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Risk of angina pectoris and plasma concentrations of vitamins A, C, and E and carotene

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The relation between risk of angina pectoris and plasma concentrations of vitamins A, C, and E and carotene was examined in a population case-control study of 110 cases of angina, identified by the Chest Pain Questionnaire, and 394 controls selected from a sample of 6000 men aged 35-54. Plasma concentrations of vitamins C and E and carotene were significantly inversely related to the risk of angina. There was no significant relation with vitamin A. Smoking was a confounding factor. The inverse relation between angina and low plasma carotene disappeared and that with plasma vitamin C was substantially reduced after adjustment for smoking. Vitamin E remained independently and inversely related to the risk of angina after adjustment for age, smoking habit, blood pressure, lipids, and relative weight. The adjusted odds ratio for angina between the lowest and highest quintiles of vitamin E concentrations was 2.68 (95% confidence interval 1.07-6.70; $p=0.02$). These findings suggest that some populations with a high incidence of coronary heart disease may benefit from eating diets rich in natural antioxidants, particularly vitamin E.

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Introduction

There is growing interest in the possible role of free radicals in the development of atheroma. Oxidative modification of low density lipoprotein (LDL) particles in the arterial subendothelium results in structural changes, which are postulated to make them more atherogenic than native LDL.¹ The most readily available naturally occurring antioxidants in food are vitamins E and C. Vitamin E is the major antioxidant in the lipid phase and protects polyunsaturated fatty acids from peroxidation. Vitamin C acts in the water-soluble compartment and has a sparing effect on vitamin E.

We have tested the hypothesis that plasma concentrations of vitamins with antioxidant properties may be related to the risk of angina and have measured the extent to which such risk is independent of classic risk factors for coronary heart disease (CHD).

Subjects and methods

The design of this case-control study has been described elsewhere.² Briefly, the population sampled was men aged 35-54 years listed on the Lothian Health Board Central Register with an address in the city of Edinburgh. A systematic sample of 6000 men was drawn from the register and surveyed by postal questionnaire, which included a self-administered version of the World Health Organisation Chest Pain Questionnaire.³ We have previously documented dietary changes in subjects who had been told that they had CHD.⁴ To avoid the confounding effect of such dietary changes we included only subjects who answered positively to the chest pain questionnaire, but who had never seen a doctor on account of these symptoms. These angina cases were compared with controls matched for age and sex drawn from the same population, who gave negative replies to the chest pain questionnaire and also had no reported history of CHD.

125 cases of angina pectoris found in the survey (response rate 83%) and 430 healthy controls (response rate 76%) attended for medical assessment between April, 1983, and April, 1984. Complete vitamin data were obtained for 110 cases of angina and 394 controls. A self-administered questionnaire recorded demographic information, medical history, smoking habit, alcohol intake, and diet. Height, weight ('Seca' weighing scales model 760), and supine blood pressure (two recordings; Hawksley random zero sphygmomanometer) were measured by one observer. A blood sample was taken from the antecubital fossa without a tourniquet for measurement of non-fasting plasma lipids, vitamins, and platelet fatty acid composition, and the samples were processed immediately in the clinic. Adipose tissue was sampled under local anaesthetic from the anterior abdominal wall.

10 ml heparinised blood for analysis of plasma vitamins was centrifuged immediately at 2000 g for 10 min at room temperature. 0.5 ml plasma was mixed with 4.5 ml washed metaphosphoric acid for the analysis of vitamin C, and the remaining plasma was frozen at -40°C in 1 ml volumes in Eppendorf tubes. Vitamin C was analysed fluorimetrically by means of iodine oxidation followed by condensation with 1,2-phenylenediamine;⁵ the coefficient of variation was 5%. Vitamins A and E and the carotene fraction

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TABLE I—RISK FACTORS FOR CHD IN CONTROLS AND CASES

	Mean (SEM)	
	Controls (n = 430)	Cases (n = 125)
Age (yr)	47.8 (0.3)	46.8 (0.6)
% current cigarette smokers	29%	46%*
Cholesterol (mmol/l)	6.27 (0.06)	6.19 (0.11)
HDL cholesterol (mmol/l)	1.18 (0.02)	1.13 (0.03)
Weight/height ² (kg/m ²)	25.2 (0.2)	25.9 (0.5)
Blood pressure (mm Hg)	137 (1)/84 (1)	140 (2)/86 (1)

Data reproduced from ref 2. *p < 0.01.

(predominantly beta-carotene) were extracted into *n*-hexane and analysed by automated high performance liquid chromatography with a 'Lichrosorb Si 60-5 μ m' column.⁶ Vitamin A and carotene peaks were detected spectrophotometrically at 313 nm and 436 nm, respectively. A fluorometric detector (excitation 290 nm, emission 330 nm) was used for the detection of vitamin E. The peaks were integrated by means of a Perkin-Elmer Sigma 10B system. The coefficients of variation for the estimations of vitamin A and E and carotene were 3.3%, 3.0%, and 1.8%, respectively. Plasma vitamin E concentration correlated strongly with circulating total cholesterol concentrations ($r = 0.61$, $p < 0.001$), so results for vitamin E are also expressed as vitamin E/cholesterol (μ mol/mmol).

All laboratory analyses were carried out on samples from cases and controls simultaneously by laboratory staff unaware of the status of individual samples.

Statistical comparisons between groups were done by means of *t* tests and analysis of variance for more than two groups. The analysis of variance for smoking categories was supplemented with pairwise *t* tests with a Bonferroni correction to allow for the examination of six sets of comparisons. For vitamin C and carotene a square-root transformation was used to compare mean values, since the distribution of the data was positively skewed (log transformation of these variables resulted in a negatively skewed distribution). Logistic regression was used to investigate the relations among angina pectoris, vitamins, fatty acids, and risk factors for CHD. The triglyceride measurements were log transformed before analysis. Each vitamin was included separately in a multiple logistic regression with all the risk factors (whether or not they were

significant) and the statistical significance of the overall trend in odds ratios is presented.

The adjusted odds ratios for angina were calculated in relation to the distribution of the vitamin concentrations in the healthy control population. The vitamin concentrations of cases, expressed as a categorical variable (1-5: quintile of the control data whose limits included the result) were fitted in a model with all the risk factors. The odds ratios were calculated as the exponential of the four coefficients that resulted, and the exponential of the confidence intervals of these coefficients were the confidence intervals of the odds ratios (95% CI). The possibility of an interaction between vitamin E and adipose linoleic acid content was examined. The statistical analyses were made by means of the BMDP package (BDMP Statistical Software 1983, University of California Press).

Results

Risk factors for CHD in angina cases and controls are shown in table 1. The only significant difference between the groups was in the proportion of cigarette smokers ($p < 0.01$). The cases were significantly shorter ($p < 0.05$) but there was no difference in weight or weight/height index.

Mean plasma concentrations of vitamin C, carotene (predominantly beta-carotene), and vitamin E adjusted for plasma cholesterol (in view of the relation between the two) were lower in angina cases than in controls (table II). There was no difference in plasma vitamin A concentrations.

We examined the relations among plasma vitamin concentrations and CHD risk factors in the healthy controls. Cigarette smokers had significantly lower levels of carotene and vitamin C than subjects who had never smoked (table III). There was no significant difference in any vitamin concentration between ex-smokers (cigarettes, pipe, and cigars) and never smokers, but ex-smokers had significantly higher vitamin C and vitamin E/cholesterol concentrations than current cigarette smokers. Pipe and cigar smokers also had significantly higher vitamin C levels than cigarette smokers.

Vitamin A and E concentrations were positively related to total cholesterol (Pearson correlation coefficients [r] 0.28 and 0.61, respectively; $p < 0.001$) and triglycerides ($r = 0.32$ and 0.41; $p < 0.001$) but not to HDL cholesterol. Plasma carotene was inversely related to non-fasting triglyceride concentration ($r = -0.25$; $p < 0.001$). The plasma concentrations of vitamin E and vitamin C were positively related to adipose linoleate content ($r = 0.31$ and 0.34; $p < 0.001$), as was vitamin E/cholesterol ($r = 0.48$; $p < 0.001$). Vitamin A was positively related to diastolic (and systolic) blood pressure ($r = 0.24$; $p < 0.001$). There were no significant correlations with age or weight/height index. The concentrations of all the vitamins showed significant seasonal trends (table IV).

The odds ratios for angina, relative to the highest quintile of the distribution of plasma vitamin concentrations in the control population are shown in table V. The odds ratio for angina in the lowest quintile of vitamin E distribution rose from 2.51 to 2.68 after adjustment for cholesterol and other CHD risk factors and the overall trend was significant ($p = 0.02$). The results were similar when vitamin E

TABLE II—PLASMA VITAMIN CONCENTRATIONS

	Mean (SEM)		p
	Controls (n = 394)	Cases (n = 110)	
Vitamin A (μ mol/l)	2.32 (0.03)	2.29 (0.05)	NS
Carotene (μ mol/l)	0.49 (0.02)	0.30 (0.03)	< 0.001
Vitamin C (μ mol/l)	35.3 (1.1)	28.1 (2.1)	< 0.01
Vitamin E (μ mol/l)	24.0 (0.3)	22.7 (0.6)	NS
Vitamin E/cholesterol (μ mol/mmol)	3.86 (0.04)	3.66 (0.08)	< 0.01

NS = not significant.

TABLE III—PLASMA VITAMIN CONCENTRATIONS IN RELATION TO REPORTED SMOKING HABIT IN CONTROLS

	Mean (SEM) in μ mol/l*				
	Vitamin A	Carotene	Vitamin C	Vitamin E	Vitamin E/ cholesterol
Never smoked (n = 132)	2.27 (0.04)	0.53 (0.03)*	40.9 (1.8)†	23.5 (0.5)	3.88 (0.06)
Ex-smokers (n = 87)	2.35 (0.05)	0.50 (0.03)	42.6 (2.4)†	25.5 (0.7)	4.12 (0.08)†
Pipe/cigar smokers (n = 44)	2.35 (0.06)	0.53 (0.05)	38.4 (3.4)†	24.3 (1.2)	3.93 (0.13)
Cigarette smokers (n = 127)	2.33 (0.05)	0.42 (0.02)	24.1 (1.9)	23.3 (0.6)	3.63 (0.07)

*Except vitamin E/cholesterol which is in μ mol/mmol.
For significance of differences (pairwise *t* test with Bonferroni correction) from cigarette smokers: *p < 0.01; †p < 0.001 after normalisation. No data on 6 subjects

TABLE IV—SEASONAL TRENDS IN PLASMA VITAMIN CONCENTRATIONS IN CONTROLS

	Highest	Lowest	Difference
Vitamin A	November	May	12% ($p < 0.001$)
Carotene	November	May	37% ($p < 0.01$)
Vitamin C	August	February	48% ($p < 0.001$)
Vitamin E	December	June	15% ($p < 0.001$)

TABLE V—ODDS RATIOS FOR ANGINA PECTORIS BY QUINTILES OF PLASMA VITAMIN CONCENTRATIONS IN CONTROLS, WITH AND WITHOUT ADJUSTMENT* FOR CHD RISK FACTORS

	Vitamin A		Carotene		Vitamin C		Vitamin E	
	μmol/l	Odds ratio (95% CI)						
<i>Quintile 1</i>	<1.93		<0.26		<13.1		<18.9	
Unadjusted	..	1.67 (0.86-3.26)	..	2.64 (1.32-5.29)	..	2.35 (1.16-4.78)	..	2.51 (1.24-5.10)
Adjusted	..	2.73 (1.24-6.02)	..	1.41 (0.63-3.13)	..	1.63 (0.76-3.49)	..	2.68 (1.07-6.70)
<i>Quintile 2</i>	1.93-2.16		0.26-0.37		13.1-23.8		19.0-21.8	
Unadjusted	..	1.11 (0.53-2.33)	..	1.42 (0.68-2.95)	..	1.66 (0.80-3.42)	..	1.04 (0.44-2.44)
Adjusted	..	1.34 (0.60-2.98)	..	1.00 (0.44-2.23)	..	1.32 (0.60-2.38)	..	1.69 (0.72-4.00)
<i>Quintile 3</i>	2.17-2.37		0.38-0.49		23.9-41.4		21.9-24.2	
Unadjusted	..	1.11 (0.54-2.27)	..	1.30 (0.70-2.17)	..	1.75 (0.79-3.92)	..	1.00 (0.43-2.35)
Adjusted	..	1.58 (0.72-3.45)	..	0.98 (0.43-2.22)	..	1.56 (0.72-3.36)	..	1.18 (0.49-2.81)
<i>Quintile 4</i>	2.38-2.68		0.50-0.67		41.5-57.3		24.3-28.1	
Unadjusted	..	1.39 (0.70-2.75)	..	1.16 (0.53-2.53)	..	0.81 (0.36-1.81)	..	1.63 (0.77-3.43)
Adjusted	..	1.84 (0.88-3.88)	..	0.95 (0.43-2.12)	..	0.87 (0.70-2.04)	..	1.64 (0.76-3.51)
<i>Quintile 5</i>	≥2.69		≥0.68		≥57.4		≥28.2	
Unadjusted	..	1.00	..	1.00	..	1.00	..	1.00
Adjusted	..	1.00	..	1.00	..	1.00	..	1.00

*By logistic regression for age, systolic and diastolic blood pressure, cholesterol, HDL cholesterol, non-fasting triglycerides, relative weight, smoking habit, and season for 105 cases and 382 controls with complete data. Linear trend after adjustment in logistic regression not statistically significant, except for vitamin E ($p=0.02$).

cholesterol ratios were used (adjusted odds ratio 2.21 [1.05-4.67]; overall trend $p<0.01$). The unadjusted odds ratios for angina subjects with the lowest concentrations of vitamin C and of carotene were 2.35 (1.16-4.78) and 2.64 (1.32-5.29), respectively, but the strength of these inverse relations was reduced and they were no longer significant ($p=0.09$ and 0.40, respectively) after adjustment for smoking and other risk factors. The odds ratios for angina by vitamin A and vitamin A/cholesterol (not shown) concentrations were not significant.

We have previously reported an inverse relation between adipose linoleic acid and platelet eicosapentaenoic acid content and the risk of angina for this population.² The inverse relation between plasma vitamin E concentration and the likelihood of angina was therefore examined in relation to these fatty acids. When they were included in the logistic analysis for vitamin E, adipose tissue linoleic acid content ($p<0.001$) and platelet eicosapentaenoic acid content ($p<0.01$) each made an independent contribution to the explanation of angina, but plasma vitamin E concentration did not ($p=0.09$). There was no significant interaction between adipose linoleic acid and vitamin E ($p=0.52$).

Discussion

In this population case-control study low plasma concentrations of vitamins E and C and carotene were related to an increased risk of angina pectoris in men. For plasma vitamin E concentration the relation remained significant after adjustment for age, blood pressure, total and HDL cholesterol, non-fasting triglycerides, relative weight, and smoking status. The association between low plasma vitamin C levels and angina was confounded by cigarette smoking. Vitamin C levels are, as we confirmed, lower in cigarette smokers than in non-smokers. In the National Health and Nutritional Examination Survey⁷ vitamin C intake and plasma concentrations were lowest in those who smoked most cigarettes. Furthermore, smoking may interfere with vitamin C absorption and increase vitamin C requirements.⁸

We found no significant difference in vitamin E/cholesterol between cigarette smokers and non-smokers,⁹ though ex-smokers had significantly higher vitamin E/cholesterol ratios than current cigarette smokers. One explanation for this finding could be a change in dietary

habit after stopping smoking; a dietary survey in this population showed that ex-smokers ate more polyunsaturated fat (vitamin E rich) than those who were still smoking cigarettes.¹⁰ Our cigarette smokers also had significantly lower concentrations of carotene than non-smokers, which confirms the findings of Stryker and colleagues.¹¹ The concentration of vitamin E, which is transported in the bloodstream by lipoprotein particles, was closely correlated with that of total cholesterol and derived LDL cholesterol.¹² There were no strong correlations between plasma concentrations of antioxidant vitamins and other classic risk factors.

Adipose tissue linoleic acid and platelet eicosapentaenoic acid content are inversely related to the risk of angina pectoris in this population.² Plasma vitamin E was positively correlated with adipose tissue linoleate,¹³ but not with platelet eicosapentaenoic acid. So subjects with low plasma vitamin E concentrations also tend to have low adipose tissue linoleate, reflecting long-term low dietary intake of this polyunsaturated essential fatty acid. A dietary survey in Scotland showed that middle-aged men have low consumption of polyunsaturated oils and cereal fibre,^{10,14} but vitamin intake was not measured. Although adipose linoleate and plasma vitamin E were both inversely related to likelihood of angina in our study, the relation between low vitamin E and angina is important since it is independent of the generally accepted CHD risk factors.

Our angina cases were defined by a self-administered WHO chest pain questionnaire and were unaware of the nature of their chest pain. They were therefore unlikely to have changed their dietary habits. Of course, a positive response to the WHO questionnaire is not the same as a doctor's diagnosis of angina, but nor are patients diagnosed by their general practitioners necessarily representative of all patients with angina. We used the WHO questionnaire as a valid and reproducible measure of angina in the population which is not subject to the bias of self-referral or the large variation between and within observers associated with doctors' diagnoses. Some of the subjects positive on this questionnaire will not have CHD, but this is a conservative bias in the study. If there is a true inverse relation between plasma vitamin concentration and risk of angina, the inclusion of subjects without angina will dilute this estimate.

The evidence from cross-cultural studies linking low plasma antioxidants with CHD has been equivocal. Our study¹³ of healthy middle-aged men in Scotland, Finland, and Italy showed no consistent relation between plasma concentrations of carotene or vitamins A, C, and E and CHD mortality statistics for these populations, though the vitamin E/cholesterol ratio was generally lower in northern Europe, where CHD mortality is higher. These studies are being extended within the MONICA framework. This study was carried out over several years and repeat analyses of men in Finland have shown pronounced rises in plasma vitamin E and C concentrations between spring 1983 and spring 1987.

Within-population studies have shown an inverse relation between vitamin consumption and cardiovascular mortality. In the USA, industrial vitamin C production is inversely related to CHD mortality over the past 20 years.¹⁵ Regional standardised mortality ratios for CHD in the UK relate inversely to calculated vitamin C intake (National Food Survey), reflecting a long-established regional gradient in lifestyle and social circumstance.¹⁶ There is a similar inverse and powerful relation between the consumption of fresh fruit and green vegetables and mortality from all cerebrovascular disease, independent of social class.¹⁷ Middle-aged Scottish men eat very little fruit and green vegetables.¹⁸ However, material deprivation predicts CHD mortality better than social class^{19,20} and preliminary data have shown that the relations between dietary factors and material deprivation are not identical to those that correlate with social class.

There have been only a few other studies of vitamins and their relation to CHD in individuals. Ramirez and Flowers²¹ found that men with significant coronary artery obstructions and regional wall kinetic abnormalities had lower leucocyte ascorbic acid levels than those with normal arteriograms, irrespective of smoking status. On the other hand, Salonen and colleagues²² found that neither plasma vitamin C nor cholesterol-adjusted vitamin E differed between those with and without CHD defined on the basis of symptoms, a history of CHD, or objective evidence of ischaemia on a bicycle ergometer exercise test. The fact that liver vitamin A content does not differ significantly between subjects dying from CHD and from accidents²³ and the possibility that patients with established CHD may change their diet, makes the interpretation of necropsy and chronic CHD studies difficult, if not impossible. Prospective studies of the relation between vitamin levels and CHD mortality in individuals, both men and women, have found no significant relations.^{24,25}

We should emphasise that the inverse relation we found may not apply to other communities with a high incidence of CHD.¹³

How could low plasma and (presumably) low tissue levels of naturally occurring antioxidants relate to CHD? Formation of foam cells from monocytes/macrophages is favoured when LDL is in the oxidised or modified form.¹ Probucol, a lipid-lowering drug with antioxidant properties, protects LDL against oxidative modification by endothelial cells in culture²⁷ and reduces aortic atheromatous lesions in a rabbit model of hyperlipidaemia, an effect not explained by changes in lipoprotein concentrations.²⁸ Whether a low concentration of naturally occurring antioxidants favours the formation of oxidised LDL has yet to be shown, but the addition of vitamin E to cell cultures blocks the oxidative modification of LDL.²⁹

An increased tendency to peroxidation of polyunsaturated fatty acids resulting from a reduction in antioxidant availability might favour thrombosis by allowing saturated fatty acids, which are more thrombotic,³⁰ to have an unbalanced effect. The myocardium may also be adversely affected after periods of ischaemia if tissue concentrations of free radical scavengers, such as vitamins E and C, are low. During ischaemic reperfusion, oxygen free radicals are produced and these may lead to further tissue damage and reperfusion injury,³¹ the development of arrhythmias,³² and depression of myocardial contractility.³³

The importance of oxidative modification of LDL and increased atherosclerosis, thrombosis, and myocardial ischaemic damage in leading to CHD may depend on the fatty acid composition of the diet. Polyunsaturated fatty acids are very vulnerable to free radical attack. Lipid peroxidation can become autocatalytic, but the chain-reaction can be prevented by the action of vitamin E. Vitamin C has a sparing activity on vitamin E. Diets low in linoleic acid tend to be low in vitamin E (and also C).

Our study cannot of course elucidate the mechanisms whereby low plasma vitamin E and C may predispose to CHD. The evidence from our retrospective study is sufficiently strong to justify further studies of essential antioxidants. An intervention trial with vitamin supplements is the most conclusive way to test the nature of the association between low plasma antioxidants and CHD.

Antioxidants are more easily destroyed by food processing than polyunsaturated fatty acids.³⁴ This fact, combined with a habitual low intake of vitamins E and C, suggests that some populations with a high incidence of CHD should supplement their eating habits with more cereals, vitamin-E-rich oils, vegetables, and fruit.

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Rapid diagnosis of tuberculous meningitis by polymerase chain reaction

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The polymerase chain reaction (PCR) in cerebrospinal fluid was compared with conventional bacteriology and an enzyme-linked immunosorbent assay (ELISA) for cerebrospinal fluid antibodies in the diagnosis of tuberculous meningitis (TBM). PCR was the most sensitive technique; it detected 15 (75%) of 20 cases of highly probable TBM (based on clinical features), 4 (57%) of 7 probable cases, and 3 (43%) of 7 possible cases. ELISA detected 11 (55%) of the highly probable cases and 2 each of the probable and possible cases. Culture was positive in only 4 of the highly probable cases. Among the controls (14 pyogenic meningitis, 3 aseptic meningitis, 34 other neurological disorders), 6 subjects tested early in the study (2 pyogenic meningitis, 4 other disorders) were PCR positive. Second DNA preparations from their stored cerebrospinal fluid samples were all PCR negative, suggesting that the false-positive results were due to cross-contamination. 18 PCR-positive TBM samples retested were all still PCR positive. The antibody ELISA was positive in 3 controls despite the use of a high cutoff value.

Lancet 1991; 337: 5-7.

Introduction

Tuberculous meningitis (TBM), the most dangerous form of extrapulmonary tuberculosis, occurs in 7-12% of tuberculous patients in developing countries.¹ Despite the availability of effective chemotherapy, the mortality and morbidity remain high. Delay in diagnosis is directly related to poor outcome; there are neurological sequelae in 20-25% of patients who do not receive early treatment.² Bacteriological methods are inadequate for early diagnosis of TBM because there are too few organisms in the cerebrospinal fluid for consistent demonstration by direct smear, and cultural identification takes 6-8 weeks. Diagnosis in the critical early stage of the disease is therefore often presumptive.

Several new techniques for the rapid diagnosis of TBM have been developed lately.^{3,4} We have developed a detection method for *Mycobacterium tuberculosis* based on the polymerase chain reaction (PCR).⁵ On initial evaluation the technique was highly specific for the *M tuberculosis* complex. Here we report a comparison in TBM on

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EXHIBIT B

Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS)

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Summary

Background Vitamin E (α -tocopherol) is thought to have a role in prevention of atherosclerosis, through inhibition of oxidation of low-density lipoprotein. Some epidemiological studies have shown an association between high dietary intake or high serum concentrations of α -tocopherol and lower rates of ischaemic heart disease. We tested the hypothesis that treatment with a high dose of α -tocopherol would reduce subsequent risk of myocardial infarction (MI) and cardiovascular death in patients with established ischaemic heart disease.

Methods In this double-blind, placebo-controlled study with stratified randomisation, 2002 patients with angiographically proven coronary atherosclerosis were enrolled and followed up for a median of 510 days (range 3–981). 1035 patients were assigned α -tocopherol (capsules containing 800 IU daily for first 546 patients; 400 IU daily for remainder); 967 received identical placebo capsules. The primary endpoints were a combination of cardiovascular death and non-fatal MI as well as non-fatal MI alone.

Findings Plasma α -tocopherol concentrations (measured in subsets of patients) rose in the actively treated group (from baseline mean 34.2 μ mol/L to 51.1 μ mol/L with 400 IU daily and 64.5 μ mol/L with 800 IU daily) but did not change in the placebo group. α -tocopherol treatment significantly reduced the risk of the primary trial endpoint of cardiovascular death and non-fatal MI (41 vs 64 events; relative risk 0.53 [95% CI 0.34–0.83; $p=0.005$]). The beneficial effects on this composite endpoint were due to a significant reduction in the risk of non-fatal MI (14 vs 41; 0.23 [0.11–0.47]; $p=0.005$); however, there was a non-significant excess of cardiovascular deaths in the α -tocopherol group (27 vs 23; 1.18 [0.62–2.27]; $p=0.61$). All-cause mortality was 36 of 1035 α -tocopherol-treated patients and 27 of 967 placebo recipients.

Interpretation We conclude that in patients with angiographically proven symptomatic coronary atherosclerosis, α -tocopherol treatment substantially

reduces the rate of non-fatal MI, with beneficial effects apparent after 1 year of treatment. The effect of α -tocopherol treatment on cardiovascular deaths requires further study.

Lancet 1996; **347**: 781–86

See Commentary page 776

Introduction

The Cambridge Heart Antioxidant Study (CHAOS) was designed to test the hypothesis that treatment with a high dose of α -tocopherol (vitamin E) would reduce the risk of myocardial infarction (MI) in patients with angiographic evidence of coronary atherosclerosis. This hypothesis developed from the idea that macrophage-mediated oxidation of low-density lipoprotein (LDL) has a central role in atherogenesis and the extensive experimental and epidemiological evidence to support this view.¹

Epidemiological studies of dietary intake and serum concentrations of α -tocopherol in relation to risk of coronary atherosclerosis have had mixed results. Three large, prospective, nested case-control studies found no correlation between serum α -tocopherol concentrations and subsequent myocardial infarction or cardiovascular death.^{2–4} However, two studies in the USA found significant risk ratios of 0.64 in men and 0.66 in women between quintiles with the highest and lowest intake.^{5,6}

The single published randomised controlled trial of α -tocopherol (in the prevention of lung cancer)⁷ found no effect on cardiovascular mortality, with a low dose of α -tocopherol (50 mg daily). An overview of epidemiological data suggested that a trial of α -tocopherol in ischaemic heart disease would need to use large doses of α -tocopherol to demonstrate any treatment effect.

In CHAOS, we studied the effects of α -tocopherol at doses of 400 IU or 800 IU daily on the risk of cardiovascular death and non-fatal MI in patients with overt clinical and angiographic coronary atherosclerosis at recruitment. These individuals are at higher risk of subsequent MI than an unselected group; the higher risk allows an adequately powered trial with a smaller sample than a primary prevention trial.

Chemical evidence of lipid oxidation is evident at all stages of atherosclerosis, especially in macrophage-rich and early atherosclerotic lesions.⁸ Steinberg⁹ therefore suggested that antioxidants might exert their greatest effect in early lesions, with a long lag time before impact on clinical events in healthy subjects. However, patients with advanced coronary atherosclerosis are at much greater risk of MI (which generally occurs as a result of rupture of mature atheromatous plaques¹⁰), and are therefore the most appropriate subjects for investigation of the clinical value of antioxidant treatment on prevention of MI. An effect of α -tocopherol in this setting would be

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support for the antioxidant hypothesis of atherogenesis and would have clinical implications for prevention of MI in high-risk individuals.

The regional structure of cardiac services in our part of the UK allowed us to design a low-cost, prospective study of α -tocopherol. Papworth Hospital is a tertiary referral centre that undertakes the majority of invasive cardiac procedures for a population of 2.2 million, with accurate follow-up in the seven referring hospitals that have coronary-care units.

Methods

CHAOS was a prospective, double-blind, placebo-controlled, randomised, single-centre trial in the East Anglian region of the UK. Patients were recruited at one centre (Papworth Hospital). The study compared two parallel groups of patients with angiographically proven coronary atherosclerosis. One group received α -tocopherol and the other placebo. The primary outcome variables were a combined endpoint of cardiovascular death and non-fatal MI, and non-fatal MI alone. Enrolment began on Oct 10, 1992, and ended on Dec 15, 1994. The analysis included all endpoints between Oct 10, 1992, and June 18, 1995. The CHAOS trial was designed to have 80% power (with $2p=0.05$) to detect a relative risk of the combined endpoint (non-fatal MI and cardiovascular death) of less than 0.75 between the treatment groups after median follow-up of 1.5 years. This calculation assumed an accrual rate of 1000 individuals per year for 2 years, and an event rate of 5% per year, which were estimated from past referral and event rates at Papworth Hospital. Blinded interim analysis was planned for safety reasons, with trial termination if the relative risk between the treatment groups was below 0.70 with $2p<0.001$ in either of the primary endpoints. The prospectively defined stopping criteria were not met in the interim analysis in June, 1994.¹¹

When the trial was designed, there were few data to guide the choice of vitamin E dose. The first 546 patients on active therapy took 800 IU daily throughout the trial. When we had adequate evidence from measurements of α -tocopherol concentrations on therapy that a lower dose would exceed physiological values, newly recruited subjects were allocated 400 IU daily. The dose was constrained by the needs to exceed a physiological concentration of α -tocopherol and to avoid interruption of recruitment through the limited supplies of study drugs available to use. There was no attempt at randomisation between the two vitamin E dosage groups, and the study was not planned to examine dose-response effects on the primary endpoints. These two groups are therefore not distinguished in this analysis.

The inclusion criterion was angiographically proven coronary atherosclerosis. More than 90% of patients had angina, evidence of reversible cardiac ischaemia or both features, although these characteristics were not required. Almost all the subjects were recruited on the day of their admission immediately after elective coronary angiography. There were no exclusion criteria except prior use of vitamin supplements containing vitamin E. Patients were prestratified by seven variables—sex, blood pressure (cutoff for systolic 160 mm Hg, diastolic 90 mm Hg), age (55 years), body-mass index (25 kg/m^2), total cholesterol (6.5 mmol/L), smoking habit, and planned therapy (medical therapy, percutaneous transluminal coronary angioplasty [PTCA], or coronary artery bypass grafting [CABG]). Randomisation was done by means of a computer programme, which used a random-number database to allocate treatment by blocks of two after clinical data had been entered. Active treatment was capsules of α -tocopherol (free 2R,4R,8R- α -tocopherol from natural sources in soya oil), 400 or 800 IU daily (268 or 537 mg) in one daily dose. The identical placebo capsules (oil only) contained a maximum of 0.4 mg α -tocopherol. 546 patients took 800 IU daily for a median of 731 days (range 3–981); 489 took 400 IU daily for 366 days (8–961); 967 took placebo for 494 days (9–965).

An initial supply of 2 months' α -tocopherol or placebo was dispensed at recruitment. Patients were asked to request all

	α -tocopherol (n=1035)	Placebo (n=967)
Age (years)*	61.8 (9.3)	61.8 (8.9)
M/F	848/187	842/125
Vessels with >75% stenosis		
0 or 1	372	370
2	253	216
3 or left main stem	393	359
Intended therapy		
CABG	402	352
Medical	355	324
PTCA	257	265
Left ventricular impairment (n=706)		
None	208	215
Mild	59	50
Moderate	60	50
Severe	34	30
Serum concentrations*		
Fasting total cholesterol (mmol/L)	5.96 (1.19)	5.81 (1.11)
α -tocopherol ($\mu\text{mol/L}$) (n=1752)	33.8 (11.0)	33.2 (10.2)
Lipid-corrected α -tocopherol	1.00 (0.28)	1.00 (0.28)
Blood pressure (mm Hg)*		
Systolic	135 (21)	133 (20)
Diastolic	79 (13)	78 (12)
Diabetic	102 (9.9%)	68 (7.0%)
Smoking status		
Current smoker	149	121
Ex-smoker (>2 years)	300	269
Non-smoker	586	577
Family history in first-degree relative <60 years old (n=521)	96 (36.6%)	105 (40.2%)
Body-mass index (kg/m^2)*	26.5 (3.5)	26.4 (3.4)
Alcohol intake (units per week) (n=1390)*	6.97 (10.3)	6.96 (10.5)
Drug treatment		
Calcium antagonist	718 (69.4%)	666 (68.9%)
β -blocker	410 (39.6%)	325 (33.7%)
Nitrate	557 (53.8%)	556 (57.5%)
Aspirin (mg daily)*	82.9 (53.3)	82.6 (55.8)

*Mean (SD). Some totals do not reach 1035 and 967 because of missing data.

