003, 2004
- CVG Lecture Series, RIVM, Bilthoven (NL), 2003-2006
- 5-month visit at the University of Minnesota, Division of Epidemiology and Community Health, Minneapolis, Minnesota (USA), 2007

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