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

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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

Parameter	Age-adjusted Crete (n=105)	Age-adjusted Zutphen (n=139)	P	Multivariable-adjusted * Crete (n=105)	Multivariable-adjusted * 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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