Table 1: Baseline characteristics of patients

follow-up study medication, which was posted to them. The time of request provided a simple index of compliance. Other management and medication were at the discretion of the physician responsible for the patient's usual care.

All patients gave informed, written consent to participation in the study, which was approved by the Huntingdon District Local Research Ethics Committee.

Two endpoints were examined—non-fatal MI alone and a combination of non-fatal MI and cardiovascular death (major cardiovascular events). Non-fatal MI and death were distinguished in the trial design, with a separate analysis of treatment effects on non-fatal MI. This distinction was made because of greater diagnostic precision in non-fatal MI allowed by examination of electrocardiography, cardiac enzyme measurements, and case notes over the hospital admission. For these cases, definite or probable MI was defined by a ratified modification of the MONICA criteria.¹² The certified cause of death was judged to be cardiovascular if the cause was classified according to the International Classification of Diseases, 9th revision, as codes 410, 427, 428, 434, or 441. Necropsy data were available in 21 (34%) of 62 cases. There was no information about cause of death in one case; this patient was censored in the survival analysis at the reported date of death and was not included in the analysis as a cardiovascular death. All events were classified by a member of the study team independently of the main analysis.

There was no planned clinic follow-up as part of the trial, because of the large geographical area served by the study centre (up to 120 km radius). A dedicated database was installed in seven coronary-care units serving the region's population, to allow tracking of admissions of study patients. In addition, we sent a questionnaire to patients and their family physicians to

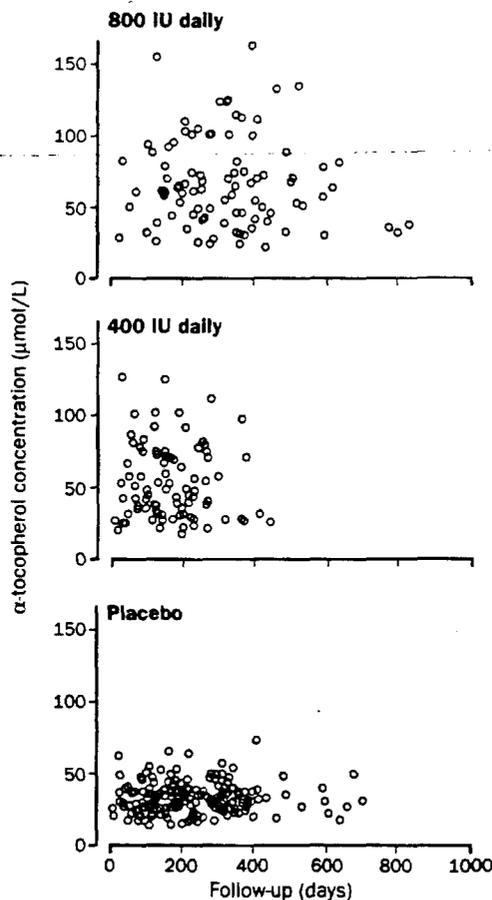


Figure 1: Serum α -tocopherol concentrations during follow-up

find out about the occurrence of study endpoints and other events. Compliance was measured as the ratio of days that study medication was requested to per-protocol days prescribed.

Serum α -tocopherol concentrations were measured by high-performance liquid chromatography¹³ at baseline in 1763 individuals and in 482 who reattended after 15–836 days (in 307 cases these patients returned for planned CABG).

Baseline continuous variables were compared by Student's *t* test and categorical variables by Pearson's χ^2 test. Bivariate correlation was with Pearson's correlation coefficient. ANOVA was used to compare the effect of treatment on follow-up serum cholesterol and α -tocopherol. A lipid-standardised serum α -tocopherol concentration was calculated as the ratio of the measured serum α -tocopherol to the concentration predicted by a linear regression equation based on serum total cholesterol. The main analysis was by intention to treat. Primary-endpoint-free survival curves were calculated by the Kaplan-Meier technique and treatment groups were compared by the log-rank test. A Cox proportional hazards regression model was used to assess the influence of all potential explanatory baseline variables on relative hazards of the primary endpoints. Ordinal variables (New York Heart Association class, number of vessels diseased, and planned management) were treated as continuous in the model. Eighteen variables were entered stepwise into a model, with significance levels for backwards removal and entry of 0.4 and 0.2. A separate per-protocol analysis was done by treating compliance with active therapy as a time-dependent variable in a Cox regression model. The difference between treatment groups in all-cause mortality was tested with χ^2 . The difference in side-effects between groups was tested by Pearson's χ^2 test, with odds ratios calculated by the approximation of Woolf.

Results

Of the 2002 patients recruited, 1035 were assigned α -tocopherol and 967 placebo (table 1). Median follow-

up was 510 days (range 3–981). There were small differences between active treatment and placebo groups in sex ratio, serum total cholesterol, systolic blood pressure, presence of diabetes, and the proportion taking β -blockers. These differences arose because there were fewer women than men and so some of the rarer stratification blocks were unbalanced when recruitment stopped. All these differences weighted risk in favour of the placebo group. Overall, the study patients were at high risk of further cardiovascular events—37.6% had triple-vessel or left-main-stem coronary disease and 24.6% had moderate or severe left ventricular dysfunction.

73.2% of all prescribed α -tocopherol or placebo were requested as follow-up medications. There was no difference between treatment groups in the proportion who were 100% compliant with the trial medication (48% placebo, 49% α -tocopherol; $p=0.76$). Complete follow-up data were available in 98% of participants. There were no differences between the groups in completeness of follow-up (98.0% placebo, 97.8% active treatment; $p=0.80$).

Baseline serum α -tocopherol concentration was 34.2 $\mu\text{mol/L}$ (95% CI 33.1–35.3; $n=226$). Mean serum α -tocopherol did not change on placebo therapy 32.4 (30.9–33.9; $n=224$) $\mu\text{mol/L}$ but increased to 51.1 $\mu\text{mol/L}$ (46.5–55.9; $n=114$) on therapy with 400 IU daily and 64.5 $\mu\text{mol/L}$ (59.6–69.5; $n=142$) with 800 IU daily. Follow-up measurements were made at 6–836 (median 266) days. There was no trend in serum α -tocopherol concentrations with time (figure 1).

Treatment did not affect serum cholesterol: the mean follow-up concentration was 5.77 mmol/L (5.51–6.04) on placebo, 5.55 mmol/L (5.29–5.81) on 400 IU daily, and 5.93 mmol/L (5.60–6.24) on 800 IU daily ($p=0.24$). The strong correlation between serum total cholesterol and α -tocopherol reported previously^{14,15} was seen for baseline concentrations in this study ($r=0.39$; $p<0.0001$). The regression equation used to predict serum α -tocopherol ($r^2=0.150$, $p<0.0001$) was:

$$\alpha\text{-tocopherol } (\mu\text{mol/L}) = 12.5 + 3.61 (\text{cholesterol [mmol/L]})$$

There was no significant association between baseline measured or corrected α -tocopherol concentration and risk of cardiovascular death or MI.

There were 50 cardiovascular deaths and 55 non-fatal MIs during the study period (table 2). 14 recipients of α -tocopherol (3/489 on 400 IU, 11/546 on 800 IU daily) and 41 placebo recipients had non-fatal MIs. Of the 50 cardiovascular deaths, 27 were in the α -tocopherol group (10/489 on 400 IU, 17/546 on 800 IU daily) and 23 in

	ICD-9 code	α -tocopherol group (n=1035)	Placebo group (n=967)
Non-fatal MI	410	14	41
Cardiovascular death			
Fatal MI	410	18	13
Left ventricular failure	428	5	8
Stroke	434	1	1
Ruptured AAA	441.3	2	0
Cardiac arrhythmia	427	1	1
Total cardiovascular deaths	..	27	23
Other causes of death			
Pulmonary embolism	415	3	1
Septicaemia	38	2	0
Bowel carcinoma	150–159	4	1
Unknown	..	0	1
Total deaths	..	36	26

AAA=abdominal aortic aneurysm.

Table 2: Distribution of non-fatal MI and deaths by certified cause in each treatment group

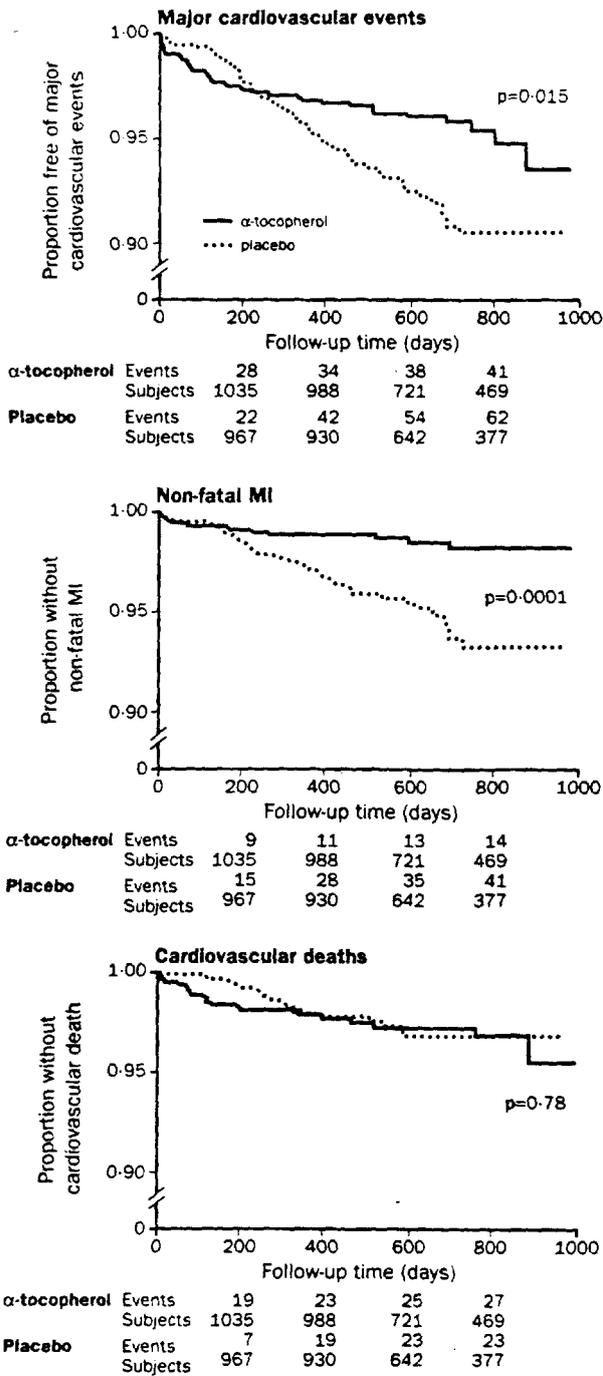


Figure 2: Kaplan-Meier survival analysis for major cardiovascular events, non-fatal MI, and cardiovascular deaths

the placebo group. Total mortality was slightly but not significantly greater in the α -tocopherol group than in the placebo group (36 [3.5%] vs 26 [2.7%], $p=0.31$).

Kaplan-Meier survival curves for the combined primary endpoint and separately for non-fatal MI and cardiovascular death are shown in figure 2. Treatment with α -tocopherol significantly reduced the rates of major cardiovascular events (log-rank $p=0.015$), and of non-fatal MI ($p=0.0001$) but had no effect on cardiovascular deaths ($p=0.78$). There was a delay in the onset of treatment benefit, with divergence of the Kaplan-Meier curves after about 200 days.

In the Cox model, treatment with α -tocopherol reduced the risks of a major cardiovascular event (relative risk 0.53

Variable	Relative risk (95% CI)	p
α -tocopherol vs placebo	0.53 (0.34-0.83)	0.005
NYHA class	1.71 (1.17-2.49)	0.005
Age	1.04 (1.01-1.07)	0.01
Diabetes	1.93 (1.04-3.60)	0.04
Current smoker	1.94 (1.02-3.69)	0.04
Atenolol vs no β -blocker	2.14 (0.86-5.34)	0.10

NYHA=New York Heart Association.

Table 3: Risk of cardiovascular death or non-fatal MI

[95% CI 0.34-0.83], $p=0.005$) and of a non-fatal MI (0.23 [0.11-0.47], $p<0.001$) but had no effect on cardiovascular death (1.18 [0.62-2.27], $p=0.61$). Table 3 shows the results for major cardiovascular events, with data for the other variables in the model for which the probability value associated with the Wald statistic was less than 0.2.

In a separate Cox regression analysis, compliance with α -tocopherol was treated as a time-dependent variable to assess the benefits of treatment taken as prescribed. The results were similar to those of the intention-to-treat analysis. The relative risk of a major cardiovascular event was 0.53 (0.35-0.81, $p=0.003$) and that of a non-fatal MI 0.32 (0.17-0.59, $p<0.001$) for individuals who were compliant with α -tocopherol treatment.

Treatment was well tolerated; only 11 (0.55%) of the 2002 patients discontinued therapy because of diarrhoea, dyspepsia, or rash. There was no significant difference between the treatment groups for these side-effects (α -tocopherol vs placebo relative risk 1.12 [0.34-3.69], $p=0.85$).

Discussion

The idea that lipid oxidation within the atherosclerotic lesion might contribute to atherogenesis was raised during the 1980s.¹⁶⁻¹⁸ Support for this idea has come from laboratory studies^{19,20} that showed oxidation of LDL particles (particularly their polyunsaturated cholesterol esters²¹) by macrophages⁸ in atheromatous plaques. The oxidation products of these reactions have various effects that may promote plaque progression and instability.^{18,19}

The CHAOS trial design took advantage of the unitary cardiac service of a large health region to examine the effects of α -tocopherol on the rate of major cardiac events in a homogeneous and stable population with established coronary disease. We found that α -tocopherol, in a higher dose than in previous studies, reduced the risk of the primary trial endpoint (a combination of death and non-fatal MI) by 47%. This benefit was due to a reduction in the risk of a non-fatal myocardial infarction of 77% and this treatment effect was apparent after about 200 days. The effects on the combined endpoint were not due to a reduction in cardiovascular death; indeed, there were more cardiovascular deaths among α -tocopherol recipients than among placebo recipients. By contrast with the delayed effects of non-fatal MI, this increased risk was due to an excess of early events (before 200 days).

Because of the study design, this trial did not have sufficient power for us to form conclusions about the reason for the disparity in treatment effects on cardiovascular death and non-fatal MI. The discrepancy may be due to chance alone or it may reflect a difference in antioxidant effects on the biological processes leading to death and those leading to non-fatal MI. Most of the deaths occurred in the early part of the follow-up period, perhaps before any putative beneficial effects on

atheromatous plaques could have occurred. Furthermore, the deaths certified (most without necropsy evidence) as due to ischaemic heart disease will include deaths due to causes less likely to be responsive to α -tocopherol treatment (eg, arrhythmias, progression of heart failure, or perioperative complications). Whether there is a true adverse effect on early mortality cannot be ascertained from these data and must await the results of longer-term multicentre trials designed with mortality as a primary endpoint.

We did not plan to study treatment effects on endpoints such as unplanned CABG, hospital admission with angina, or restenosis after PTCA. These endpoints are appropriate in prospective studies in healthy populations that seek to find out the subsequent incidence of ischaemic heart disease,⁵ but lack precision in a population who already have severe atherosclerosis and symptomatic cardiac ischaemia at baseline.

Despite the randomisation process, there were small but significant differences in the distribution of five conventional coronary risk factors between the active treatment and placebo groups. However, all these differences weighted risk in favour of the placebo group and so posed a more rigorous test for α -tocopherol treatment. This trial was not designed to examine dose-response relations in terms of the primary endpoints. The use of two doses of α -tocopherol does not obscure interpretation of the effects on primary endpoints; a similar approach in randomised controlled trials of drug treatment for hypertension allowed analysis of effects on definable cardiovascular endpoints.²² The lipid oxidation hypothesis might predict a greater benefit for α -tocopherol in patients who smoke, who are diabetic, or who have vascular hypertrophy due to hypertension. This study does not have sufficient power to allow us to draw firm conclusions about these patients, but we hoped that stratification before randomisation would reveal any trends towards greater benefit in these subgroups. Direct comparisons of this type were, however, precluded by the small number of women recruited and the consequent imbalance in stratification subgroups.

This study could not directly address the mechanism by which α -tocopherol reduces the risk of myocardial infarction. The extent of the risk reduction suggests that the benefit may be due to more than one mechanism, such as α -tocopherol-mediated reductions in platelet adhesion and aggregation,^{23,24} inhibition of vitamin-K-dependent clotting factors by the oxidised moiety vitamin-E-2-quinone,²⁵ and oxidised-LDL-mediated stimulation of endothelin production and inhibition of nitric oxide production.²⁶ However, we believe that inhibition of oxidation is likely to exert its main effects by modification of plaque enlargement or plaque rupture.

We carried out this study because of evidence that even advanced atherosclerotic lesions may be influenced by antioxidants.²⁷ The presence of large numbers of macrophage foam cells in advanced lesions may be an index of progression.²⁸ Although their numbers are variable, macrophages are found mainly at the periphery of advanced lesions, which suggests continuous peripheral recruitment of monocytes and enlargement of the lesion.²⁸ Macrophage-rich intermediate and advanced lesions and the peripheral part of ulcerated advanced lesions show chemical evidence of enhanced lipid oxidation.²⁹ The enhancement of lipid oxidation may bring about further macrophage recruitment and progressive death of

macrophage foam cells, enlarging the lipid core. Occlusive thrombosis, leading to MI, probably results from rupture of the plaque at the soft, macrophage-rich periphery.²⁹

There is a striking contrast between the clinical benefit on non-fatal MI in our study and the modest effects of α -tocopherol intake on the severity of carotid and coronary atherosclerosis as assessed by ultrasonography and angiography.^{30,31} This disparity suggests that the beneficial effects of antioxidant therapy are on the lipid composition of atheromatous plaques rather than their volume.

Our findings are the first from a prospective clinical trial to be consistent with the lipid oxidation theory of human coronary artery disease. Our findings support the use of a high dose of α -tocopherol to prevent non-fatal MI in patients with angina and coronary atherosclerosis, although there was no benefit in terms of cardiovascular death or total mortality. Further studies will be required to show the patient groups for whom these findings are applicable.

This study was supported by the East Anglia Region locally organised research scheme. We thank the Henkel Corporation (La Grange, Illinois, USA) for supply of α -tocopherol and placebo capsules; the Council for Responsible Nutrition, Thames Ditton, UK; Janine Kelleher for recruitment of patients; Michael Petch, Leonard Shapiro, and David Stone for permission to recruit patients in their care; Linda Sharples (MRC Biostatistics Unit, Cambridge) and Bianca de Stavola (London School of Hygiene and Tropical Medicine) for statistical advice and critical comments on the manuscript.

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Cyclical variation in paroxysmal supraventricular tachycardia in women

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Summary

Background Paroxysmal supraventricular tachycardia (SVT) in premenopausal women is often judged to be related to anxiety, and may be associated with the menstrual cycle. The aim of this study was to determine whether a cyclical variation of episodes of SVT exists and to correlate such variation with cyclical variation in plasma ovarian hormones.

Methods 26 women (mean age 36 [SD 8] years; with paroxysmal SVT were screened; those with regular menses who experienced at least three episodes of paroxysmal SVT in two consecutive 48-hour ambulatory ECG recordings were included. 13 patients (aged 32 [6] years) met these criteria. Patients underwent 48-hour ambulatory ECG monitoring and determination of plasma concentrations of oestradiol-17 β and progesterone on day 7, 14, 21, and 28 of their menstrual cycle.

Findings An increase in the number and duration of episodes of paroxysmal SVT was observed on day 28 as compared to day 7 of the menstrual cycle. A significant positive correlation was found between plasma progesterone and number of episodes and duration of SVT (5.6 [2.2] ng/mL; $r=0.83$, $p=0.0004$; and $r=0.82$, $p=0.0005$), while a significant inverse correlation was

found between plasma oestradiol-17 β and number of episodes and duration of SVT (155 [22] pg/mL; $r=-0.89$, $p<0.0001$; and $r=-0.81$, $p=0.0007$).

Interpretation Women with paroxysmal SVT and normal menses exhibit a cyclical variation in the occurrence of the arrhythmia with their menstrual cycle. There is a close correlation between the episodes of paroxysmal SVT and the plasma concentrations of ovarian hormones. These data suggest that changes in plasma levels of ovarian hormones (and their interaction) may be of importance in determining episodes of arrhythmia in such patients. The mechanisms of these effects are unknown.

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Introduction

Paroxysmal supraventricular tachycardia (SVT) can occur in the absence of cardiac disease. Ovarian hormones exhibit a cyclical variation in their plasma concentration during the menstrual cycle. These changes appear to be associated with changes in plasma catecholamine levels and adrenergic activity. Oestrogens play a role in neurotransmitter synthesis, uptake, and degradation involving receptors at both presynaptic as well as postsynaptic sites.^{1,2} Decreased or progestin-opposed oestrogen production is associated with increased adrenergic activity and vasomotor instability.³ An increase in plasma catecholamines may cause or facilitate the occurrence of episodes of paroxysmal SVT.

In addition, ovarian hormones may have direct effects on the cardiovascular system. Hyperpolarisation of vascular smooth muscle in dog coronary arteries after treatment with oestradiol-17 β has led to the suggestion that the hormone acts by increasing potassium conductance,⁴ while recent studies on isolated cardiac

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EXHIBIT C

Vitamin E supplementation enhances cell-mediated immunity in healthy elderly subjects¹⁻⁴

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ABSTRACT The effect of vitamin E supplementation on the immune response of healthy older adults was studied in a double-blind, placebo-controlled trial. Subjects ($n = 32$) resided in a metabolic research unit and received placebo or vitamin E (800 mg *dl*- α -tocopheryl acetate) for 30 d. Alpha-tocopherol content of plasma and peripheral blood mononuclear cells (PBMCs), delayed-type hypersensitivity skin test (DTH), mitogen-stimulated lymphocyte proliferation, as well as interleukin (IL)-1, IL-2, prostaglandin (PG) E₂, and serum lipid peroxides were evaluated before and after treatment. In the vitamin E-supplemented group 1) α -tocopherol content was significantly higher ($p < 0.0001$) in plasma and PBMCs, 2) cumulative diameter and number of positive antigen responses in DTH response were elevated ($p < 0.05$), 3) IL-2 production and mitogenic response to optimal doses of concanavalin A were increased ($p < 0.05$), and 4) PGE₂ synthesis by PBMCs ($p < 0.005$) and plasma lipid peroxides ($p < 0.001$) were reduced. Short-term vitamin E supplementation improves immune responsiveness in healthy elderly individuals; this effect appears to be mediated by a decrease in PGE₂ and/or other lipid-peroxidation products. *Am J Clin Nutr* 1990;52:557-63.

KEY WORDS Vitamin E, immune response, aging, prostaglandin

Introduction

Considerable evidence indicates that aging is associated with altered regulation of the immune system (1). Age-related functional changes have been well characterized for both humoral and cell-mediated immune responses (2-4). Although all cell types of the immune system show age-related changes, the major alterations occur in the T cells (5).

In vivo, T-cell-dependent cell-mediated functions, such as delayed type hypersensitivity skin test (DTH) (6, 7), graft vs host reaction (2), and resistance to challenge with syngeneic and allogeneic tumors and parasites (5), are depressed with age. In vitro the proliferative response of human and rodent lymphocytes to phytohemagglutinin (PHA) and concanavalin A (Con A) become depressed with age (2). Several groups showed that antigen- and mitogen-stimulated interleukin (IL)-2 production declines with age and contributes to the T-cell-mediated defects observed with aging (8-11). Cooperation between monocytes and lymphocytes is essential in antigen recognition, lymphocyte differentiation and eventual antibody production, and development of the effector state of cellular immunity, ie,

the DTH phase (12). In addition to presenting antigen, macrophages synthesize IL-1 which induces the production of IL-2 by the activated T cells.

Macrophages have a high concentration of arachidonic acid in their membrane phospholipids. Upon stimulation, macrophages release up to 50% of their arachidonic acid content in the form of oxygenated metabolites, eg, prostaglandin (PG), hydroxycosatetraenoic acid (HETE), and leukotriene (LT) (13, 14). PGE₂ was shown to suppress lymphocyte proliferation and lymphokine synthesis (15-17). Other oxidative metabolites of activated macrophages, such as H₂O₂, was also shown to suppress lymphocyte proliferation (18, 19). Increased PGE₂ production by macrophages from aged rats (20) and mice (21) was reported.

Vitamin E was shown to decrease PG production in immune cells (21, 22) and enhance cell-mediated immunity in young (22) and old (21) animals. A significant decrease of PGE₂ synthesis in spleen in old C57BL/6J mice was associated with an enhanced DTH, in vitro spleen mitogenic responses to Con A but not to PHA, and IL-2 formation (21). One of the biologic changes associated with aging is an increase in free radical formation with subsequent damage to cellular processes. Several studies investigated the free radical theory of aging and the role of antioxidants, including vitamin E, on the life expectancy of rodents (23). Vitamin E supplementation was shown to be protective against age-associated diseases such as cancer (24-26) and amyloidosis (27). An increase in the average life span of short-lived autoimmune-prone NZB/NZW mice receiving vitamin E supplements was reported by Harman (28). Furthermore, a community-based survey by Cheavance et al (29, 30) showed a positive correlation between plasma vitamin E con-

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centrations and DTH responses and a negative correlation between plasma vitamin E and the number of recent infections. Therefore, we investigated the effect of vitamin E supplementation on in vivo and in vitro indices of cell-mediated immune response in healthy elderly subjects.

Subjects and methods

Study subjects and experimental design

Thirty-two healthy men and women, ≥ 60 y of age, with no known medical illness and receiving no prescription medication, were recruited from the Boston area. Those using vitamin supplements and/or any nonsteroidal antiinflammatory drugs were excluded. All subjects passed a complete physical examination and had normal weight-for-height. Blood and urine samples from each subject were obtained for complete blood and differential counts, clinical chemistry profile, and routine urinalysis before subjects were admitted. The study was approved by the Tufts University/New England Medical Center Human Investigation Review Committee (HIRC). All volunteers signed a HIRC-approved written consent form.

Volunteers were randomly assigned to a placebo or vitamin E-supplemented group. All subjects resided and consumed their meals in the Metabolic Research Unit of the USDA Human Nutrition Research Center on Aging at Tufts University. A 3-d-cycle menu consisting of foods typical to the American diet and adequate in all nutrients was served throughout the study. The basal 3-d menu contained $20 \pm 1.5\%$ ($\bar{x} \pm SD$) protein, $31.5 \pm 2.8\%$ fat, with a ratio of polyunsaturated to saturated fatty acids (P:S) of 0.41 ± 0.09 , and $49 \pm 2\%$ carbohydrate. The amounts of fat and protein, the P:S, and all other dietary components except for carbohydrates were kept constant for all subjects. The total calorie content provided by the basal 3-d menu (1768 ± 46 kcal/d) was adjusted by changing the carbohydrate content to meet each subject's calorie requirement and to maintain the subject's weight.

Subjects were weighed weekly and their vital signs were monitored daily. Subjects were advised to continue their normal activity and to avoid excessive sun exposure. This precaution was taken because membrane lipid peroxidation and PGE₂ formation have been implicated as major components of ultraviolet-induced skin injury.

During the first 2 wk of the study all subjects received a placebo capsule with breakfast and with dinner. On days 8, 10, and 12, 40 mL fasting blood and 24-h urine samples were obtained for different in vitro immunologic tests and PGE₂ analyses as baseline or presupplementation values. On day 12, 30 mL additional blood was collected for biochemical measures. Subjects were then administered a DTH. Upon completion of the 48-h evaluation of the skin test, the placebo group continued consuming two placebo capsules containing soybean oil whereas the vitamin E group consumed two vitamin E capsules containing 400 mg *dl*- α -tocopheryl acetate in soybean oil (Hoffmann-LaRoche, Inc. Nutley, NJ) daily for 30 d. At the completion of the test period three fasting blood and 24-h urine samples were collected every other day for postsupplementation analyses (except for the first 10 subjects for whom one blood sample was collected 15 d after supplementation and the remaining two samples were collected 30 and 32 d after supplementation. The amount of blood withdrawn was similar to that collected at baseline.

The study was conducted in a double-blind fashion with the codes broken only after all data collection had been completed. The average of three baseline and three postsupplementation values for immunologic tests and PGE₂ were used in analyses.

Procedures

DTH was assessed with multitest CMI (Merieux Institute, Inc, Miami), a single-use, disposable applicator of acrylic resin with eight heads loaded with a glycerine control and the following seven recall antigens: tetanus toxoid, diphtheria toxoid, streptococcus (group C), mycobacterium tuberculosis, candida (*albicans*), trichophyton (metagrophytes), and proteus (*mirabilis*). The diameter of positive reaction was measured 24 and 48 h after administration of the test. The antigen score was calculated as the total number of positive antigens and the cumulative score was calculated as the total diameter of induration of all the positive reactions. According to the manufacturer's instructions, an induration of ≥ 2 mm was considered positive. If a positive reaction to the glycerine control was observed, the diameter of its induration was subtracted from all the other positive reactions. The test was administered by the same nurse before and after supplementation for each subject and the diameter of induration was measured by the same person before and after supplementation.

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood according to the procedure of Boyum (31). PBMCs were removed from the interface and washed twice in RPMI 1640 supplemented with 100 mg/L penicillin, 100 g/L streptomycin, 2 mmol *l*-glutamine/L, and 25 mmol HEPES/L (Gibco, Grand Island, NY). Cells were resuspended in medium and counted under a light microscope. Cell viability was assessed by using the trypan blue exclusion method. Cells were then suspended at appropriate concentrations for measurement of mitogenic lymphocyte proliferation, IL-2, IL-1, and PGE₂ formation.

Lymphocyte proliferation was measured by [³H]thymidine incorporation after stimulation with T-cell and B-cell mitogens. Dilutions of mitogens between 1 and 100 mg/L for PHA (PHA-P, Difco, Detroit) and Con A (Sigma, St Louis) and 0.015% to 0.15% for *Staphylococcus aureus* Cowan I (SAC; Zysorbin, Zymed, San Francisco) were prepared in RPMI 1640 with 100 mL fetal bovine serum (FBS)/L and optimal dilution for each mitogen was determined. One hundred microliters of each mitogen was plated in triplicate into 96-well, flat-bottomed microtiter plates (Becton Dickinson, Oxnard, CA). PBMC were suspended at 1×10^9 cells/L in RPMI 1640; 100 μ L of the cell suspension was plated with and without mitogens and incubated for 72 h at 37 °C in an atmosphere of 5% CO₂ and 95% humidity. Four hours before termination of incubation, 18.5 GBq of [³H]thymidine (specific activity 247.9 GBq/mol. New England Nuclear, Boston) in 20 μ L was added to each well. Cells were harvested onto glass microtiter filter paper by use of a cell harvester (PHD, Cambridge, MA). Filter disks were placed in minivials and counted in a liquid-scintillation counter (Beckman Instruments, Palo Alto, CA). The results are reported as corrected counts per minute (ccpm), the average cpm of mitogen-stimulated cultures minus the average cpm of cultures without mitogens.

Cells (1×10^9 /L) in RPMI with 100 mL FBS/L were cultured in 24-well flat-bottomed plates (Becton Dickinson) with Con A (10 mg/L for 48 h). Cell-free supernatant was stored at -70 °C for later analysis of IL-2. IL-2 activity was measured with a

TABLE 2

Effect of vitamin E supplementation on plasma and peripheral blood mononuclear cell (PBMC) α -tocopherol concentration in elderly subjects*

Group	Plasma α -tocopherol		PBMC α -tocopherol	
	Before	After	Before	After
	$\mu\text{mol/L}$		nmol	
Placebo	26.2 \pm 1.7 (<i>n</i> = 14)	23.9 \pm 1.4 (<i>n</i> = 14)	0.14 \pm 0.04 (<i>n</i> = 6)	0.19 \pm 0.03 (<i>n</i> = 6)
Vitamin E supplemented	25.6 \pm 1.4 (<i>n</i> = 17)	70.9 \pm 6.3† (<i>n</i> = 17)	0.12 \pm 0.02 (<i>n</i> = 5)	0.39 \pm 0.05‡ (<i>n</i> = 5)

* $\bar{x} \pm \text{SEM}$.

† Significantly different from before-treatment values: †*p* < 0.0001, ‡*p* < 0.001.

statistical significance was the same regardless of which values were analyzed. The cumulative score significantly increased in vitamin E-treated group whereas no change in cumulative score was observed in the placebo group (Table 3). Seventy-one percent of the subjects in the vitamin E-supplemented group had an increase in their cumulative score, with a mean increase of 52.8 \pm 20.0% in the vitamin E-supplemented group, compared with a 3.7 \pm 3.7% increase in the placebo group. Percent change in the supplemented group is significantly higher than that in the placebo group at *p* = 0.04. The antigen score (total number of positive responses) was also significantly increased in the vitamin E-supplemented group (2.5 \pm 0.38 before vs 3.1 \pm 0.48 after, *p* < 0.05) whereas no change was noted in the placebo group (3.2 \pm 0.45 before vs 3.3 \pm 0.42 after placebo).

Table 4 shows the mitogenic response to optimal concentrations (10 mg/L) of Con A. A significant increase in Con A-stimulated mitogenic response was observed in the vitamin E-supplemented group. No significant change in response to Con A was observed in the placebo group. No significant change was observed in response to PHA or SAC in either placebo or vitamin E-supplemented group (data not shown).

IL-2 formation in response to Con A was significantly increased in the vitamin E-supplemented group (*p* < 0.05) (Table 5). In contrast, no significant change in IL-2 production was observed in the placebo group. Although 29% of subjects in the placebo group showed an increase in IL-2 formation, 64% of the subjects in the vitamin E-supplemented group had an increase in IL-2 concentration (*p* < 0.0002 by chi-square analysis). The percent change in IL-2 concentration in the vitamin

TABLE 3

Effect of vitamin E supplementation on DTH in elderly subjects*

Group	Cumulative index	
	Before treatment	After treatment
	<i>mm</i>	
Placebo (<i>n</i> = 13)	16.5 \pm 2.2	16.9 \pm 2.1
Vitamin E supplemented (<i>n</i> = 16)	14.2 \pm 2.9	18.9 \pm 3.5†

* $\bar{x} \pm \text{SEM}$.

† Significantly different from before-treatment values *p* < 0.05 (paired Wilcoxon signed-rank test).

TABLE 4

Effect of vitamin E supplementation on con A-induced lymphocyte proliferation by PBMCs from elderly subjects*

Group	Before treatment	After treatment
	<i>ccpm</i>	
Placebo (<i>n</i> = 14)	24 478 \pm 2 729	21 954 \pm 2 908
Vitamin E supplemented (<i>n</i> = 18)	20 551 \pm 1 927	23 770 \pm 2 991†

* $\bar{x} \pm \text{SEM}$.

† Significantly different from before treatment values, *p* < 0.05 (paired Wilcoxon signed-rank test).

sis). The percent change in IL-2 concentration in the vitamin E-supplemented group (67 \pm 24%) was significantly (*p* < 0.025) higher than that in the placebo group (-7 \pm 20%). Furthermore, in the vitamin E-supplemented group, a positive correlation (*r* = 0.50, *p* = 0.056) was noted between changes in IL-2 concentration and changes in plasma α -tocopherol concentration. No significant change in endotoxin-stimulated IL-1 production was observed in either group (data not shown).

No significant change was observed in serum immunoglobulin concentrations in either the placebo or the vitamin E-supplemented group (data not shown).

Table 6 shows PGE₂ production by the placebo and vitamin E-supplemented groups before and after supplementation. There was no significant change in unstimulated cultures in either group. However, in the vitamin E-supplemented subjects but not in the placebo group, a highly significant decrease was observed in PHA-stimulated PGE₂ formation (Table 6). The percent decrease in the vitamin E group was significantly (*p* < 0.005) higher than that of the placebo group (Fig 1).

A highly significant decrease in plasma lipid peroxides was observed in the vitamin E-supplemented group with no significant change in placebo group (Table 7).

Discussion

This study represents the first double-blind, placebo-controlled trial of the effect of vitamin E supplementation on the immune response of healthy elderly individuals. Supplementation of healthy elderly individuals with 800 mg *dl*- α -tocopheryl acetate/d for 30 d significantly improved DTH, an in vivo mea-

TABLE 5

Effect of vitamin E supplementation on Con A-induced IL-2 production by PBMCs from elderly subjects*

Group	Before treatment	After treatment
	<i>kU/L</i>	
Placebo (<i>n</i> = 14)	31.8 \pm 8.3	37.5 \pm 12.5
Vitamin E supplemented (<i>n</i> = 18)	35.6 \pm 9.1	49.6 \pm 12.6†

* $\bar{x} \pm \text{SEM}$.

† Significantly different from before-treatment values, *p* < 0.05 (paired Student's *t* test).

TABLE 6
Effect of vitamin E supplementation on PGE₂ formation by PBMCs of elderly subjects*

Group	Control cultures		PHA-stimulated cultures	
	Before treatment	After treatment	Before treatment	After treatment
	<i>In pmol/L</i>			
Placebo (n = 14)	7.3 ± 1.6	6.6 ± 1.7	8.3 ± 1.6	8.4 ± 1.6
Vitamin E supplemented (n = 18)	7.8 ± 1.6	7.7 ± 1.6	9.1 ± 1.6	8.5 ± 1.5†

* $\bar{x} \pm \text{SEM}$.

† Significantly different from before-treatment values, $p < 0.0004$ (paired Student's *t* test).

sure of cell-mediated immunity, and enhanced the in vitro mitogenic response to the T-cell mitogen Con A but not to PHA or the B-cell mitogen SAC. Furthermore, a significant increase in IL-2 formation in response to Con A was observed after vitamin E supplementation. The increase in IL-2 concentration was positively correlated with changes in vitamin concentration. No effect on IL-1 formation was noted. Decreases in DTH, mitogenesis to Con A and PHA, and IL-2 production are well documented in both senescent rodents and older adults (44-46) whereas changes in B-cell response (47) and IL-1 production are equivocal (10, 46-48). The results of this clinical trial closely parallel our earlier studies in aged mice where vitamin E supplementation significantly improved DTH, mitogenic response to Con A (but not to PHA), and IL-2 formation (21).

It is interesting that the majority but not all of the vitamin E-supplemented subjects showed an enhancement of immune response. However, the responders showed improvement in all indices. The quantitative change of the immune indices was variable in the responders depending on the individual and the

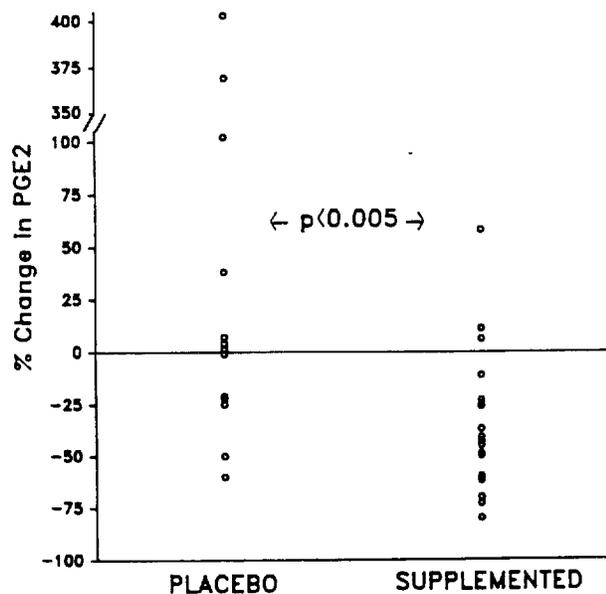


FIG 1. Effect of vitamin E supplementation on PHA-stimulated PGE₂ production by PBMCs of elderly subjects. Each circle denotes one subject.

TABLE 7
Effect of vitamin E supplementation on plasma lipid peroxide concentration in elderly subjects*

Group	Before treatment	After treatment
	$\mu\text{mol/L}$	
Placebo (n = 14)	2.26 ± 0.76	2.20 ± 0.55
Vitamin E supplemented (n = 18)	2.76 ± 0.67	1.20 ± 0.60†

* $\bar{x} \pm \text{SEM}$.

† Significantly different from before-treatment values, $p < 0.001$ (paired Student's *t* test).

type of test. The variability in response could be partly due to differences in plasma or PBMC concentration of tocopherol after supplementation. The greatest relative change after vitamin E supplementation was observed in IL-2 production followed by cumulative DTH score and mitogenic response to Con A. Changes in IL-2 were directly correlated with changes in plasma vitamin E concentration. The lack of a larger average change in Con A and PHA responses might be due to the greater variability inherent in these assays. In addition to large interindividual variation, substantial intraindividual differences were also observed in mitogen-stimulated lymphocyte-proliferation tests. All in vitro culture assays were performed in the presence of FBS, a poor source of tocopherol. This medium may diminish the effect of vitamin E during the longer culture periods used in lymphocyte-proliferation assays relative to shorter culture periods utilized for the IL-2 assay. In both our previous animal experiment (21) and the present study, vitamin E supplementation improved Con A- but not PHA-induced lymphocyte proliferation. This implies specificity of the vitamin E effect because these mitogens stimulate different T-cell populations.

The immunostimulatory effect of vitamin E might be mediated by decreases in PGE₂ production and/or decreases in other lipid-peroxidation products. PGE₂ suppresses lymphocyte proliferation and IL-2 production. Increased PGE₂ production and lipid peroxidation have been found in aged animals (21, 23). Lymphocytes from elderly individuals are also more sensitive to the inhibitory effect of PGE₂ (49). Of particular interest is our observation that PBMCs from healthy elderly subjects synthesize significantly more PGE₂ than do those of young subjects (unpublished observations, 1990). In this study we observed a significant reduction in PGE₂ production by PBMCs and in plasma lipid peroxides (TBAR) of elderly subjects supplemented with vitamin E. Because malonaldehyde is produced as a by-product of arachidonic acid metabolism, the relative contribution to the reduction in total plasma TBAR by decreases in arachidonic acid metabolism and decreases in the formation of other lipid peroxides is not clear.

PGE₂ was shown to decrease IL-1 production by monocytes (50). In this study no significant increase in IL-1 production was observed. However, vitamin E can inhibit the synthesis of lipoxygenase products (51, 52) including LT B₄, which was shown to enhance endotoxin-stimulated IL-1 production (53).

Except for a small but statistically significant increase in plasma zinc concentration, the status of other nutrients was unaffected by vitamin E supplementation. Although supplementation of elderly subjects with 440 mg zinc/d (~30 times

the recommended dietary allowance) was shown to improve DTH response (54), the increase in plasma zinc concentration in our subjects was minor (~5%) compared with the threefold increase in plasma and PBMC α -tocopherol concentrations. Furthermore, a change in plasma zinc concentration does not indicate a proportionate change in the tissue concentration of the mineral (55). A recent double-blind, placebo-controlled study by Bogden et al (55) showed no significant effect of zinc supplementation on immune response of elderly subjects.

In conclusion, our data indicate that short-term supplementation of most healthy elderly subjects treated with 800 mg *dl*- α -tocopheryl acetate significantly improves several indices of cell-mediated immunity. In view of the known effects of PGE₂ on immune function, it is plausible that the immunostimulatory effect of vitamin E is due to a reduction in PGE₂ synthesis and a concomitant increase in IL-2 production. Epidemiologic studies indicate a lower incidence of infectious disease in elderly subjects with high plasma tocopherol concentrations (30). Population groups maintaining high plasma tocopherol concentrations were also noted to possess a lower incidence of cancer (25, 56). Improved DTH response in hospitalized patients was shown to decrease sepsis and mortality (57). However, Harman and Miller (58) were not able to show a difference in antibody development against influenza virus vaccine or the incidence of infectious disease in elderly patients from a chronic-care facility supplemented with 200 or 400 mg tocopherol/d for 1 y. Unfortunately, data on the health and nutrition status, medication use, antibody concentrations, and other pertinent indices were not reported so it is difficult to interpret their findings.

It was demonstrated in young rodents that a higher than standard intake of tocopherol is necessary to promote optimal immune responsiveness (59). Although our study suggests that many elderly individuals might benefit from a supplementary intake of vitamin E, such public health recommendations can only be considered after longer-term studies with lower amounts of tocopherol are completed. This point will be especially important in determining if the immunostimulatory effect observed is due to pharmacologic or physiologic effect of vitamin E. Nevertheless, it is encouraging to note that a single nutrient supplement can enhance immune responsiveness in healthy elderly subjects consuming the recommended amounts of all nutrients. This is especially significant because dietary intervention represents the most practical approach for delaying or reversing the rate of decline of immune function with age.

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EXHIBIT D

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THE EFFECT OF VITAMIN E AND BETA CAROTENE ON THE INCIDENCE OF LUNG CANCER AND OTHER CANCERS IN MALE SMOKERS

THE ALPHA-TOCOPHEROL, BETA CAROTENE CANCER PREVENTION STUDY GROUP*

Abstract Background. Epidemiologic evidence indicates that diets high in carotenoid-rich fruits and vegetables, as well as high serum levels of vitamin E (alpha-tocopherol) and beta carotene, are associated with a reduced risk of lung cancer.

Methods. We performed a randomized, double-blind, placebo-controlled primary-prevention trial to determine whether daily supplementation with alpha-tocopherol, beta carotene, or both would reduce the incidence of lung cancer and other cancers. A total of 29,133 male smokers 50 to 69 years of age from southwestern Finland were randomly assigned to one of four regimens: alpha-tocopherol (50 mg per day) alone, beta carotene (20 mg per day) alone, both alpha-tocopherol and beta carotene, or placebo. Follow-up continued for five to eight years.

Results. Among the 876 new cases of lung cancer diagnosed during the trial, no reduction in incidence was observed among the men who received alpha-tocopherol (change in incidence as compared with those who did not, -2 percent; 95 percent confidence interval, -14 to 12 percent). Unexpectedly, we observed a higher incidence of lung cancer among the men who received beta caro-

tene than among those who did not (change in incidence, 18 percent; 95 percent confidence interval, 3 to 36 percent). We found no evidence of an interaction between alpha-tocopherol and beta carotene with respect to the incidence of lung cancer. Fewer cases of prostate cancer were diagnosed among those who received alpha-tocopherol than among those who did not. Beta carotene had little or no effect on the incidence of cancer other than lung cancer. Alpha-tocopherol had no apparent effect on total mortality, although more deaths from hemorrhagic stroke were observed among the men who received this supplement than among those who did not. Total mortality was 8 percent higher (95 percent confidence interval, 1 to 16 percent) among the participants who received beta carotene than among those who did not, primarily because there were more deaths from lung cancer and ischemic heart disease.

Conclusions. We found no reduction in the incidence of lung cancer among male smokers after five to eight years of dietary supplementation with alpha-tocopherol or beta carotene. In fact, this trial raises the possibility that these supplements may actually have harmful as well as beneficial effects. (N Engl J Med 1994;330:1029-35.)

PREVIOUS studies have suggested that higher intakes of vitamin E (alpha-tocopherol) and beta carotene may be associated with a reduced risk of lung cancer. In particular, epidemiologic studies have linked the intake of vegetables rich in beta carotene with a lower risk of cancer (especially lung cancer) and have suggested that certain micronutrients are inhibitors of cancer.^{1,2} The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study was a randomized, double-blind, placebo-controlled primary-prevention trial undertaken to determine whether supplementation with alpha-tocopherol, beta carotene, or both would reduce the incidence of lung cancer in male smokers. A secondary outcome of interest was the incidence of other cancers. Lung cancer was deemed a

particularly appropriate target for this trial because of its high incidence, its generally poor prognosis, and the existence of a well-defined high-risk population (i.e., smokers).³ In this report we describe the initial overall results of the study, which was conducted in Finland as a joint project of the National Public Health Institute of Finland and the U.S. National Cancer Institute.

METHODS

Study Design

The rationale, design, and methods of the study, the characteristics of the participants, and the measures of compliance have been described in detail elsewhere.⁴ Briefly, the participants (n = 29,133) were male smokers who were 50 through 69 years old at entry; they were recruited from the total male population of this age group in 14 geographic areas in southwestern Finland (n = 290,406). The participants were randomly assigned to one of four supplementation regimens: alpha-tocopherol alone (n = 7286), alpha-tocopherol and beta carotene (n = 7278), beta carotene alone (n = 7282), or placebo (n = 7287). Thus, a total of 14,564 men received alpha-tocopherol, and 14,560 received beta carotene. The daily dose of alpha-tocopherol was 50 mg and that of beta carotene, 20 mg. Follow-up continued for 5 to 8 years (median, 6.1), until death or April 30, 1993, with a total of 169,751 person-years contributed by

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*The participants in the study group are listed in the Appendix.

the surviving participants. This study was approved by the institutional review boards of the participating institutions, and all subjects provided informed consent before randomization.

Eligibility

Participants were recruited in 1985 through 1988 from the respondents to a postal survey ($n = 224,377$) who lived in the designated study region. To be eligible, they had to be smokers (five or more cigarettes per day at entry), 50 to 69 years old, and willing to give informed written consent. Potential participants with a history of cancer or serious disease limiting their ability to participate, those taking supplements of vitamin E, vitamin A, or beta carotene in excess of predefined doses, and those being treated with anticoagulant agents were excluded. Before their enrollment, the participants were interviewed at 1 of 14 local study centers to obtain details of their medical, dietary, smoking, and occupational histories and information about other risk factors for cancer. Each participant's dietary intake of alpha-tocopherol and beta carotene was estimated from the diet-history questionnaire⁵; levels of alpha-tocopherol and beta carotene were measured in serum samples by high-performance liquid chromatography.⁶ Participants identified after randomization as ineligible ($n = 113$) were equally distributed among the four intervention groups; they included men with preexisting cancer other than nonmelanoma skin cancer ($n = 64$), men with lung cancer identified on the base-line chest film ($n = 33$), users of vitamin supplements in excess of the study limits ($n = 15$), and 1 nonsmoker.

Randomization and Blinding

The participants at each of the 14 study sites were randomly assigned to one of the four intervention groups. Treatment assignments were based on a two-by-two factorial design that permitted assessment of the effects of the two supplements independently. Thus, half the participants received alpha-tocopherol ($n = 14,564$) and half did not ($n = 14,569$). Similarly, half received beta carotene ($n = 14,560$) and half did not ($n = 14,573$). The proportion of participants who reported yellowing of the skin at any time during active follow-up was 34 percent in the two groups that received beta carotene, as compared with 7 percent in the groups given no beta carotene; persistent yellowing of the skin (during two thirds or more of the follow-up visits) was reported by 8.8 percent of the participants who received beta carotene, as compared with 0.3 percent of those who did not. Participants and all study staff involved in the ascertainment of end points and the assignment of final diagnoses remained blinded to the participants' treatment assignments throughout the trial.

Delivery of Supplements and Assessment of Compliance

The study agents were formulated as synthetic *dl*-alpha-tocopheryl acetate (50 percent powder) and synthetic beta carotene (10 percent water-soluble beadlets); all formulations were colored with quinoline yellow. Capsules were packaged in coded blister-pack wallets in calendar format provided by Hoffmann-LaRoche (Basel, Switzerland). All participants took a single capsule daily. The participants received a new supply of capsules at each of their thrice-yearly follow-up visits. Visits began in April 1985 for some participants and were concluded in April 1993 for all. Compliance was assessed by counts of the remaining capsules at each visit, by measurement of serum alpha-tocopherol and beta carotene levels after three years of supplementation, and by measurements in random serum samples throughout the study.⁴

Assessment of End Points

Cases of lung cancer were identified through the Finnish Cancer Registry.⁷ All cases known to have been diagnosed up to April 30, 1993, are included in this report. To enhance the ascertainment of cases, a chest film was obtained at a study visit every 28 months and at each participant's exit from the study. For various reasons, the final chest film was not available for 494 of the surviving men. There were no differences among the intervention groups in the proportion of exit chest films available for analysis or in the reasons why no film was obtained. All diagnostic information for each case

of lung cancer was reviewed by the Clinical Review Committee for confirmation and staging. Clinical diagnoses were based on histologic features in 77 percent of the cases, on cytologic analysis alone in 15 percent, and on clinical data alone in 8 percent.

Cancers other than lung cancer were also identified through the Finnish Cancer Registry, with medical records reviewed by clinicians at the central study office.

Monitoring of Safety and Efficacy

Possible side effects of the interventions were assessed at each follow-up visit by means of a questionnaire covering symptoms and an interview focusing on illnesses since the most recent visit that had led to a visit to a doctor or to hospitalization. Information on morbidity unrelated to cancer was also obtained from the Finnish National Hospital Discharge Registry. Deaths ($n = 3570$) were identified from the National Death Registry, a branch of Statistics Finland. The underlying cause of death was coded by trained nosologists using the *International Classification of Diseases*, ninth revision (ICD-9), and reviewed at the study coordinating center; the death certificate was not available for four participants. In 91 percent of all deaths, the cause was based on the autopsy findings (54 percent), the inpatient diagnosis, or both.

A data and safety monitoring committee was convened twice annually throughout the study to review its progress and integrity and to evaluate unblinded data relevant to safety and efficacy.

Statistical Analysis

Analyses of trial results focused on estimating the overall effect of the two supplements on the incidence of cancer and on mortality due to cancer or other causes. Analyses were based on the intention-to-treat principle; that is, follow-up and case ascertainment continued regardless of whether participants continued in the trial. We tested for an interaction between the effects of alpha-tocopherol and beta carotene by means of a proportional-hazards model.⁸

Kaplan-Meier cumulative-incidence plots and two-sided nominal *P* values derived from the unweighted log-rank statistic⁹ are presented for each intervention separately: alpha-tocopherol as compared with no alpha-tocopherol, and beta carotene as compared with no beta carotene. The effect of intervention is expressed as the percentage change in the incidence of an end point and its 95 percent confidence interval. Computations of confidence intervals were based on the binomial distribution, derived from conditioning on the number of cases and adjustment of probabilities for the number of person-years of follow-up in the two comparison groups.^{8,9}

The preliminary data on cancers other than lung cancer are presented in the form of counts and rates of incidence according to intervention group. Two or more of the five primary cancers in a single participant were counted as separate cases in each category, but were counted only once within each category (even in the category "other cancers"). Thus, the cancer counts are not mutually exclusive. Cases of carcinoma in situ of the lung ($n = 6$) and basal-cell carcinoma of the skin ($n = 217$) were excluded from the analysis. Cause-specific data on deaths are presented in the form of counts and mortality rates in mutually exclusive cause-of-death categories according to intervention group. The categories are based on the following ICD-9 codes: cancer (140 through 208), ischemic heart disease (410 through 414), hemorrhagic stroke (430 through 432), ischemic stroke (433 through 436 and 438), other cardiovascular disease (390 through 405, 415 through 429, 437, and 440 through 459), injuries and accidents (800 through 999), and other causes (001 through 139, 210 through 389, and 460 through 799). Only cases in which cancer was the underlying cause of death were included among the deaths due to cancer.

RESULTS

Characteristics of the Participants

At study entry, the men in the cohort averaged 57.2 years of age, smoked an average of 20.4 cigarettes daily, and had smoked for an average of 35.9 years.

re were no differences among the intervention groups with respect to any characteristic or risk factor for lung cancer that we evaluated at base line (Table 1) during follow-up, except those directly related to supplementation. A total of 6131 participants stopped smoking during the trial; the numbers who quit in the two intervention groups differed by less than 26. Similarly, 9061 participants left the study for any reason, including death; the groups differed in the number of such dropouts by less than 37.

Incidence of Lung Cancer and Base-Line Alpha-Tocopherol and Beta Carotene Levels

When the placebo group was divided according to quartiles with regard to the base-line serum alpha-tocopherol or beta carotene concentration, the incidence of lung cancer was higher among the subjects in the lowest quartile group than among those in the next highest quartile group: alpha-tocopherol, 56.8 vs. 41.8; beta carotene, 53.3 vs. 43.1). There was, however, an inverse association between dietary intake of alpha-tocopherol and beta carotene at base line and the risk of lung cancer during the trial: incidence per 10,000 person-years, lowest vs. high-alpha-tocopherol, 61.4 vs. 40.6; beta carotene, 61.4 vs. 39.9).

Compliance

Compliance, estimated on the basis of residual-capsule counts, was excellent, with four out of five active participants taking more than 95 percent of their capsules. In addition, there were no differences in capsule consumption among the intervention groups (median percentage of capsules taken, 99.0 percent in each). Participants receiving active treatment accounted for 99 percent of the total follow-up, whereas the remaining 1 percent was contributed by men who died or dropped out and therefore did not consume capsules. Compliance with intervention was confirmed by the substantial increases in serum alpha-tocopherol and beta carotene concentrations in the groups receiving active agents, whereas the levels changed little in those who did not receive the agents (Table 2).

Incidence of Lung Cancer and Mortality

A total of 876 newly diagnosed cases of lung cancer and 564 deaths due to lung cancer were identified in the entire cohort. There was no evidence of an interaction between the two supplements in their effect on lung cancer (incidence per 10,000 person-years: alpha-tocopherol alone, 47.3; alpha-tocopherol and beta carotene, 55.3; beta carotene alone, 57.2; and placebo, 47.7; likelihood-ratio test for interaction: chi-square = 0.04, $P = 0.84$). Our findings regarding the influence of lung cancer and mortality from that disease according to intervention are shown in Figures 1, 2, and 3. For alpha-tocopherol recipients, the small reduction in incidence (2 percent) during the entire study was not statistically significant ($P = 0.8$ by the rank test). Among the men who received beta

Table 1. Median Base-Line Characteristics of the Participants, According to Whether They Received Alpha-Tocopherol and Beta Carotene.*

CHARACTERISTIC	ALPHA- TOCOPHEROL	NO ALPHA- TOCOPHEROL	BETA CAROTENE	NO BETA CAROTENE
No. of subjects	14,564	14,569	14,560	14,573
Age (yr)	57.2	57.1	57.3	57.0
Cigarettes smoked/day	20	20	20	20
Years of smoking	36	36	37	36
Serum cholesterol (mmol)	6.2	6.2	6.2	6.2
Body-mass index†	26.0	25.9	26.0	26.0
Total energy intake (kcal/day)	2,725	2,715	2,717	2,722
Total fat intake (g/day)	117.7	116.9	117.4	117.2
Alcohol intake (g/day)	11.1	10.9	10.9	11.1

*This was a two-by-two study, with a total of 29,133 participants. The numbers with data on the dietary-intake variables are as follows: alpha-tocopherol, 13,536; no alpha-tocopherol, 13,575; beta carotene 13,521; and no beta carotene, 13,590.

†The weight in kilograms divided by the square of the height in meters.

carotene, an excess cumulative incidence of lung cancer was observed after 18 months and increased progressively thereafter, resulting in an 18 percent difference in incidence by the end of the study (95 percent confidence interval, 3 to 36 percent; $P = 0.01$) between the participants who received beta carotene and those who did not. The results were essentially identical when the analysis was restricted to men who had no yellowing of the skin or to those with lung cancers detected radiographically during the study. Mortality due to lung cancer was also apparently higher in the groups that received beta carotene than in those that did not ($P = 0.08$). No difference associated with the presence or absence of beta carotene supplementation was observed in the case fatality rate or in the length of time from diagnosis to death.

The six cases of carcinoma in situ that were excluded from these analyses were distributed as follows: three each among participants who received alpha-tocopherol and those who did not, and two cases among participants who received beta carotene and four among those who did not. There was one new

Table 2. Serum Concentrations of Alpha-Tocopherol and Beta Carotene before and after Supplementation, According to Intervention.*

INDEX AND GROUP	NO. OF SUBJECTS	MEDIAN	20TH PERCENTILE	80TH PERCENTILE
<i>milligrams per liter</i>				
Alpha-tocopherol level				
At base line				
Alpha-tocopherol	14,472	11.5	9.3	14.2
No alpha-tocopherol	14,469	11.4	9.3	14.1
At three years				
Alpha-tocopherol	11,332	17.3	14.3	21.1
No alpha-tocopherol	11,258	12.4	10.2	15.1
Beta carotene level				
At base line				
Beta carotene	14,460	0.17	0.10	0.29
No beta carotene	14,460	0.17	0.10	0.29
At three years				
Beta carotene	11,276	3.0	1.6	4.5
No beta carotene	11,314	0.18	0.10	0.30

*To convert values for alpha-tocopherol to millimoles per liter, multiply by 2.322. To convert values for beta carotene to millimoles per liter, multiply by 1.863.

case of lung cancer among the 113 participants excluded after randomization; the man was assigned to receive alpha-tocopherol.

Other Cancers

A total of 1415 first cancers other than lung cancer were identified in 1331 subjects during the trial (basal-cell carcinoma of the skin was excluded, as were second cancers at a given site). Figure 2 shows the number of first cancers and their incidence, according to intervention group, at the five most common sites and at all other sites combined. The participants who received alpha-tocopherol had fewer cancers of the prostate and colorectum than those who did not receive alpha-tocopherol, whereas more cancers of the bladder, stomach, and other sites combined were diagnosed in the participants who received this supplement. The participants who received beta carotene had more cancers of the prostate and stomach and fewer cases of other cancers than those who did not receive beta carotene. There were two cancers other than lung cancer (melanoma and astrocytoma) among the participants who were excluded after randomization.

Mortality

Altogether, 3570 deaths occurred during the trial. Among participants receiving alpha-tocopherol, there were fewer deaths caused by ischemic heart disease and ischemic stroke than there were among those who did not receive alpha-tocopherol, but more deaths due to cancers other than lung cancer or due to hemorrhagic stroke (Fig. 3). Overall mortality was 2 percent higher in the alpha-tocopherol groups than in the groups that received no alpha-tocopherol (95 percent confidence interval, -5 to 9 percent; $P = 0.6$). There were more deaths due to lung cancer, ischemic heart disease, and ischemic and hemorrhagic stroke among recipients of beta carotene (Fig. 3). Overall mortality was 8 percent higher among the participants who received beta carotene than among those not given beta carotene (95 percent confidence interval, 1 to 16 percent; $P = 0.02$).

DISCUSSION

Our results provide no evidence of a beneficial effect of supplemental vitamin E (alpha-tocopherol) or beta carotene in terms of the prevention of lung cancer. In fact, men who received beta carotene were found to have lung cancer more frequently than those who did not receive beta carotene. These results are sufficiently strong that it is highly unlikely that 20 mg of beta carotene per day confers any material protective effect against lung cancer among smokers over a period of about six years.

The lack of reduction in the incidence of lung cancer among the men given supplemental beta carotene may be explained by bias, an inadequate duration of supplementation, the use of the wrong dose, or an inappropriate study population. Bias can be discount-

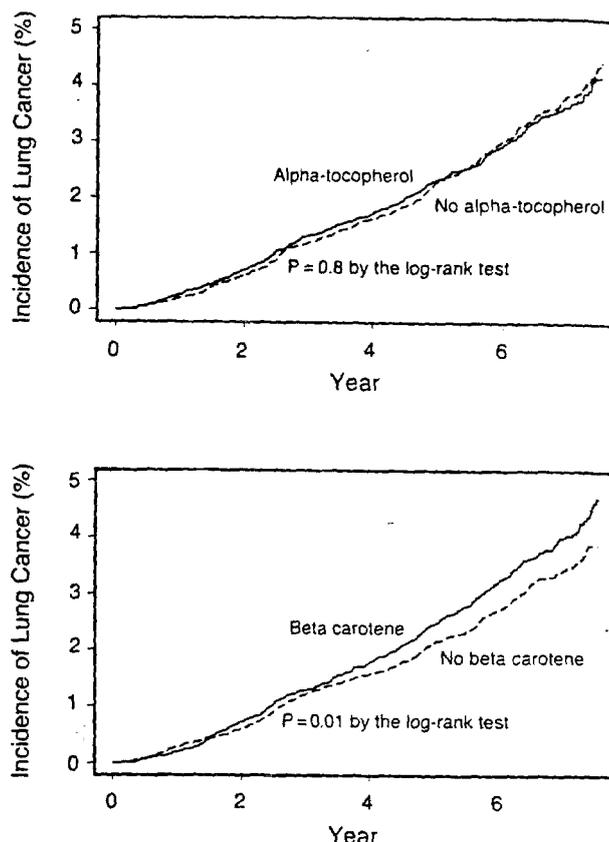


Figure 1. Kaplan-Meier Curves for the Cumulative Incidence of Lung Cancer among Participants Who Received Alpha-Tocopherol Supplements and Those Who Did Not (Upper Panel) and among Participants Who Received Beta Carotene Supplements and Those Who Did Not (Lower Panel).

Data are shown only through 7½ years of follow-up because of the small numbers of participants beyond that time.

ed, since the intervention groups were balanced in terms of all the relevant characteristics we studied. The study population was large, and case ascertainment was essentially complete. In addition, the men in the various intervention groups sought treatment at virtually the same time for lung cancer, as measured by the length of time from diagnosis to death, and even for such minor problems as yellowing of the skin. Moreover, analyses of the incidence of lung cancer that were restricted to participants who did not report yellowing of the skin or to cases diagnosed on the chest film obtained at the study examination yielded results similar to those for the entire cohort; this similarity of results essentially rules out bias caused by self-selection or by differences in diagnostic procedures.

It is plausible that the intervention period was too short to inhibit the development of cancers resulting from a lifetime of exposure to cigarette smoke and other carcinogens. Beta carotene may not be the active cancer-inhibiting component of the fruits and vegetables identified as protective in observational studies, or the intake of beta carotene may be only a nonspecific marker for lifestyles that protect against cancer.

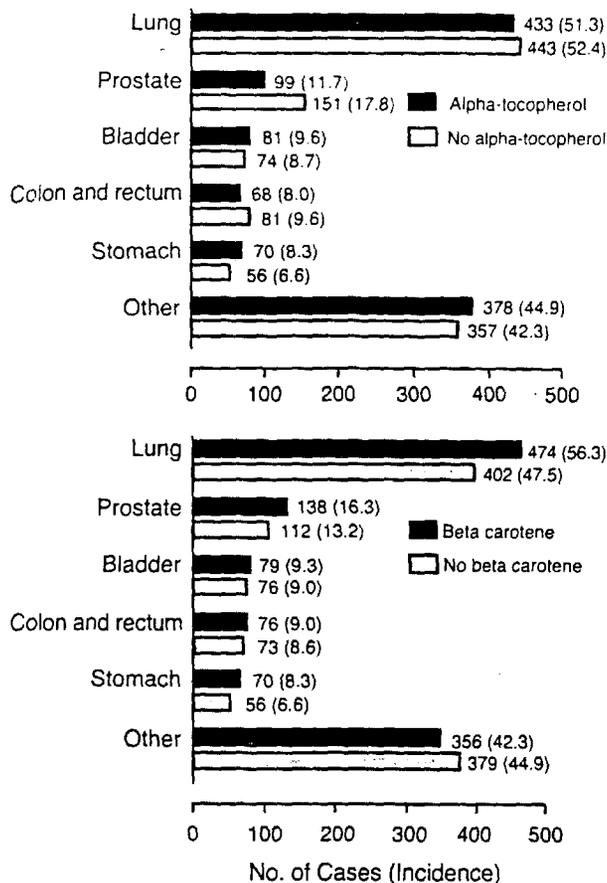


Figure 2. Number and Incidence (per 10,000 Person-Years) of Cancers, According to Site, among Participants Who Received Alpha-Tocopherol Supplements and Those Who Did Not (Upper Panel) and among Participants Who Received Beta Carotene Supplements and Those Who Did Not (Lower Panel).

Although it is conceivable that the dose we used was too low, this seems unlikely, since that dose exceeded by many times the dietary intake of beta carotene in epidemiologic studies that found a strong inverse association between the consumption of carotene-rich foods and the incidence of lung cancer.^{10,11} Finally, study findings regarded as showing supplementation to be beneficial or harmful may occur by chance.

The lack of benefit of beta carotene is particularly surprising given the substantial and consistent epidemiologic evidence of an association between a higher beta carotene intake and a lower incidence of lung cancer,¹¹⁻¹⁵ including the results of the cohort-based analysis in this study. Furthermore, a recent large trial in China found a significant reduction in mortality due to cancer among persons whose diets were supplemented daily with the combination of beta carotene (15 mg), alpha-tocopherol (30 mg), and selenium (50 µg) for 5¼ years.¹⁶

We also observed no beneficial effect of alpha-tocopherol on the incidence of lung cancer or on mortality due to this disease. At the start of the trial, the a priori evidence that alpha-tocopherol prevented

lung cancer was less substantial than that for beta carotene, and since then little additional evidence has been accumulated.¹⁷⁻¹⁹ Possible explanations for the lack of effect are similar to those for beta carotene, although the relatively low dose and the short duration of supplementation merit greater consideration in the case of alpha-tocopherol. Furthermore, we observed no interaction between alpha-tocopherol and beta carotene in their effect on the incidence of lung cancer.

The apparently protective effect of alpha-tocopherol against prostate cancer and, to a lesser extent, against colorectal cancer is intriguing. Although there was little or no evidence linking alpha-tocopherol to the incidence of cancers at either of these sites when the trial started, limited observational data consistent with these findings have now been published.²⁰⁻²² Although these results are suggestive, many comparisons with these two agents were made in these analyses, increasing the possibility that some of the apparent benefits may have occurred by chance alone. Additional data from the continued follow-up

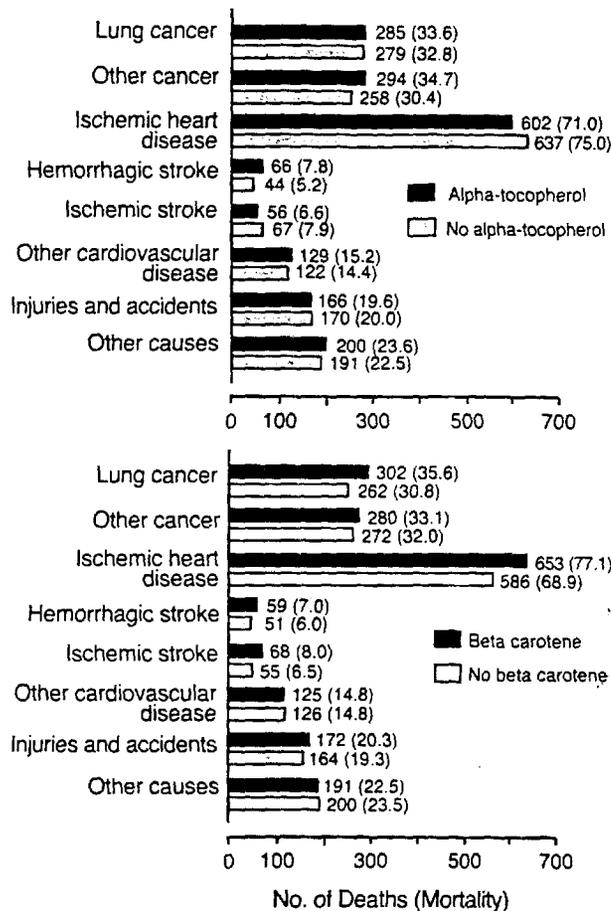


Figure 3. Deaths and Mortality Rates (per 10,000 Person-Years), According to Cause of Death, among Participants Who Received Alpha-Tocopherol Supplements and Those Who Did Not (Upper Panel) and among Participants Who Received Beta Carotene Supplements and Those Who Did Not (Lower Panel).

The cause of death was unknown for four participants.

of the participants in this and other intervention studies are needed before conclusions can be drawn about the role of alpha-tocopherol in preventing these cancers.

Our results raise the possibility that supplementation with beta carotene may be harmful in smokers. The higher mortality due to ischemic heart disease and lung cancer among the beta carotene recipients requires more detailed analysis, and information from other studies is also needed. We are aware of no other data at this time, however, that suggest harmful effects of beta carotene, whereas there are data indicating benefit.^{16,23} Furthermore, there are no known or described mechanisms of toxic effects of beta carotene, no data from studies in animals suggesting beta carotene toxicity, and no evidence of serious toxic effects of this substance in humans.²⁴ In the light of all the data available, an adverse effect of beta carotene seems unlikely; in spite of its formal statistical significance, therefore, this finding may well be due to chance.

The higher mortality due to hemorrhagic stroke among the participants receiving alpha-tocopherol also requires careful review. Alpha-tocopherol has effects on platelet function^{25,26} that could conceivably underlie this observation.

In summary, we found no overall reduction in the incidence of lung cancer or in mortality due to this disease among male smokers who received dietary supplementation with alpha-tocopherol, beta carotene, or both in this large trial in Finland. The results of this study raise the possibility that these substances may have harmful as well as beneficial effects. Longer observation of the participants in this trial and data from other studies of people at normal risk^{27,28} or high risk²⁹ for cancer will be required to determine the full spectrum of effects of these agents. Public health recommendations about supplementation with these micronutrients would be premature at this time.

APPENDIX

The participants in the Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group were as follows: *Principal investigators* — O.P. Heinonen and J.K. Huttunen, National Public Health Institute, Helsinki, Finland, and D. Albanes, National Cancer Institute, Bethesda, Md.; *Senior investigators* — J. Haapakoski, J. Palmgren, P. Pietinen, J. Pikkarainen, M. Rautalahti, and J. Virtamo, National Public Health Institute, and B.K. Edwards, P. Greenwald, A.M. Hartman, and P.R. Taylor, National Cancer Institute; *Investigators* — J. Haukka, P. Järvinen, N. Malila, and S. Rapola, National Public Health Institute; *Data management* — P. Jokinen, A. Karjalainen, J. Lauronen, J. Mutikainen, M. Sarjakoski, A. Suorsa, M. Tiainen, and M. Verkasalo, National Public Health Institute, and M. Barrett, Information Management Services, Silver Spring, Md.; *Laboratory measurements* — G. Alfthan, C. Ehnholm, C.G. Gref, and J. Sundvall, National Public Health Institute; *Nutritionists* — E. Haapa, M.L. Ovaskainen, M. Palva-Alhola, and E. Roos, National Public Health Institute; *Cancer Registry* — E. Pukkala and L. Teppo, Finnish Cancer Registry, Helsinki; *Data and Safety Monitoring Committee* — H. Frick (chairman), University of Helsinki, Helsinki, A. Pasternack, University of Tampere, Tampere, Finland, B.W. Brown, Jr., Stanford University, Palo Alto, Calif., and D.L. DeMets, University of Wisconsin, Madison; *Collaborating hospitals in Finland* — Coordinators: K. Kokkola, National Public

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North Georgia

SUE ANNE BRENNER, M.D.

EXHIBIT E

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Dietary Antioxidants and the Risk of Lung Cancer

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Olli P. Heinonen,² Demetrios Albanes,⁴ Marina Heinonen,⁵ Eero Pukkala,⁶ and
Lytty Teppo⁸

The relation between the intake of retinoids, carotenoids, vitamin E, vitamin C, and selenium and the subsequent risk of lung cancer was studied among 4,538 initially cancer-free Finnish men aged 20-69 years. During a follow-up of 20 years beginning in 1966-1972, 117 lung cancer cases were diagnosed. Inverse gradients were observed between the intake of carotenoids, vitamin E, and vitamin C and the incidence of lung cancer among nonsmokers, for whom the age-adjusted relative risks of lung cancer in the lowest tertile of intake compared with that in the highest tertile were 2.5 (p value for trend = 0.04), 3.1 ($p = 0.12$), and 3.1 ($p < 0.01$) for the three intakes, respectively. Adjustment for various potential confounding factors did not materially alter the results, and the associations did not seem to be due to preclinical cancer. In the total cohort, there was an inverse association between intake of margarine and fruits and risk of lung cancer. The relative risk of lung cancer for the lowest compared with the highest tertile of margarine intake was 4.0 ($p < 0.001$), and that for fruits was 1.8 ($p = 0.01$). These associations persisted after adjustment for the micronutrient intakes and were stronger among nonsmokers. The results suggest that carotenoids, vitamin E, and vitamin C may be protective against lung cancer among nonsmokers. Food sources rich in these micronutrients may also have other constituents with independent protective effects against lung cancer. *Am J Epidemiol* 1991;134:471-9.

diet; longitudinal studies; lung neoplasms; selenium; vitamin A; vitamin C; vitamin E

It has been hypothesized that vitamin A has anticancer effects by controlling cellular differentiation and growth and that its precursor beta-carotene, vitamin C, vitamin E, and selenium have anticancer effects because of their antioxidant and other prop-

erties (1, 2). Some, but not all, epidemiologic studies on the association between these micronutrients and the occurrence of lung cancer are in line with these hypotheses (2-7).

The most reliable evidence of an association between these micronutrients and cancer comes from cohort studies. In addition, these have given conflicting results. Most cohort studies on the intake of carotene or of foodstuffs rich in carotene as well as follow-up studies based on circulating beta-carotene levels support the hypothesis that carotene protects against lung cancer (7). In contrast, most studies based either on dietary intakes or on serum retinol levels have reported no protective effect for retinol (4).

There is relatively little information from cohort studies on the association between lung cancer and vitamins C and E and selenium. The findings concerning the relation between vitamin C and lung cancer are in-

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Abbreviation: CI, confidence interval.

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consistent. An inverse association between the occurrence of lung cancer and the intake of either vitamin C or fruits rich in vitamin C has been reported in some, but not all, studies (5). The results of cohort studies on vitamin E and lung cancer risk are also somewhat discrepant. A significant protective effect of vitamin E against lung cancer was found in one of the serum studies, but other studies have shown only weak or no associations (6). Similarly, studies based on prediagnostic serum selenium samples have reported a significant inverse association, a nonsignificant inverse association, or a nonsignificant positive association (4).

Some of the studies concerning intakes of various micronutrients and lung cancer are based on food frequency questionnaires which only partially cover the total diet of the subjects. For examination of the relative importances of micronutrients and their main food sources, however, comprehensive dietary intake data are needed. Using dietary intake data based on dietary history interviews, we studied the simultaneous relation between dietary intakes of major carotenoids, retinoids, vitamin C, vitamin E, and selenium, and that of the major food sources of these micronutrients and the subsequent development of lung cancer in a cohort of Finnish men followed up for 20 years.

MATERIALS AND METHODS

During 1966-1972, the Mobile Health Clinic of the Social Insurance Institution carried out multiphasic screening examinations in several regions of Finland (8). Altogether, 62,440 men and women aged 15 years or older were invited to take part in the study; the participation rate was 82.5 percent. As part of the main study, dietary histories were obtained from a random subsample of 10,054 participants (9, 10). This study covers the 4,538 men aged 20-69 years from whom dietary histories were obtained and who had never had cancer.

Food consumption was estimated at the baseline examination by the dietary history method covering the total habitual diet of

the subjects during the previous year (9, 11). The amounts of retinoids, carotenoids, and various tocopherol and tocotrienol compounds and the content of selenium in the diet, were based on analyses of Finnish foods (12-15). The vitamin E activities of different tocopherols and tocotrienols in alpha-tocopherol equivalents were estimated by using the factors in the paper by McLaughlin and Weinrauch (16). The intake of preformed vitamin A and the vitamin A activity of different provitamin A carotenoids were calculated in retinol equivalents according to the Food and Nutrition Board (17). The vitamin C content and that of other nutrients in food items were derived from several sources, mainly from international food composition tables. Energy intake was calculated on the basis of the amounts of protein, fat, and available carbohydrate consumed. The nutrient intakes represent amounts in raw foodstuffs. Because few men (1.4 percent) used vitamin supplements, this association with lung cancer could not be studied.

In this cohort, vegetables provided on average 70 percent and dairy products 26 percent of dietary carotenoids (i.e., provitamin A carotenoids). Dairy products and live supplied the largest proportions of retinoid (i.e., preformed vitamin A), with average proportions of 53 and 35 percent, respectively. On average, 33 percent of vitamin E was derived from potatoes, 36 percent from fruits and berries, 18 percent from vegetables, and 12 percent from dairy products. Cereals provided 32 percent of the dietary vitamin E, dairy products provided 20 percent, and margarine provided 13 percent. On average, 85 percent of the selenium was supplied by animal products (i.e., meat products, fish, eggs, and dairy products), and 12 percent by cereals.

The short- and long-term repeatability of the daily consumption of the per capita foodstuffs and micronutrients were estimated by repeating the dietary interviews 8 months and 4-7 years after the initial interviews. Overall, the short-term repeatability was high, with the intraclass correlation coefficient (r) ranging from 0.47 to 0.91.

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- and long-term repeatabilities consumption of the pertinent nd onutrients were esti- eating the dietary interviews 4- nd 4-7 years after the initial verall, the short-term repeat- igh, with the intraclass correla- nt (r) ranging from 0.47 to 0.83

for the food groups and, with one exception, from 0.53 to 0.78 for the nutrients. The repeatability of retinoid intake was low ($r = 0.16$), mainly as a result of the low repeatability of liver intake. The coefficient of long-term repeatability, with one exception, ranged from 0.28 to 0.55 for the food groups and from 0.29 to 0.58 for the nutrients. Although the short-term repeatability of margarine intake was high ($r = 0.83$), the long-term repeatability was very low ($r = 0.10$), mainly because of a rapid increase in margarine use in Finland during the follow-up.

All participants completed a premailed questionnaire checked at the baseline examination. The questionnaire yielded information about residence, social class, occupation, use of drugs (e.g., vitamin supplements), and smoking. The classification of social class was made on the basis of occupational esteem (18). Subjects were classified according to smoking status as never smokers, ex-smokers, and current smokers. The two first classes were also combined to form a class of nonsmokers. Body height and weight were measured at the baseline examination, and the body mass index (weight (kg)/height (m²)) was calculated.

Information concerning subsequent cancer incidence, available through the nationwide Finnish Cancer Registry (19), was linked to the dietary data in order to study the association between the intake of various micronutrients and the incidence of lung cancer. During the 20-year follow-up period 1967-1986, 117 lung cancer cases (*International Classification of Diseases, Seventh Revision*, codes 162-163) (20) were diagnosed, including 18 small cell carcinomas, 41 squamous cell carcinomas, 11 adenocarcinomas, and 47 other carcinomas.

The age-adjusted mean levels of several descriptive and potential confounding factors among cancer cases and noncases were estimated using the general linear model (21). The Cox proportional hazards model was used to estimate the association between the dietary factors and risk of lung cancer adjusting for age and other possible confounding factors (22). Relative risks were

computed for tertiles of intake by using the lowest tertile as the reference category. Statistical significances were tested by using the likelihood ratio test based on Cox models. The repeatability of the reported intake of micronutrients and foods was estimated with the intraclass correlation coefficient (23).

RESULTS

The baseline characteristics of lung cancer cases and noncases are presented in table 1. Men who subsequently developed lung cancer were older, thinner, more often current smokers, and from lower social classes than the other men.

The mean daily intake of energy and different food items at the baseline among subsequent lung cancer cases and noncases, adjusted for age, is shown in table 2. Among the cases, the intake of energy was higher but the intakes of vegetables, fruits and berries, and margarine were lower. In addition, the mean daily intakes of various carotenoids were lower, but the intake of retinol was higher among the cases than among controls (table 3). There was a significant inverse gradient between the age-adjusted daily intake of fruits and margarine and incidence of lung cancer; the relative risk between the lowest and highest tertiles of intake of these foods were 1.8 (p value trend = 0.01) and 4.0 ($p < 0.001$), respectively. Adjustment for smoking did not materially alter the results. The micronutrients presented only nonsignificant associations with lung cancer risk.

The possible modifying effects of smoking, social class, and energy or fat intake on the relation between micronutrient intake and occurrence of lung cancer were also studied. The strongest interaction observed with respect to smoking. The adjusted relative risk of lung cancer between tertiles of intake of carotenoids, vitamin C, and vitamin E showed an inverse association among nonsmokers, but not among smokers (table 4). Adjustment for several possible confounding factors (geographic area, social class, body mass index, height, and energy

TABLE 1. Selected characteristics* of the lung cancer cases and noncases: the cancer incidence follow-up of the Finnish Mobile Clinic Health Survey, 1967-1986

Characteristic	Cancer cases (n = 117)			Noncases (n = 4,421)		
	No.	Mean (%)	Standard deviation	No.	Mean (%)	Standard deviation
Age (years)		54.1	9.2		40.4	13.0
Height (cm)		173.3	5.7		172.7	6.7
Weight (kg)		72.2	11.2		75.0	11.1
Body mass index (kg/m ²)		24.0	3.1		25.1	3.3
Smoking						
Never	6	7.1		1,112	25.1	
Former	18	8.2		985	22.5	
Current	93	84.7		2,324	52.4	
Social class†						
I (highest)	4	3.4		174	3.9	
II (median)	47	43.7		2,272	51.3	
III (lowest)	47	42.7		1,355	30.6	
IV (farmers)	19	10.2		619	14.2	

* The means of all the characteristics except age and smoking have been adjusted for both age and smoking. Age has been adjusted for smoking, and smoking has been adjusted for age.

† One noncase with missing data was excluded.

TABLE 2. Age-adjusted mean (\pm standard deviation) daily intake of energy and various foods for cases and noncases: the cancer incidence follow-up of the Finnish Mobile Clinic Health Survey, 1967-1986

Foods (g/day)	Cases (n = 117)	Noncases (n = 4,421)
Energy (kcal/day)	3,093 \pm 1,016	3,000 \pm 925
Cereals	334 \pm 152	335 \pm 147
Potatoes	282 \pm 149	275 \pm 141
Vegetables	94 \pm 71	103 \pm 73
Green	47 \pm 36	48 \pm 36
Yellow or red	32 \pm 36	36 \pm 40
Fruits and berries	120 \pm 94	144 \pm 117
Fruits	71 \pm 68	92 \pm 96
Berries	14 \pm 17	14 \pm 19
Margarine	5 \pm 8	8 \pm 14
Dairy products	1,165 \pm 574	1,062 \pm 487
Milk	912 \pm 559	848 \pm 471
Butter	82 \pm 31	54 \pm 31
Meat and meat products	189 \pm 123	176 \pm 112
Liver	4 \pm 8	4 \pm 6
Fish	43 \pm 52	39 \pm 49
Eggs	36 \pm 27	37 \pm 32

and fat intakes) did not materially change the results. When the cancer cases diagnosed during the first 2 years of follow-up were excluded from the analyses, the results were not notably altered. The relative risk of cancer among nonsmokers in the lowest tertile of intake compared with that in the highest

was 2.0 (95 percent confidence interval (0.5-7.7) for carotenoids, 2.6 (95 percent 0.7-9.1) for vitamin E, and 2.7 (95 percent CI 0.7-10.0) for vitamin C.

Few other interactions were observed. There was an interaction between vitamin C intake and age in that the relative risk of lung cancer between the lowest and highest tertiles of intake was 1.8 (95 percent CI 0.3-7.7) among men under age 50 years and among the older men. There was also a suggestive nonsignificant interaction between intakes of energy and micronutrients. The relative risk of lung cancer between low and high intakes of various micronutrients ranged from 1.6 to 2.4 in the lowest tertile and from 0.5 to 0.7 in the highest tertile of energy intake.

Because several of the studied micronutrients had common dietary sources and correlated intakes, with the correlation coefficients ranging from 0.14 to 0.58, the association between each micronutrient and lung cancer was also examined after adjustment for intakes of the other micronutrients. This additional adjustment did not change the results appreciably, suggesting independent effects of carotenoids, vitamin E, and vitamin C on lung cancer risk among nonsmokers. The age-adjusted relative risk

the cancer incidence follow-

Noncases (n = 4,421)	
Mean (%)	Standard deviation
40.4	13.0
172.7	6.7
75.0	11.1
25.1	3.3
25.1	
22.5	
52.4	
3.9	
51.3	
30.6	
14.2	

age and smoking. Age has been

confidence interval (CI)
oids, 2.6 (95 percent CI
n[†] and 2.7 (95 percent
and C.

actions were observed.
relation between vitamin
that the relative risk of
in the lowest and highest
is 1.8 (95 percent CI 0.9-
older age 50 years and 1.0
men. There was also a
significant interaction be-
tween energy and micronutrients.
lung cancer between low
intakes of various micronutrients
was 2.4 in the lowest tertile
and 1.7 in the highest tertile of

of the studied micronutri-
diary sources and cor-
relate the correlation coeffi-
cient 0.14 to 0.58, the asso-
ciation between each micronutrient and
lung cancer was examined after adjust-
ment for the other micronutrients.
This adjustment did not change
substantially, suggesting independ-
ence. For retinoids, vitamin E, and
selenium, the cancer risk among non-
smokers was 2.4 (95 percent CI 1.4-
4.1) and 2.7 (95 percent CI 1.7-
4.1) for the lowest and highest tertiles,
respectively. For carotenoids, the
cancer risk among nonsmokers was 3.7
(95 percent CI 1.6-8.3) for the lowest
and highest tertiles, respectively. For
vitamin E, the cancer risk among non-
smokers was 1.8 (95 percent CI 0.9-
3.4) for the lowest and highest tertiles,
respectively. For vitamin C, the cancer
risk among nonsmokers was 1.8 (95 per-
cent CI 0.9-3.4) for the lowest and high-
est tertiles, respectively. For selenium,
the cancer risk among nonsmokers was 1.8
(95 percent CI 0.9-3.4) for the lowest
and highest tertiles, respectively. For
retinoids, the cancer risk among smokers
was 0.7 (95 percent CI 0.4-1.1) for the
lowest and highest tertiles, respectively.
For carotenoids, the cancer risk among
smokers was 0.7 (95 percent CI 0.4-1.1)
for the lowest and highest tertiles, respec-
tively. For vitamin E, the cancer risk
among smokers was 0.7 (95 percent CI
0.4-1.1) for the lowest and highest tertiles,
respectively. For vitamin C, the cancer
risk among smokers was 0.7 (95 percent
CI 0.4-1.1) for the lowest and highest
tertiles, respectively. For selenium, the
cancer risk among smokers was 0.7 (95
percent CI 0.4-1.1) for the lowest and
highest tertiles, respectively.

TABLE 3. Age-adjusted mean (\pm standard deviation) daily intake of different micronutrients for cases and noncases: the cancer incidence follow-up of the Finnish Mobile Clinic Health Survey, 1967-1986

Nutrient	Cases (n = 117)	Noncases (n = 4,421)
Total vitamin A (μ g) (RE)*	1,826 \pm 1,515	1,687 \pm 955
Retinoids (μ g) (RE)	1,571 \pm 1,463	1,395 \pm 845
Carotenoids (μ g) (RE)	255 \pm 257	292 \pm 301
Beta-carotene (μ g)	1,481 \pm 1,488	1,691 \pm 1,737
Alpha-carotene (μ g)	51 \pm 96	68 \pm 116
Gamma-carotene (μ g)	39 \pm 48	40 \pm 50
Lycopene (μ g)	684 \pm 850	718 \pm 895
Lutein and zeaxanthine (μ g)	1,111 \pm 484	1,160 \pm 460
Vitamin E (mg)	8.5 \pm 3.5	8.5 \pm 3.8
Alpha-tocopherol (mg)	7.1 \pm 2.9	7.2 \pm 3.2
Beta-tocopherol (mg)	0.7 \pm 0.3	0.7 \pm 0.4
Gamma-tocopherol (mg)	2.2 \pm 2.7	2.8 \pm 4.2
Delta-tocopherol (mg)	0.4 \pm 0.7	0.6 \pm 1.3
Vitamin C (mg)	81 \pm 36	83 \pm 36
Selenium (μ g)	29 \pm 19	28 \pm 17

* RE, retinol equivalents.

TABLE 4. Age-adjusted relative risk* of lung cancer between tertiles of intake of various micronutrients by smoking status: the cancer incidence follow-up of the Finnish Mobile Clinic Health Survey, 1967-1986

Smoking status and tertile	Micronutrient				
	Retinoids (RE)†	Carotenoids (RE)	Vitamin E	Vitamin C	Selenium
Nonsmokers (n = 2,121)					
Highest	1.0	1.0	1.0	1.0	1.0
Middle	1.40	3.60	1.90	2.36	1.13
Lowest	1.47	2.50	3.06	3.11	1.83
p value (trend)	0.72	0.04	0.12	<0.01	0.63
Smokers (n = 2,417)					
Highest	1.0	1.0	1.0	1.0	1.0
Middle	0.95	0.99	0.78	0.93	0.64
Lowest	0.73	1.08	0.80	0.81	0.83
p value (trend)	0.08	0.91	0.58	0.36	0.63

* Based on the Cox model including age and an interaction term between smoking status and the micronutrient.

† Retinol equivalents.

lung cancer for men with low (tertile) intakes of all three micronutrients compared with men with no intake in the lowest tertile was 3.7 (95 percent CI 0.9-16.7) among nonsmokers and 0.7 among current smokers.

An inverse association was observed between margarine intake and the incidence of lung cancer among both smokers and nonsmokers (table 5). A similar association was also observed between lung cancer and intakes of foods from plant sources rich in micronutrients, especially fruits and berries and potatoes, among nonsmokers, but not among smokers. The overall association between intake of vegetables and cancer risk

was not significant, but a relatively strong relation was observed with regard to intake of yellow and red vegetables, with a relative risk of 2.6 (95 percent CI 0.8-8.3) between the lowest and highest tertiles of intake among nonsmokers. The corresponding risks for green and other vegetables were 1.1 (95 percent CI 0.7-5.6) and 0.9 (95 percent CI 0.3-2.4), respectively. No similar associations were observed for foods from animal sources rich in these micronutrients (dairy products, meat products, fish, and eggs). These results were not changed after adjustment for various confounding factors (age, sex, class, geographic area, energy and fat

TABLE 5. Age-adjusted relative risk of lung cancer between tertiles of intake of different foods in strata of smoking status*: the cancer incidence follow-up of the Finnish Mobile Clinic Health Survey, 1967-1984

Foods	Nonsmokers (tertile) (n = 2,121)			p value for trend	Smokers (tertile) (n = 2,417)			p value for trend
	Highest	Middle	Lowest		Highest	Middle	Lowest	
Cereals	1.0	2.25	2.50	0.51	1.0	1.04	1.25	0.41
Potatoes	1.0	1.17	2.08	0.06	1.0	0.89	0.81	0.38
Vegetables	1.0	0.91	1.44	0.12	1.0	1.13	0.98	0.81
Fruits and berries	1.0	2.92	7.69	<0.001	1.0	0.82	0.98	0.89
Margarine	1.0	8.75	12.50	0.03	1.0	1.88	2.50	0.03
Dairy products	1.0	0.27	0.46	0.35	1.0	0.77	0.88	0.24
Meat products	1.0	0.72	0.88	0.74	1.0	0.71	0.75	0.47
Fish	1.0	0.64	0.50	0.75	1.0	1.01	0.50	0.86
Eggs	1.0	0.52	1.37	0.57	1.0	0.81	0.95	0.85

* Cox model including age and an interaction term between the foods and smoking status

takes, body mass index, and height) or for all foodstuffs other than the one under consideration.

To shed more light upon the nature of the observed associations between food intake and risk of lung cancer, we included the micronutrients and the foodstuffs that were their richest sources in the regression model simultaneously. The relative risk of lung cancer between the lowest and highest tertiles of intake of fruits and berries was 8.1 (95 percent CI 1.8-37.2) among nonsmokers and 1.1 (95 percent CI 0.6-2.1) among smokers, and the corresponding risks for margarine were 16.2 (95 percent CI 2.1-126.8) and 3.1 (95 percent CI 1.5-6.1), respectively.

DISCUSSION

In agreement with several previous studies (7), this study demonstrated an inverse association between lung cancer incidence and intakes of carotenoids and of red, yellow, and green vegetables. This association was apparent among nonsmokers, but not among smokers. Some previous studies have reported a similar association only among persons with low exposure to cigarette smoke, i.e., nonsmokers, ex-smokers, and light smokers (24-28). These findings support the hypothesis that the amount of carotene in common diets is not sufficient to provide a defense strong enough to protect against heavy exposure to cigarette smoke.

It should be noted that the intake of carotenoid sources was much lower in Finland than in other countries during the 1960s and 1970s (29) and that the intake among smokers in this study was especially low. On the other hand, an inverse association between intake of foodstuffs rich in carotenoids and risk of lung cancer has also been reported among current smokers, including heavy smokers (30-32). The possible modifying effect of smoking on the association between carotene intake and lung cancer risk may thus vary, depending on other, still unknown conditions.

The intake of preformed vitamin A was not related to lung cancer risk in this study. This finding is consistent with most, but not all, previous studies (4). Furthermore, in some exceptions (33), little association has been reported between the intake of vitamin A supplement and cancer risk (26, 28). The lack of association in this study may, however, also arise from the fact that intake of retinoids may be inaccurate because of unreliable information about intake of food, the richest source of retinoids.

We observed an inverse gradient among nonsmokers between the occurrence of lung cancer and the intake of vitamin C as well as with the intake of fruits, potatoes, and vegetables; together these accounted for about 80 percent of the entire vitamin C intake in the study population. Several prior studies have reported similar results, but divergent results have also been published (5). In

9 different foods in strata
death Survey, 1967-1986

smokers (n=2417)

Strata	Lowest	p value for trend
4	1.25	0.41
3	0.81	0.38
2	0.98	0.81
1	0.98	0.89
0	2.50	0.03
7	0.88	0.24
1	0.75	0.47
1	0.50	0.86
1	0.95	0.85

that the intake of carotenoids was lower in Finland than in the United States in the 1960s and 1970s (26). The association between carotenoid intake and lung cancer risk among smokers was weak. On the other hand, the association between carotenoid intake and lung cancer risk was strong in nonsmokers. The possible modifying effect of smoking on the association between carotenoid intake and lung cancer risk may be due to other, still un-

known factors. For example, the intake of vitamin A was lower in Finland than in the United States (27). The association between vitamin A intake and lung cancer risk in this study was weak, consistent with most, but not all, previous studies (4). Furthermore, with the exception of the study by Selman et al. (3), little association has been reported between the intake of vitamin A and lung cancer risk (26, 28, 34). The association between carotenoid intake and lung cancer risk in this study may be due to the fact that intake of carotenoids was more accurate because of the use of food frequency questionnaires on about intake of liver, fish, and carotenoids.

The association between carotenoid intake and lung cancer risk was weak in this study. The inverse gradient among nonsmokers between the occurrence of lung cancer and the intake of vitamin C as well as the intake of fruits, potatoes, and other foods that are rich in these nutrients accounted for over 50% of the total variance in lifetime vitamin C intake of nonsmokers.

Several prior studies have reported similar results, but divergent conclusions have been published (5). In in-

ternational comparisons, the intake of vitamin C was rather low in Finland (29), and our results therefore support the hypothesis that vitamin C may have a protective effect in populations with a relatively low intake of this vitamin (24).

In this study, we found a strong inverse association between margarine intake and risk of lung cancer among both smokers and nonsmokers. A significant correlation between margarine intake and serum alpha-tocopherol level has previously been reported in this study cohort (35), raising the possibility that the protective effect of margarine might derive from vitamin E. However, an inverse association between serum vitamin E (36) and dietary vitamin E and risk of lung cancer was observed only among nonsmokers in this study population. Only some of the previous studies on the association between dietary or serum vitamin E and risk of lung cancer have found an inverse association (6), suggesting that vitamin E may exert its protective effect against cancer only in certain circumstances. The strong association between the intake of margarine and reduced risk of cancer might also be due to factors other than vitamin E. In particular, people consuming margarine at the time of the study baseline may have been more health conscious than others, and they may therefore have been exposed to fewer lung cancer risk factors. The fact that the long-term repeatability of margarine intake was low in this cohort supports this hypothesis rather than a direct association between some substances of margarine and lung cancer risk. Diets low in margarine may also contain high levels of butter and other fats, which may possibly increase lung cancer risk (37). However, in this study the association between margarine intake and lung cancer occurrence was not due to an interaction with or confounding by ingestion of animal fat.

The independent associations observed between the intakes of carotenoids, vitamin C, and vitamin E and incidence of lung cancer among nonsmokers persisted after adjustment for several confounders, including smoking status and intake of different nutritional factors

such as fats and energy. It is nevertheless possible that the associations observed were caused by differences in factors not controlled for in the analyses. One such factor is alcohol consumption. Our results suggest that vitamin C cannot fully account for the anticancer effect observed for its main food sources (e.g., fruits and berries and potatoes). Similarly, the diminished risk of lung cancer among margarine users did not appear to be entirely due to vitamin E intake.

There are several possible explanations for our observation that the known micronutrients in vegetables, fruits, and margarine failed to account fully for the observed strong associations between the consumption of these foodstuffs and cancer risk. Fruits and vegetables contain nonnutritive substances such as terpenes, flavones, and phenols, which may be anticarcinogenic (38). It is also possible that other healthful behaviors associated with high intake of these foodstuffs may reduce risk of lung cancer. An alternative explanation is that the nonsmoking lung cancer cases with low intake of several micronutrients suffered from some kind of poor diet specifically predisposing them to cancer. Finally, it is possible that because of low reliability of the intake estimates of the micronutrients, the adjustment was not effective enough to eliminate the association due to the micronutrients.

We failed to see an inverse association between selenium intake and lung cancer risk in this study, although a strong inverse association has been demonstrated between serum selenium level and lung cancer risk in this cohort of men, which had an unusually low serum selenium level (39). The lack of association may be due to the fact that the reported dietary intake reflects selenium status less well than serum level does (2), especially in Finland where considerable changes in the dietary intake of selenium have recently occurred (40).

The findings of this longitudinal study are based on dietary histories collected up to 2 years prior to the diagnosis of cancer, thereby avoiding the potential influence of disease on either actual or reported intake.

The results did not notably change when the cancer cases occurring during the first years of follow-up were excluded, and it was thus improbable that the observed associations were influenced by the effect of preclinical cancer on dietary recall. However, several points should be made concerning the measurement, analysis, and interpretation of the dietary data presented here. First, the dietary history method has its limitations, and the consequential misclassification of subjects tends to diminish observable associations between dietary exposure and outcome (41). The strength of the associations between micronutrient intake and risk of lung cancer may also be underestimated in this study because dietary habits such as consumption of margarine have changed considerably in Finland during the 20-year follow-up period (42). For example, the consumption of margarine has increased greatly during this period. The problem is compounded by the fact that the micronutrient content of food may have changed over time; most importantly, the selenium content of food has increased gradually during the follow-up period (40, 43). Furthermore, in this study micronutrient intake was calculated solely from food sources, ignoring the contribution of vitamin supplements. Although supplement use was uncommon at the time of the baseline examination, it has increased during the follow-up period (44).

In summary, we found an inverse relation between the dietary intake of carotenoids, vitamin C, and vitamin E and the incidence of lung cancer among nonsmokers. We also found that the intake of foodstuffs rich in vitamins C and E was associated with a reduced risk of lung cancer, but that this could not be fully attributed to the protective effect of the micronutrients. Future studies should focus on the effects of different dietary patterns and modifying factors on the possible association between micronutrients and lung cancer.

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EXHIBIT F

Relations among aging, antioxidant status, and cataract¹⁻³

Allen Taylor, Paul F Jacques, and Esther M Epstein

ABSTRACT Light and oxygen are necessary for the function of the eye. However, when present in excess or in uncontrolled circumstances, they appear to be related, probably causally, to the development of cataract. Compromises of function of the lens and retina with aging are exacerbated by depleted or diminished primary antioxidant reserves, antioxidant enzyme capabilities, and diminished secondary defenses such as proteases. Smoking appears to provide an additional oxidative challenge associated with depletion of antioxidants as well as with enhanced risk for cataract formation. Poor education and lower socioeconomic status are associated with poorer nutrition and are also significantly related to increased risk for these debilities. Optimizing nutrition, including diets rich in fruit and vegetables, may provide the least costly and most practicable means to delay cataract. *Am J Clin Nutr* 1995;62(suppl):1439S-47S.

KEY WORDS Vitamin C, vitamin E, carotenoids, lutein, zeaxanthin, cataract, proteases, antioxidant enzymes

INTRODUCTION

Studies on the etiology of cataract now include intervention trials as well as laboratory and epidemiologic approaches. We review available data regarding associations between antioxidant nutrients and eye lens cataract in humans. For more thorough treatments of this topic, particularly with respect to animal studies, readers are referred to other recent reviews (1-4).

The primary function of the eye lens is to collect and focus light on the retina (Figure 1). To do so it must remain clear throughout life. The lens is located posterior to the cornea and iris and receives nutrition from the aqueous humor. Although the lens appears to be free of structure it is exquisitely designed. A single layer of epithelial cells is found directly under the anterior surface of the collagenous membrane in which it is encapsulated (Figure 2). The epithelial cells at the germinative region divide, migrate posteriorly, and differentiate into lens fibers. The fibers elaborate crystallins, the primary gene products and the predominant proteins of the lens. They also lose their organelles. New cells are formed throughout life but older cells are usually not lost. Instead they are compressed into the center or nucleus of the lens. There is a coincident dehydration of the proteins and the lens itself. Together with modifications of the protein (noted below) and other con-

stituents, these changes result in a less flexible lens with aging.

As the lens ages the proteins are photooxidatively damaged and aggregate and accumulate in lens opacities. Dysfunction of the lens due to opacification is called cataract. The term "age-related cataract" is used to distinguish lens opacification associated with old age from opacification associated with other causes, such as congenital and metabolic disorders (5).

PUBLIC HEALTH ISSUES REGARDING CATARACT

Cataract is one of the major causes of blindness throughout the world (6-8). In the United States, the prevalence of visually significant cataract increases from ~5% at age 65 y to ~40% for persons older than 75 y (9-11). In less developed countries such as India (12), China (13), and Kenya (14), cataracts are more common and develop earlier in life than in more developed countries. For example, cataract with low vision or aphakia (ie, absence of the lens, which is usually the result of cataract extraction) is ~5 times more common by age 60 y in persons in India than in the United States (11, 12). The effect of cataract on impaired vision is much greater in less developed countries, where > 90% of the cases of blindness and visual impairment are found (8, 15-18) and where there is a dearth of ophthalmologists to perform lens extractions (19, 20). Such surgery is routinely successful in restoring sight.

Given both the extent of disability caused by age-related cataract and its costs [\$5 billion/y (21) in the United States], it is urgent that we elucidate causes of cataract and identify strategies to slow the development of this disorder. It is estimated that a delay in cataract formation of ~10 y would reduce the prevalence of visually disabling cataract by ~45% (6). Such a delay would enhance the quality of life for much of the world's older population and substantially reduce both the economic burden due to disability and surgery related to cataract.

CLINICAL FEATURES OF CATARACT

There are several systems for evaluating and grading cataracts. Most of these use an assessment of extent or density and

¹ From the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging at Tufts University, Boston.

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³ Address reprint requests to A Taylor, 711 Washington Street, Boston, MA 02111.

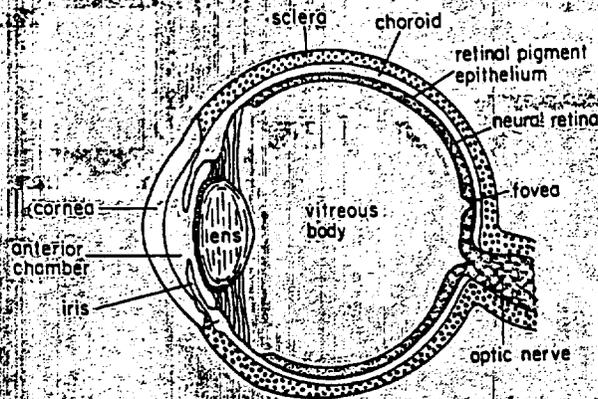


FIGURE 1. Cross section of the eye.

location of the opacity (22). Coloration or brunescence is also quantified, because these diminish visual function and contribute to cataract (23, 24). Usually evaluated are opacities in the posterior, subcapsular, nuclear, cortical, and multiple (mixed) locations (Figure 2 and Table 1).

OXIDATION AND CATARACT FORMATION

The solid mass of the lens is ~98% protein. Because these proteins undergo minimal turnover as the lens ages, they are subject to the chronic stresses of exposure to light and oxygen. Consequently, it is not surprising that these proteins are extensively damaged in aged lenses. Lens opacities develop as the damaged proteins aggregate and precipitate (1). Lipid damage to the fiber cell membrane is also associated with lens opacities (3, 26, 32). Smoking and ultraviolet light, which appear to induce oxidative stress (33-34), are also associated with elevated cataract risk (35-40) as well as with depletion of plasma ascorbate and carotenoid concentrations (33, 41).

In young lenses, damaged proteins are usually maintained at harmless amounts by defense systems. Primary defenses that directly protect the lens against the initial oxidative insult include small molecule antioxidants (eg, vitamins C and E and carotenoids) and antioxidant enzyme systems (eg, superoxide dismutase, catalase, and the glutathione redox cycle) (42-46). The lens also has secondary defense systems, which include proteolytic enzymes that selectively identify and remove dam-

aged or obsolete proteins (1, 47-53). Accumulation of photooxidized (and/or otherwise modified) proteins in older lenses indicates that protective systems are not keeping pace with the insults that damage lens proteins. This occurs in part because similar to bulk proteins, enzymes that compose some of the protective systems are damaged by photooxidation (1, 53, 54). Interactions between the primary and secondary antioxidant defense systems and putative ramifications of these relations on cataract risk are summarized in Figure 3.

ASSOCIATIONS BETWEEN ANTIOXIDANTS AND CATARACT

Many cell-free, in vitro, and animal studies addressed putative roles for antioxidants in maintenance of lens and retina function. These were reviewed recently and inspired the epidemiologic work described below (1, 3).

More than 10 epidemiologic studies examined the associations between cataract and antioxidant nutrients (20, 25-31, 58-60). Seven of the studies were retrospective case-control studies comparing nutrient concentrations in cataract patients with those in similarly aged individuals with clear lenses (25-29, 31, 60). Our ability to interpret data from retrospective studies such as these is limited by the concurrent assessment of lens status and nutrient concentrations. Prior diagnosis of cataract might influence the behavior of subjects, including diet consumed, and it might also bias reporting of usual diet. Three other studies assessed nutrient concentrations, supplement use, or both and then followed subjects with intact lenses for 8 (30, 59) and 5 y (20), respectively. Prospective studies such as these are less prone to bias because assessment of exposure is performed before the outcome is present. These prospective studies did not directly assess lens status, but used cataract extraction, or reported diagnosis of cataract, as a measure of cataract risk. Extraction may not be a good measure of cataract incidence (development of new cataract) because it incorporates components of both incidence and progression in severity of existing cataract. However, extraction is the result of visually disabling cataract and is the endpoint that we wish to prevent. Although Hankinson et al (30) measured nutrient intake over a 4-y period, Knekt et al (59) used only one measure of serum antioxidant status and Seddon et al (20) used only one measure of supplement use. One measure may not provide an accurate assessment of usual, long-term nutrient concentrations, which may be the best nutritional correlate of cataract (61).

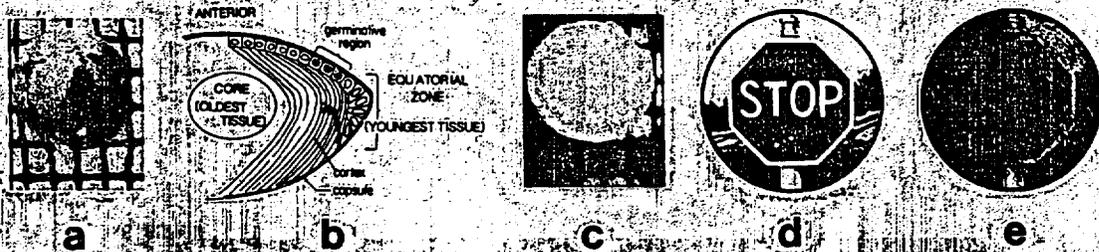


FIGURE 2. Clear and cataractous lens. a) Clear lens allows an unobstructed view of the wire grid placed behind it. b) The structure of the lens. The anterior surface of the lens has a unicellular layer of epithelial cells (youngest tissue). Cells at the anterior equatorial region divide and migrate to the cortex as they are overlaid by less mature cells. These cells produce a majority of the crystallins. As development and maturation proceed, the cells denudecate and elongate. Tissue originally found in the embryonic lens is found in the core (oldest tissue). c) The cataractous lens prohibits viewing the wire grid behind it. d) Artist's view through a clear, uncolored young lens. The image is clear and crisp. e) As in d, the image is partially obscured, and the field is darkened as the result of browning of the lens that accompanies aging.

TABLE 1
Cataract risk ratio: high versus low intake and plasma concentrations for vitamin C¹

Reference ² and nutrient source	Cataract type						
	Mixed	Cortical	PSC ³	Nuclear	Any	Advanced	Extraction
Robertson et al (25); n = 304; supplement	—	—	—	—	—	0.30 ⁴	—
Jacques and Chylack (26); n = 112 ⁵	—	—	—	—	—	—	—
Intake	—	0.27	0.09 ⁴	—	0.25	—	—
Plasma	—	0.27	0.09 ⁴	—	0.29	—	—
Leske et al (27); n = 1380; intake	0.72	0.80	0.73	0.48 ⁴	—	—	—
Jacques et al. ⁶ ; n = 294; supplement	—	0.63	—	0.18 ^{4,7}	0.22 ^{4,7}	0.17 ^{4,7}	—
Mares-Perlman et al (28); n = 1980; supplement	—	1.8 ⁴	—	0.7 ⁴	—	—	—
Mohan et al (29); n = 1990 plasma	1.87 ⁴	—	—	—	—	—	—
Hankinson et al (30); n = 50 828	—	—	—	—	—	—	0.98
Intake	—	—	—	—	—	—	—
Supplement	—	—	—	—	—	—	0.55 ^{4,8}
Vitale et al (31); n = 671; plasma	—	1.01	—	1.31	—	—	—

¹ Risk ratio represents the prevalence odds ratio for all studies except Hankinson et al (30); for Hankinson et al, it represents the incidence rate ratio for cataract extraction.

² Studies reporting estimates of odds ratio or relative risk.

³ Posterior subcapsular.

⁴ Significant ratio, $P < 0.05$.

⁵ Significant ratio, $P < 0.1$.

⁶ Jacques et al, unpublished observations, 1994.

⁷ For persons consuming ascorbate supplements > 10 y, this is the only study in which long-term nutrient intake was assessed (Jacques et al, unpublished); any \geq grade 1, advanced \geq grade 2.

⁸ There was a 45% reduction in rate of cataract surgery for women who used specific vitamin C supplements for ≥ 10 y.

Vitamin C

Vitamin C is probably the most effective and least toxic water-soluble antioxidant identified in mammalian systems (62, 63). Lens concentrations of vitamin C ($\mu\text{mol/L}$) are manyfold higher than those in plasma or other tissues (64). However, vitamin C concentrations are compromised with aging, cataractogenesis, or both (19, 47). Interest in the utility of vitamin C has been fueled by observations that concentrations of this vitamin in eye tissue are related to dietary intake in humans and animals and that concentrations of vitamin C in the lens increased with dietary supplements beyond concentrations achieved in persons who already consumed more than two times the recommended dietary allowance (RDA; 120 mg/d) for vitamin C (19, 47, 64). Although biochemically plausible, there are no data to show that vitamin C induces damage in the lens *in vitro* (54, 65, 66).

Vitamin C was considered in eight published studies (25–31, 60) and one preliminary report (25) and observed to be inversely associated with at least one type of cataract in seven of these studies (Table 1). Jacques and Chylack (26) observed that persons with high plasma vitamin C concentrations ($> 90 \mu\text{mol/L}$) had less than one-third the prevalence of early cataract as did persons with low plasma vitamin C ($< 40 \mu\text{mol/L}$); however, this difference was not statistically significant [risk ratio (RR): 0.29, 95% CI: 0.06, 1.32] after age, sex, race, and history of diabetes were adjusted for. Jacques and Chylack (26) observed similar relations between intake of vitamin C and cataract prevalence. In persons with higher vitamin C intakes ($> 490 \text{ mg/d}$), the prevalence of cataract was 25% of the prevalence in persons with lower intakes ($< 125 \text{ mg/d}$) (RR: 0.25; CI: 0.06, 1.09). In this and subsequent studies, statistically significant decreases in risk of cataract were noted for posterior subcapsular and advanced cataract (58).

This decreased risk of cataract is corroborated by data from other studies. Robertson et al (25) compared case subjects (with cataracts that impaired vision) with age- and sex-matched control subjects who were either free of cataract or had minimal opacities that did not impair vision. The prevalence of cataract in persons who consumed daily vitamin C supplements of $> 300 \text{ mg/d}$ was approximately one-third the prevalence in persons who did not consume vitamin C supplements (RR: 0.30; CI: 0.24, 0.77). Leske et al (27) observed that persons with vitamin C intake in the highest 20% had a 52% lower prevalence for nuclear cataract (RR: 0.48; CI: 0.24, 0.99) compared with persons who had intakes among the lowest 20% after age and sex were controlled for. Weaker inverse associations were noted for other types of cataract. After controlling for nine potential confounders including age, diabetes, smoking, and energy intake, Hankinson et al (30) did not observe an association between total vitamin C intake and rate of cataract surgery (RR: 0.98; CI: 0.72, 1.32) in a large prospective study of women when they compared women with high intakes (median = 705 mg/d) with women with low intakes (median = 70 mg/d). However, Hankinson et al did note that women who consumed vitamin C supplements for ≥ 10 y had a 45% reduced rate of cataract surgery (RR: 0.55; CI: 0.32, 0.96). Age-adjusted analyses (Jacques PF, Taylor A, Hankinson SE, et al, unpublished observations, 1994; Table 1) based on 165 women with high vitamin C intake (mean = 294 mg/d) and 136 women with low vitamin C intake (mean = 77 mg/d) showed that the women who took vitamin C supplements for ≥ 10 y had a $> 70\%$ lower prevalence of early opacities (RR: 0.27; CI 0.11, 0.67) and a $> 80\%$ lower risk of advanced opacities (RR: 0.19; CI: 0.05, 0.80) at any site compared with women who did not use vitamin C supplements.

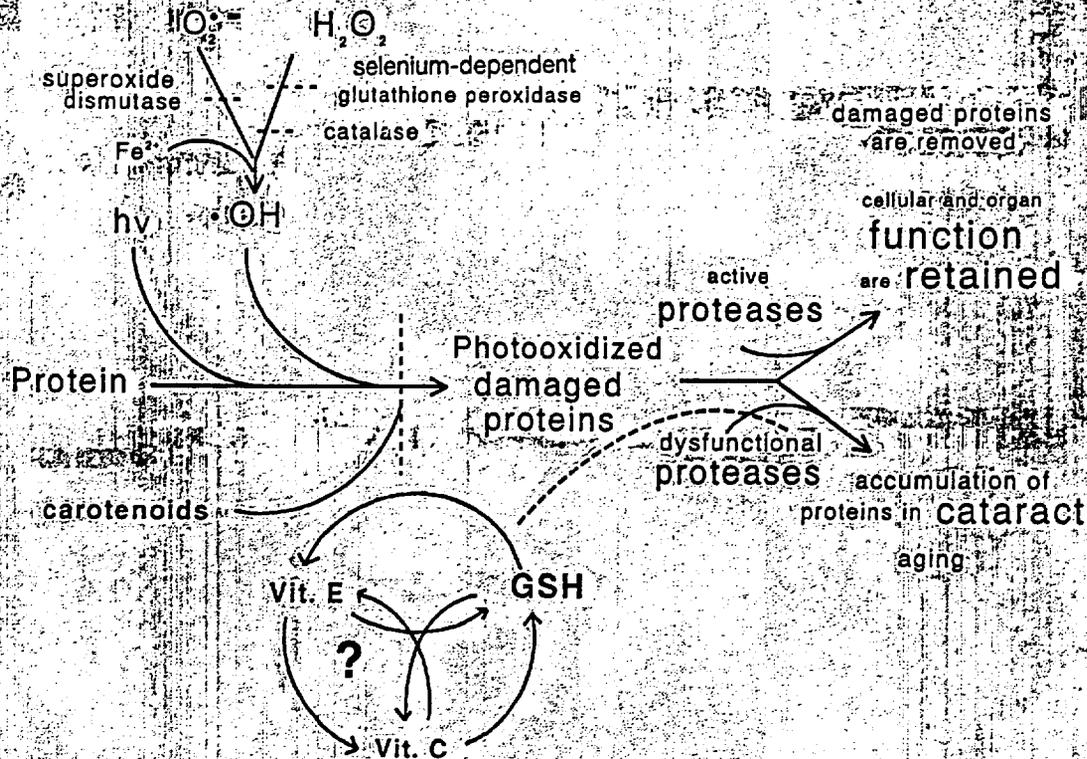


FIGURE 3. Proposed interactions among lens or retinal constituents, oxidants, light, antioxidants, antioxidant enzymes, and proteases. The constituent proteins in the lens are extremely long-lived. In both lens and retina they are subject to damage by light and various forms of oxygen. Such damage is limited by antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase, and peroxidase) that convert active oxygen to less damaging species. Direct protection may also be offered by antioxidants [vitamin C, vitamin E, carotenoids, and glutathione (GSH)]. Amounts of reduced forms of some, but not all, of these molecules are determined by interaction between them and the environment (55-57). With aging, antioxidant concentrations in some eye tissues (lens) are diminished and antioxidant enzymes may be at reduced catalytic competence, resulting in increased damage. Proteins that are obsolete or damaged are reduced to their constituent amino acids if proteolytic activity is sufficient. If not, damaged proteins may accumulate, aggregate, and precipitate in cataracts in the lens or in lipofuscin in the retina. Older tissues frequently show lower proteolytic activity. H_2O_2 , hydrogen peroxide; O_2^- , superoxide radical; $HO\cdot$, hydroxyl radical; the dashed lines indicate sites where antioxidants might protect against damage to proteins and proteases.

Compared with the data noted above, Mares-Perimán et al (28) reported that past use of supplements containing vitamin C was associated with a reduced prevalence of nuclear cataract (RR: 0.7; CI: 0.5, 1.0) but an increased prevalence of cortical cataract (adjusted RR: 1.8; CI: 1.2, 2.9) after age, sex, smoking, and history of heavy alcohol consumption were controlled for (Table 1). Mohan et al (29) also noted an 87% increased prevalence of mixed cataract (RR: 1.87; CI: 1.29, 2.69) with posterior subcapsular and nuclear involvement for each standard deviation increase in plasma vitamin C concentrations. Vitale et al (31) observed that persons with plasma concentrations $> 80 \mu\text{mol/L}$ and $< 60 \mu\text{mol/L}$ had similar prevalences of both nuclear (RR: 1.31; CI: 0.61, 2.39) and cortical (RR: 1.01; CI: 0.45, 2.26) cataract after age, sex, and diabetes were controlled for. Similarly, no differences in cataract prevalence were observed between persons with high ($> 261 \text{ mg/d}$) and low ($< 115 \text{ mg/d}$) vitamin C intakes. One other study observed no association between prevalence of cataract and vitamin C intake (60).

Vitamin E

Vitamin E, a natural, lipid-soluble antioxidant, can inhibit lipid peroxidation (67) and appears to stabilize lens cell mem-

branes (68). Vitamin E may affect ascorbate regeneration (see legend to Figure 3) and also enhances glutathione recycling, perhaps helping to maintain concentrations of reduced glutathione in the lens and aqueous humor (69). The vitamin E content of human retinal pigmented epithelial cells and neural retina increases significantly with aging and it has been suggested that it may be partitioned into the nonpolar lipofuscin (70, 71) with an attendant increase in the vitamin E requirement of the aging retina.

Three studies assessing plasma vitamin E concentrations also reported significant inverse associations with cataract (Table 2). Knekt et al (59) followed a cohort of 141 Finns for 15 y and identified 47 patients admitted to ophthalmologic wards for advanced cataract. They selected two control subjects per patient who were matched for age, sex, and municipality. These investigators reported that persons with serum vitamin E concentrations $> 20 \mu\text{mol/L}$ had about one-half the rate of subsequent cataract surgery (RR: 0.53; CI: 0.24, 1.1) compared with persons with vitamin E concentrations below this concentration. Vitale et al (31) observed the age-, sex-, and diabetes-adjusted prevalence of nuclear cataract to be $\sim 50\%$ less (RR: 0.52; CI: 0.27, 0.99) in persons with plasma vitamin E concentrations $> 29.7 \mu\text{mol/L}$ compared with persons with con-

TABLE 2
Cataract risk ratio: high versus low intake and plasma concentrations for vitamin E¹

Reference ² and nutrient source	Cataract type					
	Mixed	Cortical	PSC ³	Nuclear	Any	Advanced ⁴ Extraction
Robertson et al (25); n = 104; supplement	—	—	—	—	—	0.44 ⁵
Jacques and Chylack (26); n = 112	—	—	—	—	—	—
Intake	—	0.37	0.40	—	0.45	—
Plasma	—	0.84	0.33	—	0.83	—
Supplement	—	—	—	—	0.33 ⁵	—
Leske et al (27); n = 1380; intake	0.58 ⁶	0.59 ⁶	1.18	0.66	—	—
Mares-Perleman et al (28); n = 1980; supplement	—	1.2	—	0.9	—	—
Vitale et al (31); n = 671; plasma	—	0.96	—	0.52 ⁶	—	—
Hankinson et al (30); n = 50 828; intake	—	—	—	—	—	0.96
Knekt et al (59); n = 141; serum	—	—	—	—	—	0.5

¹ Risk ratio represents the prevalence odds ratio for all studies except Hankinson et al (30); for Hankinson et al it represents the incidence rate ratio for cataract extraction.

² Studies reporting estimates of odds ratio or relative risk.

³ Posterior subcapsular.

⁴ Significant ratio, P < 0.05.

⁵ From Jacques and Chylack; unpublished observation.

centrations < 18.6 μmol/L. A similar comparison showed that the prevalence of cortical cataract did not differ between those with high and low plasma vitamin E concentrations (RR: 0.96; CI: 0.52, 0.1.78). Jacques and Chylack (26) also observed the prevalence of posterior subcapsular cataract to be 67% lower (RR: 0.33; CI: 0.03, 4.13) in persons with plasma vitamin E concentrations > 35 μmol/L relative to persons with concentrations < 21 μmol/L after age, sex, race, and diabetes were adjusted for. Prevalence of any early cataract (RR: 0.83; CI: 0.20, 3.40) or cortical cataract (RR: 0.84; CI: 0.20, 3.60) did not differ between those with high and low plasma concentrations. Plasma vitamin E was also inversely associated with prevalence of cataract in a large Italian study after age and sex were adjusted for, but the relation was no longer statistically significant after other factors such as education, sunlight exposure, and family history of cataract were adjusted for (60). One other study observed no association between cataract and plasma vitamin E concentrations (29).

Vitamin E intake was inversely correlated with cataract risk in two studies (Table 3). Robertson et al (25) found among

age- and sex-matched case and control subjects that the prevalence of cataract was 56% lower (RR: 0.44; CI: 0.24, 0.77) in persons who consumed vitamin E supplements (> 400 IU/d) than in persons not consuming supplements. Jacques and Chylack (unpublished observations, 1988) also observed a 67% reduction (RR: 0.33; CI: 0.12, 0.96) in prevalence of cataract for users of vitamin E supplements after age, sex, race, and diabetes were adjusted for. These investigators observed a nonsignificant inverse association when they related total vitamin E intake (combined dietary and supplemental intake) to cataract prevalence (26). Persons with vitamin E intakes > 35.7 mg/d had a 55% lower prevalence of early cataract (RR: 0.45; CI: 0.12, 1.79) than did persons with intakes < 8.4 mg/d. Leske et al (27) also observed that vitamin E intake was inversely associated with prevalence of cataract after controlling for age and sex. Persons with vitamin E intakes in the highest 20% had an ~40% lower prevalence of cortical (RR: 0.59; CI: 0.36, 0.97) and mixed (RR: 0.58; CI: 0.37, 0.93) cataract relative to persons with intakes in the lowest 20%.

TABLE 3
Cataract risk ratio: high versus low intake and plasma concentrations of carotenoids¹

Reference ² and nutrient source	Cataract type					
	Mixed	Cortical	PSC ³	Nuclear	Any	Advanced ⁴ Extraction
Jacques and Chylack (26); n = 112	—	—	—	—	—	—
Intake	—	1.00	0.71	—	0.91	—
Plasma	—	0.14 ⁵	0.18	—	0.18	—
Vitale et al (31); n = 671; plasma	—	0.72	—	1.57	—	—
Hankinson et al (30); n = 50 828; intake	—	—	—	—	—	0.73 ⁵
Knekt et al (59); n = 141; serum	—	—	—	—	—	0.6

¹ Risk ratio represents the prevalence odds ratio for all studies except Hankinson et al (30); for Hankinson et al it represents the incidence rate ratio for cataract extraction.

² Studies reporting estimates of odds ratio or relative risk.

³ Posterior subcapsular.

⁴ Total carotenoids.

⁵ Significant ratio, P < 0.05.

⁶ β-carotene.

In contrast with the studies noted above, Mares-Perlman et al (28) observed only weak, nonsignificant associations between vitamin E supplement use and nuclear (RR: 0.9; CI: 0.6, 1.5) and cortical (RR: 1.2; CI: 0.6, 2.3) cataract. Hankinson et al (30) found no association between vitamin E intake and cataract surgery. Women with high vitamin E intakes (median = 210 mg/d) had a rate of cataract surgery (RR: 0.96; CI: 0.72, 1.29) similar to women with low intakes (median = 3.3 mg/d).

Carotenoids

The carotenoids, similar to vitamin E, are natural, lipid-soluble antioxidants (67). β -Carotene is the best known carotenoid because of its importance as a vitamin A precursor. It exhibits particularly strong antioxidant activity at low partial pressures of oxygen (15 mm Hg) (72). Partial pressure of oxygen in the core of the lens is \approx 20 mm Hg (73). However, it is only one of \approx 400 naturally occurring carotenoids (74) and other carotenoids may have similar or greater antioxidant potential (75, 76). In addition to β -carotene, α -carotene, lutein, and lycopene are important carotenoid components of the human diet (77). Carotenoids, lutein, and zeaxanthin have been identified in the lens at concentrations of \approx 10 ng/g wet wt (78, 79). β -Carotene is not present at measurable concentrations. There are no laboratory data relating carotenoids to cataract formation.

Jacques and Chylack (26) observed that persons with high plasma total carotenoid concentrations ($>$ 3.3 μ mol/L) had less than one-fifth the prevalence of cataract compared with persons with low plasma carotenoid concentrations ($<$ 1.7 μ mol/L) (RR: 0.18; CI: 0.03, 1.03) after age, sex, race, and diabetes were adjusted for (Table 3). However, they did not observe an association between carotene intake and cataract prevalence (26). Persons with carotene intakes $>$ 18 700 IU/d had the same prevalence of cataract as those with intakes $<$ 5677 IU/d

(RR: 0.91; CI: 0.23, 3.78). Knekt et al (59) reported that in age- and sex-matched case and control subjects, persons with serum β -carotene concentrations $>$ \approx 0.1 μ mol/L had a 40% reduction in the rate of cataract surgery compared with persons with concentrations below this amount (RR: 0.59; CI: 0.26, 1.25). Hankinson et al (30) reported that the multivariate-adjusted rate of cataract surgery was \approx 30% lower (RR: 0.73; CI: 0.55, 0.97) for women with high carotene intakes (median = 14 558 IU/d) compared with women with low intakes of this nutrient (median = 2935 IU/d). However, although cataract surgery was inversely associated with total carotene intake, it was not strongly associated with consumption of carotene-rich foods, such as carrots. Rather, cataract surgery was associated with lower intakes of foods such as spinach that are rich in lutein and zeaxanthin carotenoids, rather than β -carotene.

Vitale et al (31) also examined the relations between plasma β -carotene concentrations and age-, sex-, and diabetes-adjusted prevalence of cortical and nuclear cataract. Although the data suggested a weak inverse association between plasma β -carotene and cortical cataract and a weak positive association between this nutrient and nuclear cataract, neither association was statistically significant. Persons with plasma β -carotene concentrations $>$ 0.88 μ mol/L had a 28% lower prevalence of cortical cataract (RR: 0.72; CI: 0.37, 1.42) and a 57% higher prevalence of nuclear cataract (RR: 1.57; CI: 0.84, 2.93) compared with persons with concentrations $<$ 0.33 μ mol/L.

Antioxidant nutrient combinations

Combinations of multiple antioxidant nutrients were also considered (Table 4) because of possible synergistic effects of antioxidant nutrients on cataract risk (Figure 3). The first, and perhaps most important, study in terms of revealing the utility of diet indicates a significant fivefold decrease in RR for cataract between persons consuming \geq 1.5 servings of fruit,

TABLE 4

Cataract risk ratio: high versus low intake and plasma nutrient index using multiple antioxidant nutrients^a

Reference ^b and nutrient source	Cataract type						
	Mixed	Cortical	PSC ^c	Nuclear	Any	Advanced	Extraction
Robertson et al (25); n = 304; supplement	—	—	—	—	0.32 ^d	—	—
Jacques and Chylack (26); n = 112	—	—	—	—	—	—	—
Intake	—	0.37	0.33	—	0.45	—	—
Plasma	—	0.16 ^d	0.00 ^d	—	0.17 ^d	—	—
Fruit and vegetables ^e	—	0.20 ^d	0.08 ^d	—	0.18 ^d	—	—
Leske et al (27); n = 1380	—	—	—	—	—	—	—
Intake	0.39 ^d	0.42 ^d	0.89	0.52	—	—	—
Supplement ^f	0.70 ^d	0.52 ^d	0.40 ^d	0.55 ^d	—	—	—
Mohan et al (29); n = 1990; plasma	0.12 ^d	—	0.23 ^d	—	—	—	—
Hankinson et al (30); n = 50 828; intake	—	—	—	—	—	—	0.76
Knekt et al (59); n = 141; serum	—	—	—	—	—	—	0.4 ^d
Mares-Perlman et al (28); n = 1980; supplement ^g	—	1.6 ^d	0.8	0.6 ^d	—	—	—
Vitale et al (31); n = 671; plasma	—	1.17	—	0.96	—	—	—
Seddon et al (20); n = 17 744; supplement ^g	—	—	—	—	0.73 ^d	—	0.79

^a Risk ratio represents the prevalence odds ratio for all studies except Hankinson et al (30); for Hankinson et al, it represents the incidence rate ratio for cataract extraction and includes women who used supplements. For nonusers of supplements, the ratio is 0.60 (CI: 0.45, 0.81).

^b Studies reporting estimates of odds ratio or relative risk.

^c Posterior subcapsular.

^d Significant ratio, $P < 0.05$.

^e For persons who consume at least 3.5 servings of fruit and vegetables per day.

^f For multivitamin supplement users.

vegetables, or both daily (26). Jacques and Chylack (26) also found that the adjusted prevalence of all types of cataract was 40% (RR: 0.62; CI: 0.12, 1.77) and 80% (RR: 0.16; CI: 0.04, 0.82) lower for persons with moderate and high antioxidant index scores (based on combined plasma vitamin C, vitamin E, and carotenoid concentrations), respectively, compared with persons with low scores.

Using a similar index based on combined antioxidant nutrient intakes (vitamin C, vitamin E, carotene, and riboflavin), Leske et al (27) found that persons with high scores had 60% lower adjusted prevalence of cortical (RR: 0.42; CI: 0.18, 0.97) and mixed (RR: 0.39; CI: 0.19, 0.80) cataract compared with those who had low scores. However, Robertson et al (25) found no enhanced benefit to persons taking both vitamin E and vitamin C supplements compared with persons who took only either vitamin C or vitamin E.

Mohan et al (29) constructed a somewhat more complex antioxidant scale that included red blood cell concentrations of glutathione peroxidase and glucose-6-phosphate dehydrogenase and plasma concentrations of vitamin C and vitamin E. Even though they failed to see any protective associations with any of these individual factors, and even reported a positive association between plasma vitamin C and prevalence of cataract, they found that persons with high antioxidant index scores had a substantially lower prevalence of cataract involving the posterior subcapsular region (RR: 0.23, CI: 0.06, 0.88) or mixed cataract with posterior subcapsular and nuclear components (RR: 0.12; CI: 0.03, 0.56) after multivariate adjustment.

Hankinson et al (30) calculated an antioxidant score based on intakes of carotene, vitamin C, vitamin E, and riboflavin and observed a 24% reduction in the adjusted rate of cataract surgery in women with high antioxidant scores relative to women with low scores (RR: 0.76; CI: 0.57, 1.03). Knekt et al (59) observed that the rate of cataract surgery for persons with high concentrations of both serum vitamin E and β -carotene appeared lower than the rate for persons with either high vitamin E or high β -carotene concentrations. Persons with high serum concentrations of either nutrient had a rate of cataract surgery that was 40% less than that for persons with low concentrations of both nutrients (RR: 0.38; CI: 0.15, 1.0). Vitale et al (31) also examined the relation between antioxidant scores (based on plasma concentrations of vitamin C, vitamin E, and β -carotene) and prevalence of cataract, but did not see evidence of any association. The age, sex, and diabetes-

adjusted RRs were close to 1 for both nuclear (RR: 0.96; CI: 0.54, 1.70) and cortical (RR: 1.17; CI: 0.62, 2.20) cataract.

Relations between multiple antioxidant nutrients and cataract risk are further supported by multivitamin and food data. Leske et al (27) found that use of multivitamin supplements was associated with decreased prevalence for each type of cataract: 60%, 48%, 45%, and 30%, respectively, for posterior subcapsular (RR: 0.40; CI: 0.21, 0.77), cortical (RR: 0.52; CI: 0.36, 0.72), nuclear (RR: 0.55; CI: 0.33, 0.92), and mixed (RR: 0.70; CI: 0.51, 0.97) cataracts. Seddon et al (20) also observed reduced risk for incident cataract for users of multivitamins (RR: 0.73; CI: 0.54, 0.99).

Intervention trials

To date only one intervention trial designed to assess the effect of vitamin supplements on cataract risk has been completed. Sperduto et al (80) took advantage of two ongoing, randomized, double-blind vitamin and cancer trials to assess the effect of vitamin supplements on cataract prevalence. The trials were conducted in ~4000 participants aged 45-74 y from rural communes in Linxian, China. Participants in one trial received either a multivitamin supplement or placebo. In the second trial, a more complex factorial design was used to evaluate the effects of four different vitamin and mineral combinations: retinol (5000 IU) and zinc (22 mg), riboflavin (3 mg) and niacin (40 mg), vitamin C (120 mg) and molybdenum (30 μ g), and vitamin E (30 mg), β -carotene (15 mg), and selenium (50 μ g). At the end of the 5-y follow-up, the investigators conducted eye examinations to determine the prevalence of cataract.

In the first trial there was a significant 43% reduction in the prevalence of nuclear cataract for persons aged 65-74 y who were receiving the multivitamin supplement (RR: 0.57; CI: 0.36, 0.90; Table 5). The second trial showed a significantly reduced prevalence of nuclear cataract in persons receiving the riboflavin and niacin supplement relative to those persons not receiving this supplement (RR: 0.59; CI: 0.45, 0.79). The effect was strongest in those aged 65-74 y (RR: 0.45; CI: 0.31, 0.64). However, the riboflavin and niacin supplement appeared to increase the risk of posterior subcapsular cataract (RR: 2.64; CI: 1.31, 5.35). The results further suggested a protective effect of the retinol and zinc supplement (RR: 0.77; CI: 0.58, 1.02) and the vitamin C and molybdenum supplement (RR: 0.78; CI: 0.59, 1.04) on prevalence of nuclear cataract.

TABLE 5
Cataract risk ratio: intervention trials

Reference and nutrient source	Mixed	Cortical	PSC ¹	Nuclear	Any	Advanced	Extraction
Sperduto (80); n = 4000							
Multivitamin	—	1.05	1.41	0.80	—	—	—
Riboflavin and niacin	—	1.08	2.64 ²	0.59 ²	—	—	—
Retinol and zinc	—	1.08	0.59	0.77	—	—	—
Vitamin C and molybdenum	—	0.92	1.25	0.78	—	—	—
Selenium, vitamin E, and β -carotene	—	0.96	1.56	1.19	—	—	—

¹Posterior subcapsular

²Subjects 65-74 y of age

Significant ratio, $P < 0.05$

CONCLUSIONS

Age-related eye diseases can be devastating in terms of personal quality of life, as well as national public health and economics. Although it is too early to declare that increased consumption or intake of nutrients is associated with diminished risk of cataract at any one location in the eye, it seems that nutrient intake can be optimized to delay cataract. Optimization of nutrition can be achieved through better diets and, perhaps, with the aid of supplements once appropriate amounts of specifically beneficial nutrients are defined. Cataract develops slowly and available data indicate that future studies will be most informative if they correlate long-term dietary or supplemental intake and risk of cataract. Because poverty, low educational status, and smoking are frequently associated with enhanced risk of these eye problems and many other maladies, solutions for these societal problems should be a priority. Longitudinal and intervention trials should be conducted. ■

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EXHIBIT G



CONSUMPTION OF CAROTENOID-RICH VEGETABLES INCREASES T-LYMPHOCYTE PROLIFERATION AND PLASMA LEVELS OF CAROTENOID OXIDATION PRODUCTS.

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Interpretive Summary:

Cancer, cardiovascular disease, and age-related macular degeneration are less common in people who eat diets rich in fruits and vegetables. Many scientists believe that carotenoids (the prominent yellow, orange and red pigments in plant foods) are among the plant components providing protection. We studied 12 men and women who ate 5 servings of carotenoid-rich vegetables a day for three weeks to determine whether 1.) food carotenoids could be increased in plasma and in a tissue (colon cells), 2.) immune status would be improved, 3.) DNA and plasma lipids would be protected from damage caused by oxidation. The vegetables (sweet potato, kale, tomato juice) provided three major carotenoids -- beta-carotene, lutein, and lycopene, respectively. The level of total carotenoids in the diet was increased about 6 fold over levels typically consumed in the Americana diet. Levels of all three carotenoids increased in subjects' plasma and in their colon cells. Immune status, measured as the ability of T-lymphocytes to proliferate when challenged, was improved during the time of treatment. This effect remained for three weeks after the treatment ended suggesting prolonged benefit. However, the carotenoid-rich vegetables did not protect DNA and plasma lipids from normal, oxidative processes. We conclude that carotenoids from common vegetables are absorbed and incorporated into tissues. Carotenoids or other plant components appear to boost the immune system. This information will be useful to consumers, plant scientists, and nutritionists interested in promoting health through diet.

Keywords:

carotenoid beta carotene lycopene lutein vegetables fruits diet nutrition bioavailability transport humans adults cancer heart disease antioxidant oxidation oxidative stress lipoproteins disease prevention

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EXHIBIT H

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BETA-CAROTENE AND LUTEIN PROTECT THE PLASMA MEMBRANE OF HEPG2 HUMAN LIVER CELLS AGAINST OXIDANT-INDUCED DAMAGE

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Interpretive Summary:

This research addressed the question of how carotenoids might lower the risk for certain chronic diseases by testing the antioxidant activity of beta-carotene (BC) and lutein (lut). BC and lut are found in all human blood in relatively high concentrations. The test model was a human liver cell line, HEP-G2. The cells were exposed to several conditions of potential injury due to "oxidative" stress. The results support the concept that both BC and lut protect human cells against damage. In some of the tests, the carotenoids were as effective as the reference substance, vitamin E (alpha-tocopherol), which is recognized as an effective cell protector (antioxidant). The results also demonstrated that the protective effect of BC and lutein was not due to being converted to vitamin A (VA) since lut (unlike BC) has no such potential. Thus, this research involving human cells provides data which supports the general hypothesis that BC and lut protect cells by serving as antioxidants. These findings will benefit other scientists working in the area. In addition, the results will add to our knowledge which in the future may help prevent or delay chronic diseases in our aging U.S. population as well as other individuals.

Keywords:

zinc phytate carotenoids beta carotene gene expression retinol human cell lines cell culture molecular biology micronutrients requirements interactions antioxidants lipid peroxidation retinol-binding protein hhrim121422

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EXHIBIT I

Homocysteine and Coronary Artery Disease in French Canadian Subjects: Relation With Vitamins B₁₂, B₆, Pyridoxal Phosphate, and Folate

Karl Dalery, MD, Suzanne Lussier-Cacan, PhD, Jacob Selhub, PhD, Jean Davignon, MD, Yves Latour, MD, and Jacques Genest Jr, MD

termed plasma levels of homocysteine in 584 subjects (380 men and 204 women) from a utility company in the province of Québec, Canada, and in 150 subjects (123 men and 27 women) with radiographically documented coronary artery disease age <60 years). Plasma levels of vitamins B₁₂, pyridoxal phosphate (a vitamin B₆ derivative), and folate were also determined. Mean homocysteine levels were higher ($p < 0.05$) in the bottom quartiles for vitamin B₁₂, and pyridoxal phosphate. A significant correlation was noted between homocysteine levels and folate and vitamin B₁₂ levels. No significant correlation was found between plasma homocysteine levels and lipids and lipoprotein cholesterol, glucose, or the presence of hypertension or cigarette smoking in healthy subjects or in patients with CAD. Control men had higher homocysteine levels than control women ($p < 0.01$). Men and women with CAD had higher levels of homocysteine than controls (11.7 ± 5.8 vs 9.7 ± 4.9 nmol/l [$p < 0.001$] and 12.0 ± 6.3 vs 7.6 ± 4.1 nmol/l, $p < 0.01$, respectively). Women and men with CAD had similar homocysteine levels. The proportion of subjects with CAD having homocysteine levels >90th

percentile of controls was 18.1% for men and 44.4% for women (both $p < 0.01$). Significantly lower pyridoxal phosphate levels were seen in subjects with CAD, men and women combined (27.7 ± 29.5 vs 42.1 ± 38.4 ng/ml, $p < 0.005$). No significant differences were observed for B₁₂, folate, or total B₆. Multivariate analysis reveals that an elevated homocysteine level is a risk factor for CAD in French Canadian men and women and that reduced levels of pyridoxal phosphate, folate, and vitamin B₁₂ may contribute to elevated plasma homocysteine levels. We conclude that in our subjects of French Canadian descent, plasma levels of homocysteine are influenced by levels of folate, vitamin B₁₂, and pyridoxal phosphate. In healthy men, mean homocysteine levels are higher than in healthy women. Men and women with CAD had significantly higher homocysteine levels than controls and this elevation is independent of traditional risk factors. Prospective studies are needed to determine the role of homocysteine in CAD. The influence of treatment of elevated homocysteine levels on cardiovascular morbidity and mortality must be assessed.

(Am J Cardiol 1995;75:1107-1111)

homocysteine is a sulfur-containing amino acid formed during the processing of methionine. Mild to moderate elevations of homocysteine (in its reduced form, homocystine or the homocysteine-cystine mixed disulfide, free or protein bound, collectively referred to as homocyst(e)ine) have been associated with vascular disease in retrospective case-control studies.¹⁻⁹ The strength of association with peripheral vascular or cerebrovascular disease appears stronger than for coronary artery disease (CAD).¹⁰ In 1 prospective study, elevated homo-

homocysteine levels (>95th percentile) were associated with a threefold increase in relative risk of developing CAD.¹¹ However, in a prospective study of Finnish subjects, homocysteine levels in CAD cases were not significantly higher than those in controls.¹² Plasma homocysteine levels are modulated by the rate of sulfoconjugation of homocysteine to produce cysteine through a series of steps in the pyridoxal phosphate-dependent cystathionine β -synthase pathway, or by re-methylation to methionine, involving vitamins B₁₂ and folic acid as the methyl donor.¹³ Deficiencies of several enzymes involved in homocyst(e)ine metabolism are associated with homocystinuria, a genetic disorder characterized by severe neurologic manifestations, widespread vascular thrombosis, and premature death.¹⁴ It has been suggested that partial deficiencies of cystathionine β -synthase, or 5', 10'-methylene-tetrahydrofolate reductase are associated with vascular disease and, in some cases, with mild to moderate elevations of homocysteine. A genetic basis for elevated homocysteine levels has been suggested from family studies of patients with CAD.^{15,16} The study of a large cohort of subjects from the Framingham Study has shown that folic acid and vitamins B₁₂ and B₆ are important determinants of plasma homocysteine levels in a healthy population.¹⁷

Cardiovascular Genetics Laboratory and the Hyperlipidemia and Atherosclerosis Research Group, Clinical Research Institute of Montreal, and the Department of Medicine and Cardiology Services, St. Joseph Hospital, Montréal, Québec, Canada, and the Vitamin Metabolism Laboratory, Human Nutrition Research Center on Aging, Boston University, Boston, Massachusetts. This study was supported by a Medical Research Council of Canada scholarship to Jacques Genest Jr., and by a grant from the Fondation des Maladies du Cœur du Québec, Québec, Canada, and by Grant U11 from the Medical Research Council of Canada/CIBA-GEIGY Foundation, Montréal, Québec, Canada. Manuscript received October 16, 1994; revised manuscript received and accepted April 4, 1995.

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TABLE 1 Characteristics of Healthy Control Subjects and Patients With Coronary Artery Disease

	Men		Women	
	Controls (n = 380)	CAD (n = 123)	Controls (n = 204)	CAD (n = 27)
Age (yr)	38.6 ± 7.7	48.6 ± 7.0*	36.3 ± 6.9	50.5 ± 7.3*
Body mass index (kg/m ²)	24.4 ± 2.4	27.8 ± 3.7*	22.5 ± 2.5	27.2 ± 4.9*
Cholesterol (mmol/L)	5.10 ± 0.96	6.12 ± 1.48*	4.77 ± 0.74	6.24 ± 1.6*
LDL cholesterol (mmol/L)	3.19 ± 0.85	3.95 ± 1.31*	2.77 ± 0.67	4.18 ± 1.55*
HDL cholesterol (mmol/L)	1.22 ± 0.28	0.88 ± 0.19*	1.51 ± 0.33	1.07 ± 0.35*
Triglycerides (mmol/L)	1.33 ± 0.78	2.64 ± 2.09*	0.94 ± 0.48	2.17 ± 1.55*

*Significantly different from corresponding values in control groups at p < 0.001, by gender. Values are expressed as mean ± SD. CAD = coronary artery disease; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

This study examines homocysteine levels in patients with premature CAD and a group of healthy subjects, and determines the relation between plasma levels of vitamins B₁₂, B₆ (and its metabolite pyridoxal phosphate), folic acid, and homocysteine.

METHODS

Healthy subjects: A sample of white-collar workers aged between 20 and 59 years, employed by a major utility company, was used as a reference group. Screening procedures for this group are detailed elsewhere.¹⁸ Subjects were selected on the basis of being healthy and free of major CAD risk factors except for cigarette smoking. Plasma samples were stored at -70°C until analysis of homocysteine and related vitamins was performed. On the basis of the exclusion criteria and the availability of stored plasma samples, 584 persons (380 men and 204 women) formed the reference group.

Patients with coronary artery disease: Patients were selected from the cardiology clinic of the Clinical Research Institute of Montréal or referred from the cardiology services of the Hôtel-Dieu Hospital in Montréal. All study subjects selected were <60 years of age at the time of sampling and had angiographically documented CAD (>50% stenosis of a major epicardial coronary artery) or a documented myocardial infarction by enzymatic and electrocardiographic criteria.¹⁹ Patients underwent blood sampling according to a strict protocol. All CAD subjects (123 men and 27 women) had blood samples drawn as outpatients ≥8 weeks after discharge from the hospital for elective cardiac catheterization, myocardial infarction, coronary angioplasty, or coronary artery bypass surgery.

Clinical variables: Data were obtained on cigarette smoking, the presence of hypertension, diabetes, and a family history of premature CAD as defined by the National Cholesterol Education Program.²⁰ The use of medications was recorded; patients taking lipid-lowering medications were asked to discontinue their use for a period of ≥2 months before blood sampling. A family history was determined on all patients, including age, presence of vascular disease, hypertension, diabetes, and cause of death in all relatives for 3 generations. Height

and weight were recorded for all patients and body mass index calculated (weight [kg]/height² [m]). Blood pressure was recorded with the patient sitting for ≥5 minutes. Diet evaluation was performed on the basis of a 24-hour dietary recall. Patients were asked to resume their usual diet after discharge from the hospital and, after quantitation, all patients were given an American Heart Association phase I or II diet; advice was given and follow-up conducted by a professional dietitian.

Biochemical variables: Fasting blood samples were collected in ethylenediaminetetraacetic acid-containing tubes (final concentration 1.5 mg/ml) and kept on ice. Plasma was separated within 2 hours of sampling by centrifugation (20 minutes, 4°C, 3,000 rpm) and multiple 1 ml aliquots were stored at -70°C for further studies. Plasma lipoproteins were separated under standard conditions by ultracentrifugation (density = 1.006 g/ml) and heparin-manganese precipitation of the apolipoprotein B-containing lipoproteins. Plasma and lipoprotein cholesterol and triglycerides were measured enzymatically on an automated autoanalyzer (Roche Cobas Mira S, Hoffman-La Roche, Basel, Switzerland) as previously described.²¹ The laboratory participates in and meets the requirements of the Centers for Disease Control cholesterol standardization program. Total plasma homocysteine levels were determined on frozen samples by high-pressure liquid chromatography according to the technique of Araki and Sako.²¹ This method measures total (free and protein-bound) homocysteine. Plasma folic acid levels were determined using a microbial assay, vitamin B₁₂ with a commercial radioimmunoassay kit (Ciba-Corning, Medfield, Massachusetts) as previously described,¹⁷ and pyridoxal-5'-phosphate by the tyrosine decarboxylase method as described by Selhub et al.¹⁷ A complete vitamin profile was obtained on 518 healthy subjects and 113 patients with CAD; lack of plasma samples accounted for the missing values.

Statistical analysis: Data were analyzed using the SAS software package (Statistical Analysis Software, SAS Institute, Cary, North Carolina). All values were calculated as mean ± SD for all groups and separately by gender. Group means were compared by the *t* test using Satterthwaite's approximation when variances were unequal. An F statistic was computed to test for equality of variances. The relation between variables was evaluated by linear regression. Nonparametric data were compared with the rank-sum test; discrete variable frequencies were compared using chi-square analysis. A stepwise discriminant analysis was performed by inserting all parameters that were significantly different between CAD subjects and the control group.

RESULTS

We examined 734 patients and controls. Data were analyzed for CAD subjects and controls first, and then by gender. Because significant differences in several bio-

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Chemical variables were noted between men and women, all results are presented by gender unless otherwise noted. The characteristics of control subjects and CAD patients are listed in Table I. There were 380 men and 204 women in the control group, and 123 men and 27 women in the CAD group. Mean age was 38.6 ± 7.7 and 48.6 ± 7.0 years for men in the control and CAD group, respectively ($p < 0.001$). Mean age was 36.3 ± 6.9 and 50.5 ± 7.3 years for healthy women and women with CAD, respectively ($p < 0.001$). Body mass index was 24.4 ± 2.4 and 27.8 ± 3.7 for control and CAD men, 22.5 ± 2.5 and 27.2 ± 4.9 for control and CAD women, respectively ($p < 0.05$ for men and women). Because these

demographic variables were significantly different between patients and controls, we analyzed the data first by univariate analysis and then by stepwise discriminant analysis.

As documented in many previous studies, plasma lipoprotein levels differed significantly between CAD and control subjects. We did not correct for age or other confounding variables (medication, weight, or diet) when calculating the lipid and lipoprotein cholesterol data. Total cholesterol levels were higher in patients than in controls (5.10 ± 0.96 vs 6.12 ± 1.48 mmol/L for men in the control and CAD groups, respectively; 4.77 ± 0.74 vs 6.24 ± 1.6 mmol/L for women in the control and CAD groups, respectively). Similarly, low-density lipoprotein cholesterol levels were higher in patients than in controls (3.19 ± 0.85 vs 3.95 ± 1.31 mmol/L for men in the control and CAD groups, respectively; 2.77 ± 0.67 vs 4.18 ± 1.55 mmol/L for women in the control and CAD groups, respectively). HDL cholesterol levels were higher in healthy men and women (1.22 ± 0.28 mmol/L and 1.51 ± 0.33 mmol/L, respectively) than in men and women with CAD (0.88 ± 0.19 and 1.07 ± 0.35 mmol/L, respectively). Triglyceride values were 1.33 ± 0.78 and 2.64 ± 2.09 mmol/L for men in the control and CAD groups, respectively. Healthy women had triglyceride levels of 0.94 ± 0.48 vs 2.17 ± 1.55 mmol/L for women in the CAD group. Significant differences were found for all these variables when comparisons were made by gender between patients and controls ($p < 0.001$). Healthy men and women differed significantly with regard to age, body mass index, and every lipid trait measured ($p < 0.001$), whereas the only difference observed between men and women with CAD was the higher plasma HDL cholesterol levels in women ($p < 0.02$).

Table II lists plasma homocysteine and vitamin levels in healthy subjects and patients with CAD (men and women analyzed separately). Folate levels did not differ significantly between control and CAD subjects ($p = NS$, healthy subjects vs patients with CAD, by gender). Similarly, vitamin B₁₂ and B₆ levels were not significantly different between healthy subjects and patients with CAD (vitamin B₆ data not shown). Healthy men had significantly higher pyridoxal phosphate levels than men

	Men		Women	
	Controls	CAD	Controls	CAD
Folate (ng/ml)	$3.8 \pm 2.3^{\ddagger}$ (378)	4.1 ± 2.9 (118)	4.3 ± 2.9 (204)	4.4 ± 3.3 (27)
Vitamin B ₁₂ (pg/ml)	323 ± 139 (379)	296 ± 130 (122)	320 ± 157 (204)	369 ± 168 (27)
Pyridoxal phosphate (pmol/ml)	42 ± 38 (331)	$29 \pm 32^*$ (94)	42 ± 51 (190)	28 ± 30 (21)
Homocysteine (μmol/L)	$9.7 \pm 4.9^{\S}$ (380)	$11.7 \pm 5.8^{\dagger}$ (123)	7.6 ± 4.1 (204)	$12.0 \pm 6.3^*$ (27)
Log (homocysteine)	$0.94 \pm 0.20^{\S}$ (380)	$1.03 \pm 0.18^{\dagger}$ (123)	0.84 ± 0.19 (204)	$1.03 \pm 0.22^{\dagger}$ (27)

Values are expressed as mean \pm SD; numbers of patients in parentheses.
 Difference from control subgroup: * $p < 0.01$; $^{\dagger}p < 0.001$.
 Difference between control women and men: $^{\ddagger}p < 0.05$; $^{\S}p < 0.0001$.
 CAD = coronary artery disease.

with CAD (42 ± 38 vs 29 ± 32 pmol/ml, respectively, $p < 0.01$). A similar trend was seen in women (42 ± 51 vs 28 ± 30 pmol/ml, $p = NS$). When patients with CAD were pooled and compared with controls, pyridoxal phosphate levels differed significantly ($p < 0.005$).

The mean \pm SD plasma total homocysteine concentration for the group of healthy subjects ($n = 504$) was 8.97 ± 4.71 μmol/L (median 7.67, range 2.00 to 35.25), and for patients with CAD ($n = 150$) the mean homocysteine level was 11.7 ± 5.8 μmol/L (median 10.49, range 4.40 to 43.27). The frequency distribution for the overall groups, healthy subjects, men and women, and men and women with CAD was skewed in both groups (Figure 1). Analyses were therefore conducted on log-transformed as well as untransformed data and separated by gender. Homocysteine levels were 9.7 ± 4.9 and 11.7 ± 5.8 μmol/L for control and CAD men, respectively ($p < 0.001$), and 7.6 ± 4.1 and 12.0 ± 6.3 μmol/L in control and CAD women, respectively ($p < 0.01$). In addition, healthy men had higher homocysteine levels than healthy women ($p < 0.001$), but no such difference was observed between CAD men and women.

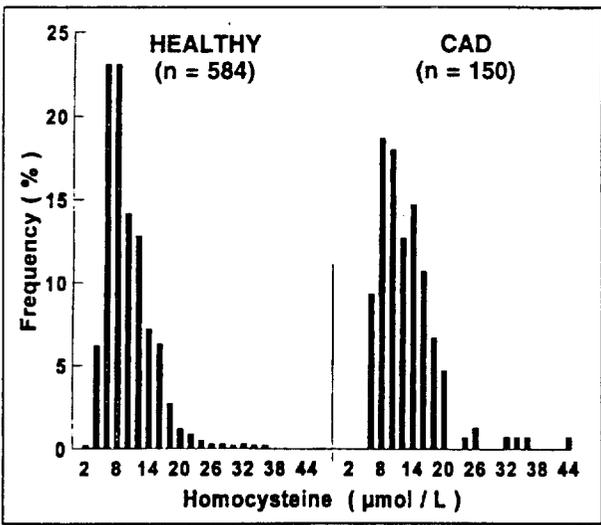


FIGURE 1. Mean plasma homocysteine levels in healthy subjects and in patients with coronary artery disease (CAD).

TABLE III Correlations Between Homocysteine Levels and Biologic Parameters in Healthy Subjects and in Patients With Coronary Artery Disease

	Controls			CAD		
	All (n = 584)	Men (n = 380)	Women (n = 204)	All (n = 150)	Men (n = 123)	Women (n = 27)
Age	0.083	0.074	0.006	0.053	0.087	-0.095
Body mass index	0.111*	0.065	-0.015	-0.014	-0.018	0.003
Glucose	0.023	-0.017	-0.003	0.000	0.019	-0.012
Cholesterol	0.113*	0.052	0.157	-0.031	0.015	-0.213
LDL cholesterol	0.115*	0.056	0.111	-0.058	-0.030	-0.163
HDL cholesterol	-0.048	0.002	0.122	-0.080	-0.076	-0.133
Triglycerides	0.090	0.033	0.049	-0.031	0.052	-0.075
Folate	-0.184†	-0.165*	-0.183*	-0.223*	-0.198	-0.294
Vitamin B ₁₂	-0.173†	-0.187†	-0.169	-0.009	-0.057	0.127
Pyridoxal phosphate	-0.002	-0.075	0.107	-0.043	-0.023	-0.131

*p < 0.01; †p < 0.001.
Abbreviations as in Table I.

Table III lists correlation coefficients between homocysteine and other biochemical variables. A significant correlation was found in the pooled control groups. No significant correlation was found between homocysteine and lipoprotein cholesterol levels. Plasma homocysteine levels were significantly and negatively correlated with folic acid in patients with CAD, whereas homocysteine was negatively correlated with folate and vitamin B₁₂ levels in control subjects.

The 90th percentile level for homocysteine in control subjects was used to define an elevated homocysteine level in patients with CAD. The 90th percentile in women and men was 12.16 and 15.55 μmol/L, respectively. The prevalence of CAD patients—men and women with homocysteine levels >90th percentile—was 18.1% for men and 44.4% for women. A significant number of patients with high homocysteine levels had lower folate levels than those with normal levels; no such difference was observed with vitamin B₁₂.

To ascertain whether homocysteine was a risk factor for CAD, independent of lipoprotein cholesterol, body mass index, and glucose, we performed a stepwise discriminant analysis using a forward selection procedure. The results reveal that even after taking into account age, lipoprotein parameters, and the presence of other risk factors, homocysteine was still an independent risk factor between patients and controls (chi-square = 5.85, p = 0.0155).

DISCUSSION

Our data suggest that homocysteine levels in a healthy population are modulated in part by plasma levels of vitamins B₁₂, folate, and pyridoxal phosphate. In control groups (both men and women), the overall effect of vitamin levels on homocysteine levels was seen in the bottom quartile of vitamins B₁₂, folate, and pyridoxal phosphate. Our previous work¹⁷ reveals that the relation between homocysteine levels and the various vitamins involved in its metabolism is not linear, but rather that low levels of these vitamins may be a major determinant of homocysteine levels in populations. Population norms for homocysteine may therefore have to be based on normal levels of these 3 vitamins as was done by Selhub et al.¹⁷ We were unable to show significant differences in

folate and vitamins B₁₂ and B₆ between patients with CAD and control subjects, partly because of the large biologic variability noted in our study subjects (healthy and CAD combined). A significant difference was observed in pyridoxal phosphate levels between CAD subjects and controls (levels were lower in CAD subjects). This effect was seen predominantly in men. Despite these findings, it is premature to recommend nutritional supplementation in vitamins B₆, B₁₂, or folate on the basis of their potential beneficial effect of decreasing elevated homocysteine levels. Our current concept is that a proper diet should supply the daily requirements of these vitamins.

Homocysteine and vascular disease: With regard to previous studies of the association between homocysteine levels and CAD,^{6,9,11} our case-control study provides data on a large number of well-characterized control subjects and patients with CAD.

Several groups of investigators have reported an association between the presence of elevated plasma levels of homocysteine and the presence of CAD, and cerebrovascular and peripheral vascular disease.¹⁻⁹ Most investigations have used a retrospective case-control design. Until clinical measurement of baseline levels of homocysteine became feasible by sensitive methods, the diagnosis of abnormal metabolism of homocysteine relied on methionine loading. Our previous studies^{6,15} were performed in a population of patients with CAD and controls. As seen in other studies, no association was seen between homocysteine and any of the conventional risk factors for CAD (age, cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, smoking, or hypertension). An inverse relation was observed with folate and vitamin B₁₂ levels. From a review of published studies on homocysteine and vascular disease, an elevated homocysteine level appears to be a greater risk for peripheral vascular and cerebrovascular disease than for CAD.¹⁰ In the present study, we have determined plasma homocysteine levels in 150 patients with premature CAD and 584 control subjects of French Canadian descent. Our data reveal that control men have significantly higher homocysteine levels than control women. We also determined that men and women with premature CAD had significantly higher homocysteine levels

EXHIBIT J

High dose B-vitamin treatment of hyperhomocysteinemia in dialysis patients

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High dose B-vitamin treatment of hyperhomocysteinemia in dialysis patients. Hyperhomocysteinemia, an arteriosclerotic risk factor, persists in 75% of dialysis patients despite routine low dose supplementation with the B-vitamin co-factors/substrates for homocysteine (Hcy) metabolism and normal or supernormal plasma status of these vitamins [1, *Pharmacology* 114:93, 1995]. We conducted a placebo-controlled eight-week trial of the effect on plasma homocysteine of adding supraphysiologic dose folic acid (15 mg/day), B-6 (100 mg/day), and B-12 (1 mg/day) to the usual daily dosing of 1 mg folic acid, 10 mg B-6, and 12 µg B-12 in 27 hyperhomocysteinemic dialysis patients. Total plasma homocysteine was measured at baseline, and after four and eight weeks. Blinded analyses revealed no evidence of toxicity in the group randomized to supraphysiologic dose B-vitamin supplementation. Plasma homocysteine was significantly reduced after both four weeks (-29.8% vs. -2.0%; $P = 0.0024$) and eight weeks (-25.8% vs. +0.6%; $P = 0.0009$) of active versus placebo treatment. Also, 5 of 15 treated versus 0 of 12 placebo group patients had their plasma Hcy reduced to within the normative range ($< 15 \mu\text{mol/liter}$). Supraphysiologic doses of B-vitamins may be required to correct hyperhomocysteinemia in dialysis patients.

Atherothrombotic sequelae such as myocardial infarction (MI) and stroke remain the leading cause of mortality in end-stage renal disease (ESRD) patients on maintenance dialysis [1]. Despite their reportedly high prevalence [2], the predictive value of traditional cardiovascular disease (CVD) risk factors in determining CVD outcomes in ESRD remains limited [3].

Homocysteine (Hcy) is a sulfur-containing amino acid product of methionine metabolism. A review [4] of 20 retrospective case control studies involving ~1800 patients and an equal number of controls indicated that moderate hyperhomocysteinemia, either fasting or after oral methionine loading, was an independent risk factor for premature coronary heart, cerebrovascular, or peripheral vascular disease. Two large prospective studies subsequently confirmed that moderate hyperhomocysteinemia was an independent predictor of incident MI, after controlling for a wide range of known CVD risk factors [5, 6]. Most recently, population-based data from the original Framingham Study cohort revealed that plasma Hcy levels in the upper quartile were associated with a

two-fold increased risk for extensive extracranial carotid arteriosclerosis, which persisted after adjustment for age, smoking, hypertension, and dyslipidemia [7].

Plasma Hcy levels determined as the acid-soluble mixed disulfide homocysteine-cysteine were first shown to be increased in chronic renal failure by Wilcken and Gupta [8]. Subsequent case control studies have revealed markedly elevated plasma levels of acid-soluble [9], protein-bound [10], or total Hcy [11, 12] in ESRD patients on peritoneal or hemodialysis, and non-dialyzed uremic patients with varying degrees of renal impairment. We recently confirmed these case control findings, and also demonstrated that hyperhomocysteinemia was more prevalent, with a dramatically greater prevalence odds than any of the traditional CVD risk factors, in maintenance dialysis patients [13, 14]. Furthermore, hyperhomocysteinemia persisted in the dialysis patients despite daily, near physiologic dose supplementation (that is, 2 to 5 × the US RDA) with the B-vitamin co-factors/substrates for Hcy metabolism (folate, B-6, B-12), and normal or supernormal plasma status of these vitamins [13, 14].

Folic acid supplementation at doses of between 0.65 and 5.0 mg per day routinely normalizes fasting total plasma Hcy in persons who have intact renal function, and are not B-12 deficient [15-17]. B-6 supplementation is required to reduce or normalize the post-methionine loading increase in Hcy levels above fasting levels [18]. These findings are consistent with what is understood about the metabolism of Hcy via the methylation and transsulfuration pathways [19]. In remethylation, the primary methyl donor for the conversion of Hcy to methionine is provided by the reduction of methylenetetrahydrofolate to 5-methyltetrahydrofolate, the major circulating form of folate, by the enzyme methylenetetrahydrofolate reductase (MTHFR). 5-Methyltetrahydrofolate serves as a one-carbon donor for the vitamin B-12-dependent remethylation of Hcy to methionine catalyzed by methionine synthase. The remethylation enzymes have a low K_m for Hcy, and function to maintain normal fasting homocysteinemia. In the transsulfuration pathway, Hcy condenses with serine to form cystathionine in an irreversible reaction catalyzed by the pyridoxal-5'-phosphate (PLP)-containing enzyme, cystathionine beta synthase (CBS). CBS has a high K_m for Hcy, and functions to maintain normal postprandial or post-methionine loading homocysteinemia [19]. These pathways appear to be disrupted most commonly by deficiencies of three micronutrients [4, 5, 7; 15-18,

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20), two of which are cofactors (vitamins B-6 and B-12), and one which is a substrate (folate).

A comprehensive review of the published clinical trial literature failed to uncover a single report in which mean fasting total Hcy levels were reduced to within the normal range (that is, $< 15 \mu\text{mol/liter}$; [20]) by any combination of B-vitamins in hyperhomocysteinemic maintenance dialysis patients [21–23]. In addition, none of these studies [21–23] were placebo-controlled so their internal validity may have been compromised by regression to the mean. Accordingly, we conducted a randomized eight-week trial of the effect on plasma Hcy of adding supraphysiologic dose folic acid, along with B-6, and B-12, versus placebo, to the usual dialysis dosing of these vitamins in hyperhomocysteinemic maintenance dialysis patients.

Methods

Study population

Thirty-two maintenance dialysis patients involved in an ongoing case-control studies of hyperhomocysteinemia and traditional cardiovascular disease risk factors [13, 14] were recruited from four Brown University-affiliated outpatient dialysis centers for the present study. Exclusion criteria included age > 75 or < 30 years old, malnutrition (that is, albumin $< 2.0 \text{ mg/dl}$ or protein catabolic rate < 0.8), rejection of a cadaveric or living-related donor kidney transplant within six months of the current study, and current use of antifolate or anticonvulsant medications. All participants had fasting total plasma Hcy levels $\geq 16 \mu\text{mol/liter}$ when determined as part of the initial case-control studies. Nearly 50% of those patients with screening Hcy levels $\geq 16 \mu\text{mol/liter}$ (32 of 63) agreed to participate. The study protocol was approved by the Rhode Island Hospital Institutional Review Board, and all participants gave their written informed consent prior to participation. Individuals were block randomized into two groups of 16 on the basis of fasting Hcy $> 30 \mu\text{mol/liter}$, fasting folate $< 6 \text{ ng/ml}$, sex, mode of dialysis (peritoneal vs. hemodialysis), and race. Subsequent to randomization, but prior to actual initiation of the intervention study, four subjects in the placebo group dropped out due to lack of continued interest, while one subject in the active treatment group could not participate due to intercurrent coronary artery bypass graft surgery. Twenty-seven subjects completed four or eight weeks of the study, 12 randomized to the placebo group, and 15 randomized to the active treatment group. One subject in the placebo group missed the blood draws after four weeks of treatment due to hospitalization for a below knee amputation, but remained in the study, and underwent the post-eight week blood draws. An additional subject in the active treatment group hospitalized for an exacerbation of chronic peptic ulcer disease was unavailable for the post-eight week blood draws, but underwent the blood draws after four weeks of treatment. Characteristics of the ($N = 27$) participants who completed all or part of the intervention study were: (a) mean age ($\pm \text{SD}$) $56 (\pm 13)$ years, range 35 to 73 years; (b) etiology of ESRD: diabetic nephropathy ($N = 5$), hypertensive nephrosclerosis ($N = 6$), glomerulonephritis ($N = 7$), polycystic kidney disease ($N = 2$), and unknown/other ($N = 7$); (c) sex: 14 men, 13 women; (d) mode of dialysis: 20 hemodialysis, 7 peritoneal dialysis; (e) race: 22 white, 5 black. Baseline Hcy data (visits 1 and 2) were available from 31 of the original 32 patients who initially agreed to participate. There was no differ-

ence in baseline Hcy levels between the 4 dropouts and the 27 patients who completed all or part of the study ($30.6 \mu\text{mol/liter}$ vs. $29.5 \mu\text{mol/liter}$, respectively, $P = 0.638$ by Mann-Whitney U -test).

Laboratory and clinical data acquisition

Phlebotomy was performed non-fasting, pre-dialysis (hemodialysis) or random dwell (peritoneal dialysis), twice 48 to 72 hours apart at baseline, and after four weeks and eight weeks of active or placebo treatment. Three of these six blood draws were synchronized with the patients' routine monthly phlebotomy to decrease participant burden. All whole bloods were immediately cooled at 4°C , and the plasma or serum separated within four hours in a refrigerated centrifuge. Total homocysteine (Hcy), the sum of the acid-soluble (that is, reduced Hcy, homocystine disulfide, and homocysteine-cysteine mixed disulfide) and protein-bound moieties, was determined in EDTA plasma by a modification of the fluorimetric high performance liquid chromatography (HPLC) method originally described by Araki and Sako [24]. In brief, this assay involves the following steps: reduction of the sample with tri-*n*-butylphosphine, precipitation of proteins, alkalization of the supernatant with sodium borate, derivitization with SBDF (7-fluoro-2-oxa-1,3-diazole-4-sulphonate), followed by ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid, sodium salt) and HPLC separation with fluorescence detection. SBDF is a fluorescence probe for compounds containing SH groups, and ANTS is used as an internal standard because of its natural fluorescence at 515 nanometers when excited at 385 nanometers, the same wavelengths used for the detection of the SH/SBDF adducts. The inter- and intra-assay coefficients of variation for this assay are routinely $< 5\%$. Plasma pyridoxal 5'-phosphate (the active metabolic form of B-6) was assessed enzymatically using tyrosine decarboxylase [25]. Plasma folate was measured by a 96-well plate microbial (*Lactobacillus casei*) assay [26], and plasma B-12 with a (Magic) radioassay from CIBA-Corning. To minimize inter-assay variability, the Hcy and vitamin assays were performed "batched" for each patient from all six study visits in thawed plasma aliquots that had been cryopreserved at -70°C for ≤ 12 weeks. Routine monthly serum clinical chemistry profiles and hematocrit determinations were performed using standard methods. A symptoms questionnaire was administered to each participant at the end of the study. Eight week compliance with the treatment and placebo regimen was assessed by pill count. All data collection was performed blinded to treatment group assignment.

Treatment regimens

The treatment design was randomized and placebo-controlled, and the treatment group assignment code was not broken until data entry and analysis were completed. Treatment consisted of 15 mg folic acid, 100 mg B-6, 1 mg B-12, and cellulose fiber, or matched placebos containing only cellulose fiber, orally administered in single tablets taken once daily in the evening. All participants continued their prescribed daily oral supplementation with 1 mg folic acid, $\pm 10 \text{ mg}$ B-6, and $12 \mu\text{g}$ B-12, throughout the study.

Statistical analyses

A priori, we estimated that the study had $> 90\%$ power to detect the expected effect size of 0.30 (that is, a 30% lowering of plasma Hcy with active treatment relative to 0% lowering with placebo

Table 1. Key baseline patient characteristics

	Placebo (N = 12)	Active (N = 15)	P value ^a
Hcy ($\mu\text{mol/liter}$)	29.6 \pm 6.3 ^a	29.5 \pm 10.0	0.733
Folate (ng/ml)	49.3 \pm 25.6	32.5 \pm 25.1	0.079
N (%) Hemodialysis	8 (66.7)	12 (80.0)	0.731
N (%) Men	7 (58.3)	7 (46.7)	0.830
N (%) White	10 (83.3)	12 (80.0)	0.782

^a Mean of two baseline values \pm standard deviation

^b Two-tailed value based on Mann-Whitney U-test or chi square test with continuity correction

treatment) given $N = 12$ subjects per group. Active versus placebo group baseline differences were evaluated by Mann-Whitney U-tests, and chi-square tests. Means of the two baseline minus two four-week or two eight-week ("delta") Hcy levels were compared by unpaired *t*-tests and Mann-Whitney U-tests. In addition, linear regression models were constructed with baseline minus four or eight week Hcy levels as the outcome variable, and treatment group, baseline Hcy, folate, and PLP as the independent variables. Mean baseline minus eight-week ("delta") alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, and hematocrit values in the active versus placebo groups were also compared by unpaired *t*-tests. Differences in reported symptom frequency in the active versus placebo treated groups were assessed by chi square analyses. Reported *P* values are based on two-tailed calculations. All statistical analyses, including the block randomization procedure outlined earlier, were performed using SAS [27].

Results

Key baseline characteristics in the two groups were comparable, indicating that block randomization was successful (Table 1). Eight week compliance by pill count was 75.1% in the active treatment and 86.7% in the placebo groups, respectively. Significant increases (Table 2) in mean plasma B-vitamin levels in the active treatment group provided confirmation of the pill count compliance data. Active versus placebo treatment also resulted in a significant mean reduction in total plasma Hcy of -29.8% versus -2.0% ($P = 0.0024$) at four weeks, and -25.8% versus $+0.6\%$ ($P = 0.0009$) at eight weeks, by unpaired *t*-tests (Table 2). These findings were confirmed by Mann-Whitney U-tests (4 weeks, $P = 0.0028$; 8 weeks, $P = 0.0012$). Linear regression analyses further demonstrated that active treatment was the strongest independent predictor of both baseline minus four weeks (beta = -7.21 , $t = 3.358$; $P = 0.003$) or eight weeks (beta = -6.32 , $t = 3.833$; $P = 0.001$) Hcy levels in models that included treatment group assignment, and mean baseline folate, PLP, and Hcy. This indicates that after adjustment for treatment group assignment and mean baseline vitamin status and Hcy levels, Hcy levels were 7.21 $\mu\text{mol/liter}$ lower at four weeks and 6.32 $\mu\text{mol/liter}$ lower at eight weeks in the active treatment group compared with the control group, and this effect was highly significant. A trend ($P = 0.086$; chi square with continuity correction) for "normalization" (that is, reduction to $< 15 \mu\text{mol/liter}$) of plasma Hcy was observed in the active treatment (5 of 15) versus placebo group (0 of 12). Active treatment was not associated with an increased frequency of any specific symptoms (Table 3), or adverse changes in hematocrit, AST, ALT, or creatinine (Table 4).

Our study design mimicked the randomized trial [12] that is, monthly determination of non-fasting plasma/serum chemistries, and the placebo group provided confirmation [12] that non-fasting pre-hemodialysis or random dwell (peritoneal dialysis) plasma Hcy is a stable outcome measure. The data reported here provide the first placebo-controlled demonstration that much larger daily doses of B-vitamins (that is, folic acid, B-6, and B-12) can significantly augment the Hcy-lowering effect of the near physiologic dose B-vitamin supplements usually given to maintenance dialysis patients. Furthermore, we found no evidence of toxicity associated with this supraphysiologic dose B-vitamin treatment regimen, based upon blinded analyses of routine monthly clinical chemistry profiles and responses to a comprehensive symptoms questionnaire. More prolonged studies of comparable or higher dose folic acid-based Hcy-lowering regimens need to be performed in larger groups of maintenance dialysis patients to confirm and extend the current findings.

As anticipated due to their routinely prescribed near physiologic dose supplementation, mean baseline folate levels in both groups of patients (treated 32.5 ng/ml; placebo 49.3 ng/ml) far exceeded the 90th percentile folate levels in age, sex, and race matched Framingham Study controls with normal renal function [13, 14]. It remains unclear why hyperhomocysteinemic maintenance dialysis patients often respond inadequately [10-14; 21-23] to doses of folic acid alone, or in combination with B-6 and B-12, that consistently normalize plasma Hcy in hyperhomocysteinemic persons with intact renal function who are not homozygous for severe Hcy-metabolizing enzyme deficiencies [15-18]. Given the extensive plasma protein-binding (~ 70 to 80% of total [28]) and resultant limited renal filtration of unbound Hcy, loss of urinary Hcy excretion is probably not responsible for this refractory hyperhomocysteinemia. Indeed, there is evidence that loss of the normally avid tubular reabsorption of the freely filterable unbound fraction of Hcy offsets the decline in its glomerular filtration in ESRD [12]. Substantiation of the hypothesis that there may be considerable metabolism of Hcy by normal renal parenchyma [28] has recently been provided by *in vivo* data from an established rat model [29]. Determination of arteriovenous Hcy differences across the rat kidney, along with simultaneous assessment of renal plasma flow, urine flow, and urinary Hcy concentration, revealed substantial Hcy uptake by normal rat kidneys, and confirmed that urinary Hcy excretion is minimal [29]. These data suggest that loss of the large Hcy-metabolizing capacity of normal kidneys may be an important determinant of the refractory hyperhomocysteinemia commonly observed in ESRD. Livant et al [30] recently reported sizable folate losses into the dialysate, and the presence of an unidentified heat-stable inhibitor(s) of folate conjugase in hemodialysis patients. Folate conjugase cleaves glutamyl residues of polyglutamyl folates and this alteration of chain lengths is believed to be crucial to the function of both folate requiring, and perhaps other co-factor dependent co-enzymes [31]. Inhibition of folate conjugase may result in the accumulation of long chain length polyglutamated folates despite folate supplementation in ESRD patients. Maintenance of a high ratio of long versus short chain length folates could adversely affect the activity of Hcy-metabolizing enzymes (such as CBS).

Table 2. Effect of active vs placebo treatment on plasma homocysteine and B-vitamin levels

Group	0 weeks	4 weeks	4-0 weeks % change	8 weeks	8-0 weeks % change
Treated (N = 15)					
Hcy $\mu\text{mol/liter}$	29.5 \pm 10.0 ^a	20.7 \pm 8.0	-29.8 ^b	21.9 \pm 7.7	-25.8 ^{cd}
Folate ng/ml	32.5 \pm 25.1	926.8 \pm 574.9	+2751.7 ^e	707.6 \pm 507.2	+2077.2 ^f
PLP pmol/ml	67.2 \pm 81.0	200.6 \pm 204.2	+198.5 ^g	183.6 \pm 146.4	+173.2 ^h
B-12 pg/ml	468.6 \pm 308.6	1271.7 \pm 466.7	+171.4 ⁱ	1348.4 \pm 563.9	+187.8 ^j
Placebo (N = 12)					
Hcy	29.6 \pm 6.3	29.2 \pm 6.2 ^l	-2.0	29.8 \pm 6.3	+0.6
Folate	49.3 \pm 25.6	47.1 \pm 32.9	-4.5	53.8 \pm 67.2	+9.1
PLP	112.6 \pm 88.8	133.3 \pm 91.4	+18.4	134.9 \pm 100.4	+19.8
B-12	649.7 \pm 244.0	638.9 \pm 261.9	-1.7	527.3 \pm 159.0	-18.8

^a Mean \pm standard deviation for the average of two values at each time point

^b Two-tailed $P = 0.0024$ vs. placebo change at 4 weeks based on unpaired t -test

^c One subject in the treated group had blood drawn at 4 weeks but not 8 weeks, so ($N = 14$)

^d Two-tailed $P = 0.0009$ vs. placebo change at 8 weeks based on unpaired t -test

^e Two-tailed $P = 0.0001$ vs. placebo change at 4 weeks based on unpaired t -test

^f Two-tailed $P = 0.0002$ vs. placebo change at 8 weeks based on unpaired t -test

^g Two-tailed $P = 0.070$ vs. placebo change at 4 weeks based on unpaired t -test

^h Two-tailed $P = 0.045$ vs. placebo change at 8 weeks based on unpaired t -test

ⁱ Two-tailed $P = 0.0001$ vs. placebo change at 4 weeks based on unpaired t -test

^j Two-tailed $P = 0.0003$ vs. placebo change at 8 weeks based on unpaired t -test

^k One subject in the placebo group had blood drawn at 8 weeks but not 4 weeks, so ($N = 11$).

Table 3. Frequency of symptoms active vs. placebo treatment groups

Symptom	Active (N = 14)	Placebo (N = 12)	P value ^a
Nausea	1	2	0.887
Heartburn	2	3	0.848
Diarrhea	5	2	0.517
Constipation	3	0	0.276
Rash	0	0	—
Itching	5	9	0.105
Muscle aches	4	3	0.811
Muscle spasms	2	3	0.848
Tiredness/weakness	5	3	0.802
Fainting	0	0	—
Nervousness	2	1	0.887
Headaches	4	3	0.811
Sleep problems	6	6	0.976
Tingling in digits	4	3	0.811
Chest pain	0	2	0.594
Rapid heart beat	2	0	0.532
Nightmares	0	0	—

^a Two-tailed value based on chi square comparison of reported symptoms during the 8th week of active vs. placebo treatment.

which is PLP-dependent. Dr. C. Krumdieck, unpublished observation). Accordingly, supraphysiologic dose folic acid supplementation might be required to replace dialysate losses of folate, favorably alter the folate chain length ratio, and promote Hcy metabolism in ESRD. Finally, Hultberg et al [12] have shown that post-methionine load Hcy levels are increased in ESRD patients versus controls, suggesting a possible defect in B-6 dependent transsulfuration of Hcy in ESRD. An increased B-6 requirement in ESRD would be less discernible by evaluating fasting Hcy levels [19], as was done in previous case control [13, 14] and intervention [21] studies.

A burgeoning amount of experimental data have linked hyperhomocysteinemia to atherothrombosis [32-39]. Putative pathomechanisms include endothelial cell injury [32, 33], enhanced low density lipoprotein oxidation [34], increased thromboxane-medi-

Table 4. Changes in liver transaminases, creatinine, and hematocrit over eight weeks in the active vs. placebo treatment groups

	Active (N = 14)	Placebo (N = 12)	P value ^a
Δ AST ^b mg/dl	-1.3 \pm 5.4	-2.3 \pm 7.2	0.700
Δ ALT mg/dl	-1.9 \pm 10.2	-2.3 \pm 8.5	0.900
Δ Hematocrit %	-0.4 \pm 1.5	-0.04 \pm 1.6	0.614
Δ Creatinine mg/dl	+1.7 \pm 2.4	-0.7 \pm 3.0	0.390

^a Two-tailed value based on unpaired t -test.

^b Δ = mean \pm standard deviation of 8-week minus 0-week values.

ated platelet aggregation [35], inhibition of cell surface thrombomodulin expression and protein C activation [36], enhancement of lipoprotein (a)-fibrin binding [37], and promotion of smooth muscle cell proliferation [38]. Rolland et al [39] recently described an elegant model of dietary-induced hyperhomocysteinemia and vascular damage in the mini-pig. Future studies using this model may elucidate the *in vivo* relevance of the studies cited above [32-38]. Regardless of the specific pathomechanism(s), prospective observational data [8, 6], and Hcy-lowering dietary intervention (that is, B-vitamin and/or betaine supplementation, and restriction of methionine intake) data [40] suggest that hyperhomocysteinemia is associated with an increased incidence of atherothrombotic events in non-uremic populations. Preliminary subgroup analyses further indicate that hyperhomocysteinemia may also be linked to prevalent atherothrombosis in uremic patients not yet dialysis-dependent [11], and incident atherothrombosis in renal transplant recipients [41].

Despite a group mean lowering of ~26 to 30% in plasma Hcy, only 5/15 of the total number of treated patients, and 3 of 12 whose baseline Hcy was $> 20 \mu\text{mol/liter}$ had their Hcy levels reduced to within the accepted "normative range" (that is, $< 15 \mu\text{mol/liter}$; [20]) in the present study. Prevalence data from the Framingham Study have revealed that there is already an increased risk for extracranial carotid arteriosclerosis $\geq 25\%$ (a marker for significant generalized arteriosclerosis [42]) at plasma

Hcy levels $\geq 11.1 \mu\text{mol/liter}$ [7]. Clearly, more effective therapeutic regimens (higher dose combinations of dietary supplements and/or improved dialysis removal of Hcy) that truly normalize total plasma Hcy in maintenance dialysis patients need to be developed. As reviewed by Butterworth and Tamura [43], folic acid has been safely administered orally at doses of up to 60 mg/day for two years, and 500 mg/day for two weeks. More recent data indicated no apparent toxicity of folic acid at 400 mg/day given for two months to a child with homocystinuria due to homozygous methylenetetrahydrofolate reductase deficiency [44]. Controlled studies of the Hcy-lowering efficacy of folic acid given in excess of 15 mg/day may be warranted in hyperhomocysteinemic maintenance dialysis patients. Identification of a safe, effective chronic Hcy-lowering regimen could lead to placebo-controlled interventional trials for primary and/or secondary prevention of cardiovascular disease in maintenance dialysis patients. Such clinical trials would evaluate the hypothesis that hyperhomocysteinemia contributes to atherothrombosis and the findings might be relevant to other hyperhomocysteinemic patient populations.

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EXHIBIT K

Colloquium: Homocyst(e)ine, Vitamins and Arterial Occlusive Diseases

Relationship between Plasma Homocysteine, Vitamin Status and Extracranial Carotid-Artery Stenosis in the Framingham Study Population^{1,2}

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ABSTRACT Recent studies demonstrated associations between occlusive vascular disease and hyperhomocysteinemia of both genetic and nutritional origin. In the present study we analyzed plasma samples from the 20th biannual examination of the Framingham Heart Study cohort to determine distribution of plasma homocysteine concentrations with emphasis on relationships to B vitamins and prevalence of carotid artery stenosis. Results showed that homocysteine exhibited strong inverse association with plasma folate and weaker associations with plasma vitamin B-12 and pyridoxal-5'-phosphate (PLP). Homocysteine was also inversely associated with intakes of folate and vitamin B-6, but not vitamin B-12. Prevalence of high homocysteine (>14 $\mu\text{mol/l}$) was 29.3% in this cohort, and inadequate plasma concentrations of one or more B vitamins appear to contribute to 67% of the cases of high homocysteine. Prevalence of stenosis $\geq 25\%$ was 43% in men and 34% in women with an odds ratio of 2.0 for individuals in the highest homocysteine quartile ($\geq 14.4 \mu\text{mol/l}$) compared with those in the lowest quartile ($\leq 9.1 \mu\text{mol/l}$), after adjustment for sex, age, high density lipoprotein cholesterol, systolic blood pressure and cigarette smoking ($P_{\text{trend}} < 0.001$). Plasma concentrations of folate and pyridoxal-5'-phosphate and folate intake were inversely associated with extracranial carotid stenosis after adjustment for age, sex and other risk factors. *J. Nutr.* 126: 1258S-1265S, 1996.

INDEXING KEY WORDS:

• homocysteine • folic acid • pyridoxal phosphate
• vitamin B-6 • vitamin B-12 • arteriosclerosis

Almost 25 years ago McCully (1969) reported a child dying of homocystinuria, cystathioninuria, methylmalonic aciduria, secondary to abnormal cobalamin metabolism, exhibited arterial lesions were strikingly similar to those seen in patients with cystathionine beta synthase deficiency. These observations led to the proposal that the markedly elevated plasma homocysteine concentrations found in patients with homocystinuria were responsible for the development of premature occlusive vascular disease. In recent years this association between plasma homocysteine concentration and atherosclerosis has become the subject of a number of studies with growing clinical

¹ Presented as part of the colloquium "Homocyst(e)ine and Arterial Occlusive Diseases" given at the Experimental Biology and Medicine '95 meeting, Atlanta, GA, on April 13, 1995. This symposium was sponsored by the American Institute of Nutrition. Guest speakers at the symposium were M. R. Malinow, Oregon Regional Primate Research Center, Beaverton, OR, and M. J. Stampfer, Harvard School of Public Health, Cambridge, MA.

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(for reviews see Mudd et al. 1989, Ueland and Refsum 1989, Ueland et al. 1992). A literature survey by Ueland et al. (1992) identified a total of 21 studies involving over 1500 patients with occlusive (cardiovascular; peripheral and cerebrovascular) vascular disease and over 1500 respective controls. Sixteen of these studies reported significantly higher mean plasma homocysteine concentrations in patients than in respective controls ($P < 0.05$ to < 0.001). The mean patient:control homocysteine ratio according to this survey is 1.31, which indicates that the elevation of homocysteine in these patients is mild and certainly not as severe as that seen in homocystinuric patients.

Clarke et al. (1991), who relied on the methionine loading test to discriminate between (mildly) hyperhomocysteinemic and normal individuals, reported that the prevalence of hyperhomocysteinemia was 42% among patients with cerebral vascular disease, 28% among patients with peripheral vascular disease and 10% among patients with cardiovascular disease. They estimated the risk of premature occlusive vascular disease to be about 30 times greater for people with hyperhomocysteinemia relative to normal controls. A recent prospective investigation of participants in the Physicians' Health Study showed that the risk of myocardial infarction within 5 years for individuals with no prior history of vascular disease was 3.4-fold greater for those with elevated plasma homocysteine concentrations than for those with normal plasma homocysteine levels (Stampfer et al. 1992). The association between homocysteine and vascular disease in this study and in earlier studies (Clarke et al. 1991, Genest et al. 1990) is independent of other known vascular disease risk factors, such as age, diabetes, hypertension, body mass index, total and high density lipoprotein (HDL) cholesterol. In addition, carotid ultrasound has previously been used to examine the association between homocysteine and arteriosclerosis. Obligate heterozygotes for cystathionine β -synthase deficiency were shown to have a greater prevalence of carotid arteriosclerosis than normal controls (Rubba et al. 1990), and postmethionine load plasma homocysteine levels were associated with asymptomatic carotid arteriosclerosis in a combined sample of obligate heterozygotes for cystathionine β -synthase deficiency and a similar number of controls (Clarke et al. 1992). A third study of asymptomatic individuals in a middle-aged, population-based cohort demonstrated that those with carotid artery wall thickness above the 90th percentile of the population distribution had significantly higher fasting plasma homocysteine levels than controls with carotid artery wall thickness below the 75th percentile (Malinow et al. 1993).

This paper is a summary of our studies (Selhub et al. 1993, Selhub et al. 1995) on the elderly survivors of the Framingham Study cohort. We examined, in this elderly population, the distribution of plasma homocysteine levels, the relationship between plasma homo-

cysteine concentration and plasma and intake levels of folate, vitamin B-12 and vitamin B-6 and the relation between carotid stenosis and plasma concentrations of homocysteine, folate, vitamin B-12 and pyridoxal-5'-phosphate.

MATERIALS AND METHODS

Subjects. Participants were members of the original Framingham Heart Study cohort, a population-based sample of 5209 men and women originally examined in 1948-1952 (Dawber et al. 1957) and followed prospectively to the present to assess the occurrence of vascular disease. This study was based on 1401 survivors of the original cohort who participated in the 20th biennial examination (1989-1990). Homocysteine and carotid ultrasound measures were available for 1041 individuals (418 men and 623 women), aged 67-96 years old at the time of data collection. Informed consent was obtained from all participants. The protocols for this study were approved by the Human Investigations Review Committee at New England Medical Center and by the Institutional Review Board for Human Research at Boston University Medical Center.

Biochemical determinations. Blood was drawn non-fasting and plasma total cholesterol and HDL cholesterol were determined in the Framingham Heart Study laboratory using enzymatic methods (McNamara and Schaefer 1987, Warnick et al. 1982). Low density lipoprotein cholesterol was not determined because the blood samples were taken in a nonfasting state. Plasma samples stored frozen at -80°C were used for the determination of total homocysteine by the method of Araki and Sako (1987), plasma folate by a microbial assay using a 96-well plate and manganese supplementation as described by Tamura et al. (1990), vitamin B-12 using a [Magic] radioassay kit from Ciba-Corning (Medfield, MA) and pyridoxal-5'-phosphate by the tyrosine decarboxylase method as described by Camp et al. (1983). Because of insufficient plasma volume, vitamin measures were not available for all subjects.

Nutrient intake. Members of the Framingham cohort received a semiquantitative food frequency questionnaire (Willett et al. 1985) by mail when they were scheduled for their 20th biennial examination. Subjects returned the completed questionnaire at the time of their examination. Estimated folate and vitamin B-6 intakes corresponded well to the respective folate and PLP plasma concentrations. Vitamin B-12 intake was not, however, correlated to plasma vitamin B-12 (Jacques et al. 1993, Willett et al. 1985).

Measurement of carotid stenosis. At the 20th biennial examination, participants underwent a carotid doppler examination with Ultrasonix, high resolution, real-time scanner equipped with a 7.5-MHz imaging transducer, a 4-MHz pulse wave Doppler transducer

and a 4-MHz continuous wave transducer. For this report, we classified individuals into two categories based on the maximum percent diameter stenosis of the more diseased artery: 0–24% stenosis or 25–100% stenosis.

Statistical methods. To describe the associations between plasma homocysteine concentrations and the B vitamins, we grouped subjects into deciles of plasma vitamin concentration and vitamin intake. We calculated the geometric mean plasma homocysteine concentrations in each vitamin decile and plotted these values and their 95% confidence intervals (CI) at the median vitamin level within each decile. We adjusted mean homocysteine levels for age, sex and concentration or intake of the other B vitamins by analysis of covariance (Kleinbaum et al. 1988) with all covariates set to their respective sample means. We also adjusted all vitamin intakes for energy (Willett 1990).

To examine the association between the occurrence of high plasma homocysteine concentrations and these vitamins, we defined high homocysteine as concentrations $>14.0 \mu\text{mol/l}$ (the 90th percentile for homocysteine among those subjects whose three plasma vitamins levels were above the 70th percentile). We developed a B vitamin index to describe the joint relationships of the three vitamins included in these analyses to homocysteine levels. The indices had five categories based on percentile values for each nutrient. We classified individuals with all three vitamins above the 70th percentile into the reference category (Category 1). Category 2 included individuals with all three vitamins above the 50th but at least one below the 70th percentiles; Category 3 included those with at least one vitamin above and one vitamin below the 50th percentile; Category 4 included those with all three vitamins below the 50th percentile but at least one above the 30th percentile; and Category 5 included individuals with all vitamins below the 30th percentile. We determined mean homocysteine concentration, the prevalence of high homocysteine, the prevalence rate ratio for high homocysteine and attributable proportion (attributable risk percent) (Rothman 1986) within each vitamin index category. We also estimated population attributable proportion, which represents the proportion of cases with high homocysteine in the population that can be attributed to low plasma vitamin concentrations or vitamin intake.

To graphically describe the relation of homocysteine to stenosis, we classified men and women into quartiles of homocysteine concentration. Within each quartile, we computed the prevalence of carotid stenosis $\geq 25\%$ and plotted the prevalence estimates at the sex-specific median homocysteine concentration for that quartile. To adjust for other risk factors for carotid stenosis, logistic regression was used with stenosis $\geq 25\%$ as the dependent variable. Homocysteine quartiles were modeled by using indicator variables to represent the three highest quartiles, and relative risk of stenosis for each quartile compared with the lowest quartile was esti-

mated as the odds ratio derived as the antilogarithm of the logistic regression coefficients. To examine the association between the nutritional determinants of plasma homocysteine and stenosis, we also divided subjects into quartiles for each vitamin measure and represented them in the regression models as indicator variables using the highest plasma vitamin quartile as the reference category to estimate the relative risk of lower nutrient levels. If not otherwise noted, statistical significance refers to $P < 0.05$.

RESULTS

Homocysteine distribution and prevalence of high homocysteine concentrations

The mean homocysteine concentration for all subjects was $11.9 \mu\text{mol/l}$ (median = $11.6 \mu\text{mol/l}$). Values ranged from 3.5 to $66.9 \mu\text{mol/l}$. Homocysteine concentration was higher in men than in women and increased with age (Table 1). The increase with age remained highly significant ($P < 0.001$) for men and women after adjustment for plasma vitamin concentrations, but the difference between men and women was no longer statistically significant.

We defined high homocysteine as concentrations greater than the 90th percentile among subjects with all plasma vitamin levels greater than the 70th percentile ($14.0 \mu\text{mol/l}$). Prevalence of high homocysteine was 29.3% for the entire cohort and over 40% for individuals aged 80 years and older.

Mean homocysteine concentration by vitamin status and intake

Folate. Mean plasma homocysteine concentrations for subjects in the two lowest deciles of plasma folate (below 4.8 nmol/l) were 15.6 and $13.7 \mu\text{mol/l}$. These were significantly greater than the mean for subjects in the highest decile, which was $11.0 \mu\text{mol/l}$ ($P < 0.01$) (Fig. 1a). Mean homocysteine concentrations for subjects in the three lowest deciles of folate intake ($<253 \mu\text{g/d}$) were 13.7 , 12.9 and $13.2 \mu\text{mol/l}$, respectively, and were significantly greater than the mean for subjects in the highest intake decile, which was $10.4 \mu\text{mol/l}$ ($P < 0.01$) (Fig. 2a).

Vitamin B-12. Mean homocysteine concentrations were significantly elevated for subjects in the lowest decile for vitamin B-12 relative to subjects in the highest decile ($P < 0.01$). Mean homocysteine concentrations were 15.4 and $10.9 \mu\text{mol/l}$ for subjects in the lowest and highest vitamin B-12 deciles (Fig. 1b). Subjects in the lowest vitamin B-12 decile had vitamin B-12 concentrations below 139 pmol/l . Vitamin B-12 intake appeared unrelated to mean homocysteine concentration even though subjects in the fifth decile had

TABLE 1

Mean homocysteine and B vitamin status and intake by age and sex

Age	n	Plasma concentrations					Nutrient intake/ 4200 kJ			
		Homocysteine	Homocysteine	Folate	Vitamin B-12	PLP	Folate	Vitamin B-12	Vitamin B-6	
		$\mu\text{mol/l}$	% elevated	nmol/l	pmol/l	nmol/l	μg	μg	mg	
67-74	239	11.8	25.3	9.3	265	52.6	174	3.7	1.4	
75-79	110	11.9	26.7	9.5	260	49.6	180	3.8	1.3	
80+	108	14.1	48.3	10.0	255	47.6	204	4.8	1.4	
trend (age)	—	<0.001	<0.001	0.41	0.62	0.36	0.02	0.02	0.81	
men	67-74	310	10.7	19.5	10.4	302	59.9	214	4.0	1.6
	75-79	204	11.9	28.9	10.2	289	52.2	220	4.4	1.5
	80+	189	13.2	41.4	9.7	290	52.1	199	4.9	1.5
trend (age)	—	<0.001	<0.001	0.60	0.47	0.13	0.23	0.03	0.55	
women	—	<0.003	<0.09	0.19	0.001	0.08	<0.001	0.07	0.02	

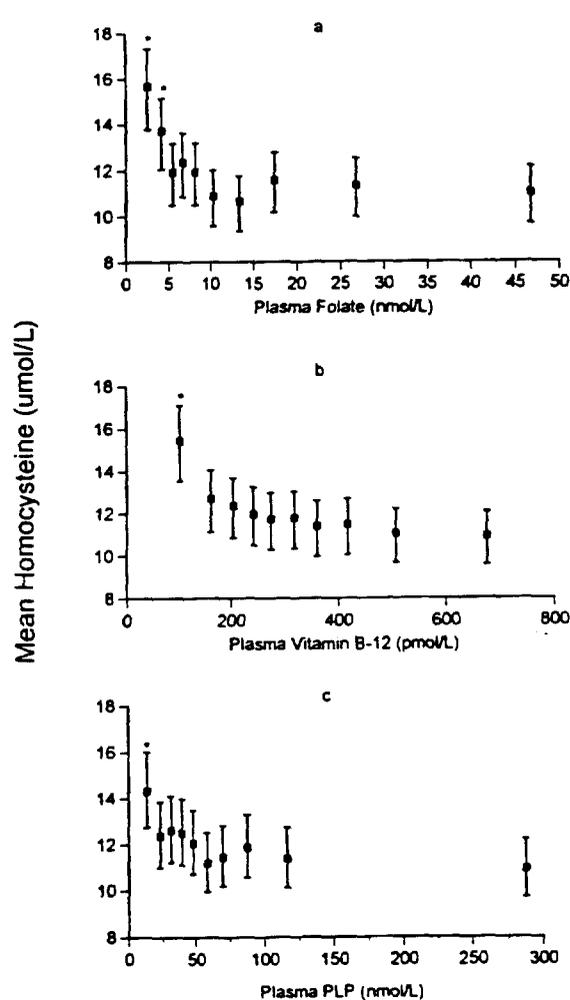


FIGURE 1 Mean plasma homocysteine concentrations (and 95% CI) by deciles of plasma folate (a), vitamin B-12 (b) and PLP (c) concentrations. Means are adjusted for age, sex and other plasma vitamins. *Significantly different from mean in the highest decile, $P < 0.05$; **significantly different from mean in the highest decile, $P < 0.01$.

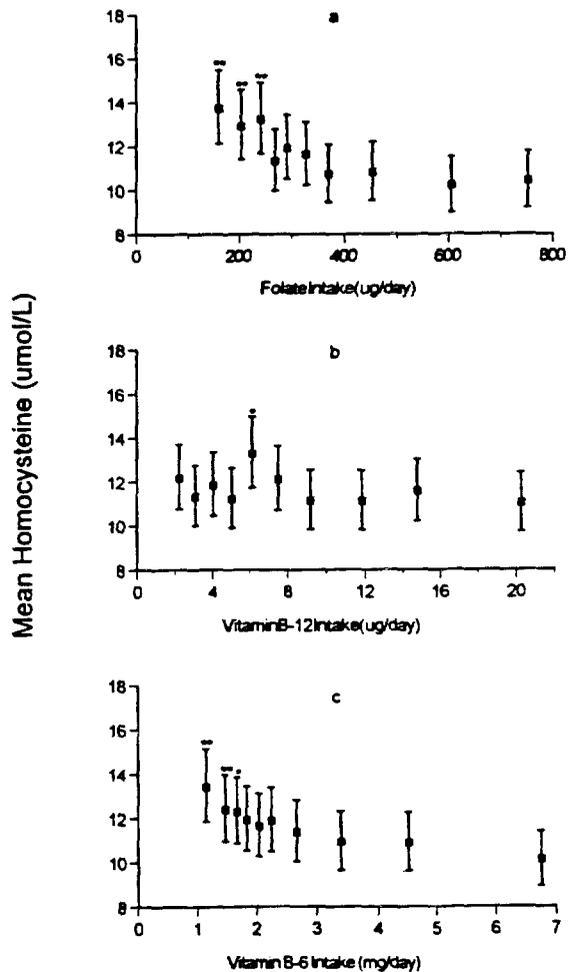


FIGURE 2 Mean plasma homocysteine concentrations (and 95% CI) by deciles of intake of folate (a), vitamin B-6 (b) and vitamin B-12 (c). Means are adjusted for age, sex and other vitamin intakes. *Significantly different from mean in the highest decile, $P < 0.05$; **significantly different from mean in the highest decile, $P < 0.01$.

TABLE 2
Elevated homocysteine concentrations by B vitamin status

B vitamin index ¹	n	Mean homocysteine	Prevalence	Prevalence rate ratio	Attributable	Population attributable
		$\mu\text{mol/l}$	%		%	%
Highest	1	89	9.4	10.1	1	0.0
	2	128	9.8	12.5	1.2	19.2
	3	534	11.9*	28.7*	2.8	64.8
	4	144	14.9*	52.1*	5.2	80.6
Lowest	5	70	16.5*	58.6*	5.8	82.8
						(total) 66.9

¹ Index combines plasma folate, vitamin B-12 and pyridoxal-5'-phosphate (PLP) concentrations: High (1) = all three B vitamins > 70th percentile; 2 = all vitamins > 50th, at least 1 < 70th percentile; 3 = vitamins above and below the 50th percentile; 4 = all vitamins < 50th percentile, at least 1 > 30th percentile; low (5) = all three vitamins < 30th percentile.

* Significantly different from Category 1, $P < 0.01$.

significantly higher homocysteine concentrations than subjects in the highest decile ($P < 0.05$) (Fig. 2b).

Vitamin B-6. Mean homocysteine concentrations were significantly elevated for subjects in the lowest decile for PLP relative to subjects in the highest decile for this vitamin ($P < 0.01$). Mean homocysteine concentrations were 14.3 and 10.9 $\mu\text{mol/l}$ for subjects in the lowest and highest PLP deciles (Fig. 1c). Subjects in the lowest decile had PLP concentrations below 18.1 nmol/l. For vitamin B-6 intake, mean homocysteine concentrations were significantly elevated in the lowest two deciles ($P < 0.01$) and the third decile ($P < 0.05$). Mean homocysteine concentrations were 13.4, 12.4 and 12.3 $\mu\text{mol/l}$ for subjects in the lowest three deciles; the mean in the highest decile was 10.1 $\mu\text{mol/l}$ (Fig. 2c). Subjects in the lowest three intake deciles reported consuming less than 1.75 mg/d.

Homocysteine concentrations by overall vitamin status

Mean homocysteine and the prevalence of high homocysteine increased dramatically across categories of the B vitamin index (Table 2). Mean homocysteine concentration was 75 and 55% greater in the lowest relative to the highest index category for the plasma index. The prevalence of high homocysteine was almost six-fold greater among subjects in the lowest index category compared with subjects in the highest category for plasma index. Sixty-seven percent of the cases of high homocysteine in this cohort of older subjects were associated with at least one vitamin concentration below the 70th percentile. Although the prevalence of high homocysteine was substantially greater in lower vitamin categories (4 and 5) than in the middle category, this latter category contributed the largest share

Relationship between plasma homocysteine and prevalence of extracranial stenosis

The prevalence of extracranial carotid stenosis $\geq 25\%$ was approximately 43 and 34% in men and women, respectively. Figure 3 shows the age-adjusted prevalence of stenosis across quartiles of plasma homocysteine levels. In men, the prevalence of stenosis $\geq 25\%$ was 27% (95% CI: 17–38%) in the lowest homocysteine quartile and 58% (95% CI: 49–67%) in the highest quartiles ($P_{\text{trend}} < 0.001$). The relation in women was not as striking as that in men; prevalence of stenosis $\geq 25\%$ ranged from 31% (95% CI: 24–38%) to 39% (95% CI: 31–47%) across homocysteine quartiles ($P_{\text{trend}} = 0.03$). Although the risk of stenosis appeared to increase in the second homocysteine quartile (9.1–11.3 $\mu\text{mol/l}$) among men, it did not appear

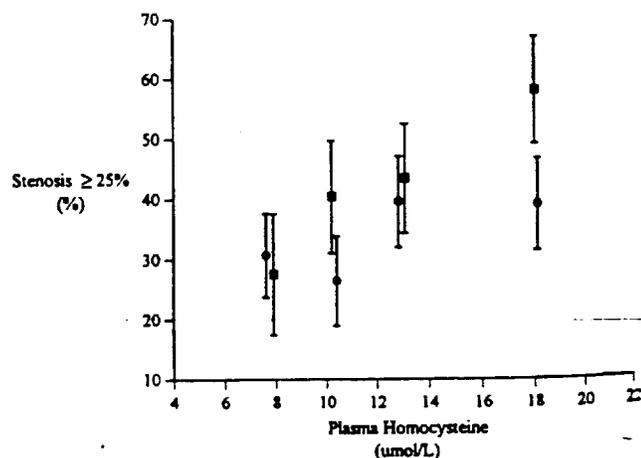


FIGURE 3 Age-adjusted prevalence and 95% CI of maximum extracranial carotid artery diameter stenosis $\geq 25\%$ by quartile of plasma homocysteine concentration in men (■) and women (●). Homocysteine quartile cutoff values were

TABLE 3

Odds ratio of maximal extracranial carotid artery diameter stenosis $\geq 25\%$ by quartile of plasma homocysteine concentration

Homocysteine quartile $\mu\text{mol/l}$	Age and sex adjusted			Multiple risk factor adjustment ¹		
	Odds ratio	95% CI ²	P value	Odds ratio	95% CI	P value
≤ 9.1	1.0			1.0		
9.2–11.3	1.1	0.8, 1.6	0.60	1.1	0.8, 1.6	0.58
11.4–14.3	1.6	1.1, 2.4	0.009	1.6	1.1, 2.3	0.02
≥ 14.4	2.1	1.5, 3.0	<0.001	2.0	1.4, 2.9	<0.001
P_{trend}			<0.001			<0.001

¹ Adjusted for sex, age, total:HDL cholesterol ratio, smoking status and systolic blood pressure for 1041 individuals.² CI = confidence interval.

to increase until the third homocysteine quartile (11.4–14.3 $\mu\text{mol/l}$) among women. Although the prevalence of stenosis appeared somewhat greater among men than women in the upper quartiles of homocysteine, a test of interaction between sex and homocysteine indicated that the trends for prevalence of stenosis $\geq 25\%$ were not significantly different for men and women ($P = 0.07$).

The age and sex adjusted odds ratios for men and women combined were significantly increased in the third (odds ratio = 1.6; 95% confidence interval: 1.1–2.4) and fourth (odds ratio = 2.1; 95% confidence interval: 1.5–3.0) quartiles of homocysteine ($\geq 14.4 \mu\text{mol/l}$)

relative to the lowest quartile ($\leq 9.1 \mu\text{mol/l}$) (Table 3). Adjustment for other risk factors had little effect on the odds ratios.

The associations between carotid stenosis and the plasma vitamins are shown in Table 4. The prevalence of stenosis $\geq 25\%$ was inversely associated with both folate ($P_{\text{trend}} < 0.001$) and pyridoxal-5'-phosphate ($P_{\text{trend}} = 0.03$) after adjustment for age, sex and other risk factors. The odds ratio for stenosis was 1.9 (95% CI: 1.3–2.7) in the lowest folate quartile and 1.6 (95% confidence interval: 1.1–2.4) in the lowest pyridoxal-5'-phosphate quartile. Plasma vitamin B-12 exhibited a weak association with stenosis ($P_{\text{trend}} = 0.11$). The odds

TABLE 4

Odds ratios of maximal extracranial carotid artery diameter stenosis $\geq 25\%$ by quartile of plasma vitamins

Vitamin	n	Multiple risk factor adjustment ¹			Multiple risk factor adjustment plus homocysteine		
		Odds ratio	95% CI ²	P value	Odds ratio	95% CI	P value
Folate, $\mu\text{g/l}^3$	1027						
<2.51		1.9	1.3, 2.7	0.001	1.5	1.0, 2.3	0.04
2.51–4.31		1.4	1.0, 2.0	0.08	1.3	0.9, 1.9	0.24
4.32–7.92		1.2	0.8, 1.8	0.28	1.2	0.8, 1.8	0.35
≥ 7.93		1.0			1.0		
P_{trend}				<0.001			0.05
Vitamin B-12, ng/l^3	881						
<290		1.4	0.9, 2.1	0.11	1.2	0.8, 1.8	0.41
290–405		1.4	0.9, 2.0	0.14	1.2	0.8, 1.8	0.36
406–572		1.3	0.9, 2.0	0.16	1.3	0.9, 1.9	0.24
≥ 573		1.0			1.0		
P_{trend}				0.11			0.47
Pyridoxal-5'-phosphate, nmol/l	967						
<31.91		1.6	1.1, 2.4	0.02	1.3	0.9, 2.0	0.15
31.91–52.19		1.1	0.7, 1.6	0.67	1.0	0.6, 1.4	0.80
52.20–89.80		1.2	0.8, 1.7	0.48	1.1	0.7, 1.6	0.71
≥ 89.81		1.0			1.0		
P_{trend}				0.03			0.23

¹ Adjusted for sex, age, total:HDL cholesterol ratio, smoking status, and systolic blood pressure.² CI = confidence interval.³ To convert to SI units, multiply folate values by 2.266 to get values in nmol/l and vitamin B-12 values by 0.7378 to get values in pmol/l .

ratio for stenosis was 1.4 (95% confidence interval: 0.9–2.1) in the lowest vitamin B-12 quartile compared with the highest quartile. Adjustment for homocysteine diminished the strength of plasma vitamin associations, but the elevated prevalence of stenosis in the lowest plasma folate quartile remained evident (odds ratio: 1.5; 95% CI: 1.0–2.3).

DISCUSSION

These data suggest an important role for nutritional status in homocysteine metabolism. We have demonstrated strong, nonlinear, inverse associations between homocysteine concentrations and plasma concentrations of folate, vitamin B-12 and vitamin B-6. We observed that individuals with low levels of each of these vitamins had high plasma homocysteine concentrations, whereas those with moderate vitamin levels had dramatically lower homocysteine concentrations. Homocysteine levels did not differ substantially between individuals with moderate and high vitamin concentrations.

The results for folate and vitamin B-6 intake data are consistent with those for the plasma vitamins. Although it is risky to attribute discrete quantitative values based on this method of dietary assessment (Willett et al. 1985), it still may be worth noting that homocysteine concentrations were elevated among individuals with folate intakes up to 280 $\mu\text{g}/\text{d}$, which is higher than the current RDA of 200 and 180 $\mu\text{g}/\text{d}$ for adult men and women, and vitamin B-6 intakes as high as 1.92 mg/d, which is less than the RDA of 2.0 mg/d for men but greater than the RDA of 1.6 mg/d for women.

Adequate levels of all three vitamins may be needed to obtain an optimal homocysteine concentration. Using the index based on levels of all three vitamins, we estimated that approximately two thirds of the cases of elevated homocysteine concentration in this cohort were associated with low or moderate plasma levels of one or more of the three vitamins.

Our data also provide evidence that plasma homocysteine levels are associated with extracranial carotid stenosis in a population-based, elderly cohort. We observed that risk of stenosis $\geq 25\%$ was increased at homocysteine concentrations previously believed to be normal based on levels of homocysteine among normative samples. As in our previous analysis, we defined elevated plasma homocysteine as concentrations $> 14 \mu\text{mol}/\text{l}$ (90th percentile among individuals with apparently adequate folate, vitamin B-12 and vitamin B-6 status). Stampfer et al. (1992) defined elevated homocysteine as concentrations $> 15.8 \mu\text{mol}/\text{l}$ (95th percentile among nondiseased control subjects). Joosten et al. (1993) defined elevated homocysteine as concentrations $> 13.9 \mu\text{mol}/\text{l}$ (mean plus 2 SD among healthy young controls). Genest and co-workers (1990) reported 90th and 95th percentile values of 15.0 and 19.0 $\mu\text{mol}/\text{l}$

among their normal controls. In the present study we observed that risk of stenosis was elevated at levels of homocysteine between 11.4 and 14.3 $\mu\text{mol}/\text{l}$. These data will require us to reconsider the current beliefs regarding standards for elevated homocysteine.

We have also examined the relations between specific nutritional determinants of hyperhomocysteinemia and stenosis in this elderly cohort. We further demonstrated that folate and pyridoxal-5'-phosphate were linked to stenosis, in large part, because of their regulation of plasma homocysteine levels as indicated by the diminished odds ratios between stenosis and these vitamins after adjustment for homocysteine levels. Although there was some residual association between plasma folate and stenosis after adjustment for homocysteine, the likelihood ratio test statistic would suggest that addition of folate to a model containing homocysteine did not add any significant contribution. It is likely that measurement error and biological variability in both folate and homocysteine might explain the residual folate association.

We demonstrated that the majority of these elderly individuals with elevated homocysteine concentrations have insufficient status of folate, vitamin B-12 or vitamin B-6, and others have demonstrated that innocuous vitamin supplementation regimens (including folate, vitamin B-12 and vitamin B-6) effectively lower moderately elevated plasma homocysteine levels to the normal range (Brattstrom et al. 1988, Brattstrom et al. 1990, Dudman et al. 1993, Franken et al. 1994, Glueck et al. 1995, Lindgren et al. 1995, Ubbink et al. 1993, Ubbink et al. 1994). Results of our present study provide the rationale for a randomized, controlled trial of the effect of homocysteine lowering vitamin therapy on vascular disease morbidity and mortality in hyperhomocysteinemic, elderly individuals.

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EXHIBIT L

Colloquium: Homocyst(e)ine, Vitamins and Arterial Occlusive Diseases

Relationship among Homocyst(e)ine, Vitamin B-12 and Cardiac Disease in the Elderly: Association between Vitamin B-12 Deficiency and Decreased Left Ventricular Ejection Fraction^{1,2}

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ABSTRACT We evaluated the association of moderate hyperhomocyst(e)inemia and vitamin B-12 status with coronary artery disease (CAD) and left ventricular ejection fraction in 367 elderly patients undergoing coronary angiography. The extent of CAD was scored, left ventricular ejection fraction was assessed and vitamins B-12 and folate and the metabolites homocyst(e)ine, methylmalonic acid and 2-methylcitric acid were measured. There was no significant trend in change in homocyst(e)ine as the extent of CAD increased. There was an association between vitamin B-12 deficiency, i.e., vitamin B-12 < 221 pmol/l and homocyst(e)ine > 16 nmol/ml and low left ventricular ejection fraction ($P = 0.014$). Of 105 samples, selected for vitamin B-12 < 221 pmol/l or high normal vitamin B-12 and folate levels, metabolites including methylmalonic acid revealed a specific diagnosis of vitamin B-12 deficiency in 18 patients. The trend among these vitamin B-12-deficient patients and low left ventricular ejection fraction was significant ($P = 0.028$). In vitro studies on rat heart revealed that nitrous oxide in the presence of 200 μ M/l methionine reduced contractility of the heart. In conclusion, vitamin B-12-deficient patients had significantly lower left ventricular ejection fractions than nonvitamin B-12-deficient patients. Whether low left ventricular ejection fraction results in malabsorption of vitamin B-12 and vitamin B-12 deficiency, or conversely, whether vitamin B-12 and its marker, elevated homocyst(e)ine, depress left ventricular function warrants further evaluation. *J. Nutr.* 126: 1249S-1253S, 1996.

INDEXING KEY WORDS:

- homocyst(e)ine • vitamin B-12
- cardiac disease • elderly • methylmalonic acid

Homocysteine is a sulfur-containing amino acid that is metabolized by transsulfuration to cysteine via cystathionine or by remethylation to methionine. Interest in homocysteine was greatly enhanced by the discovery of an inborn error of metabolism that resulted in homocystinuria and premature death. Blood homocyst(e)ine levels are extremely elevated in homocystinuria and may attain 200 nmol/ml (Malinow 1990). Characteristic arterial changes include intimal hyperplasia and fibrosis, degeneration of the internal elastic lamina and medial hyperplasia. Thrombosis commonly occurs in arteries and veins, and thromboembolism is a frequent cause of death (McCully 1969).

Moderate hyperhomocyst(e)inemia, characterized by homocyst(e)ine levels in the range of 14 or 16–30 nmol/ml, may result from a variety of causes. These include heterozygote enzyme deficiencies, such as cystathionine Beta synthase deficiency (Boers et al. 1985), ther-

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molabile methylenetetrahydrofolate reductase (Kang et al. 1993), vitamin B-6 and early vitamin B-12 and folate deficiency and renal insufficiency (Soria et al. 1990). In late vitamin B-12 and folate deficiency, levels reach those in the range of severe hyperhomocyst(e)linemia. Multiple studies have associated moderate hyperhomocyst(e)linemia with premature cerebrovascular, peripheral and coronary artery disease (CAD) (Brattstrom et al. 1984, Clarke et al. 1991, Coull et al. 1990, Genest et al. 1990, Israelsson et al. 1988, Kang et al. 1986, Malinow et al. 1989).

The aim of this study was to evaluate whether moderate hyperhomocyst(e)linemia and vitamin B-12 status were associated with CAD and left ventricular ejection fraction among a group of elderly patients.

MATERIALS AND METHODS

Data were collected on 367 patients undergoing coronary angiography at Maimonides Medical Center. The extent of CAD was scored using proximal and diffuse indices (Friesinger et al. 1970, Ringqvist et al. 1983). The coronary artery proximal score index ranged from 1 to 7. A score of 1 was assigned when proximal disease was <70% diameter stenosis, in each of the three major coronary arteries: the left anterior descending (LAD),^{*} the left circumflex (CX) and the right coronary artery (RCA) as well as <50% stenosis in the left main coronary artery (LMCA). A score of 2 was given for single vessel proximal disease ($\geq 70\%$ stenosis) in either the CX or RCA, 3 for proximal disease in the LAD, 4 for CX and RCA disease, 5 for LAD and CX or RCA disease, 6 for LMCA disease ($\geq 50\%$ stenosis) and 7 for triple vessel disease with or without LMCA disease. The coronary artery diffuse, or Friesinger score index ranged from 0 to 15. Each of the three major coronary arteries was scored separately. The scores are as follows: 0: no arteriographic abnormalities; 1: trivial luminal irregularity; 2: localized narrowing in the lumen between 50 and 90%; 3: multiple narrowings in the same vessel; 4: narrowing greater than 90%; and 5: total obstruction. Ejection fractions were qualitatively assessed in patients undergoing contrast ventriculograms ($n = 349$) by the interventional cardiologist at the time of the study.

At the time of the angiogram, fasting blood was collected on ice; plasma and serum were separated and frozen at -20°C . The protocol was approved by the Institutional Research Committee. Plasma homocyst(e)line levels, which include free and protein bound homocysteine, homocystine and the homocysteine-cysteine mixed disulfide were measured by Malinow by high performance liquid chromatography using electrochemical detection

^{*} Abbreviations used: CAD, coronary artery disease; CI, confidence interval; CX, circumflex; LAD, left anterior descending; LMCA, left main coronary artery; RCA, right coronary artery.

(Genest et al. 1990, Malinow et al. 1989). Serum vitamin B-12 and folate were measured by radioassay (Becton Dickinson, Orangeburg, NY) or by fluorometric enzyme-linked assay (Baxter Diagnostics, Deerfield, IL). The correlation between assays was 0.97 and 0.9 for vitamin B-12 and folate, respectively. One hundred five serum samples, selected for vitamin B-12 < 221 pmol/l ($n = 66$) or for high normal vitamin B-12 and folate levels, were assayed for methylmalonic acid and 2-methylcitric acid at the University of Colorado by Stabler and Allen by gas chromatography and mass spectrometry (Allen et al. 1993, Stabler et al. 1986).

Possible direct effects of L-methionine and nitrous oxide on heart function were evaluated by monitoring the changes in responses of the isolated rat heart, using an in vitro heart chamber (Pagala et al. 1992). The heart was isolated from anesthetized rats and continuously perfused with oxygenated solution at 10 ml per minute at 37°C . The heart was mounted vertically in the chamber between a fixed hook at the bottom and a movable hook attached to a force transducer at the top. The transducer monitored the tension generated by the spontaneously beating heart. The electrocardiogram was monitored by using the perfusing solution touching the apex of the ventricle as an active electrode and a platinum wire touching the auricular region as a reference electrode. Left ventricular pressure was monitored using a Millar microtip catheter introduced directly into the left ventricle. The protocol was approved by the Institutional Research and Animal Care and Use Committees.

Statistics. Means were contrasted using Student's *t* test and analysis of variance. Spearman rank correlated continuous and ordinal variables. Ejection fractions were categorized as low (<40%), moderately low (40–49%) and normal ($\geq 50\%$). Multivariate adjusted odds ratios were obtained from unconditional logistic regression models.

RESULTS

The mean age of the patients was 73 ± 6.7 (SD) y and 51.5% were men. Forty nine percent had a history of hypertension, 26% had a history of diabetes and 33% had a history of hypercholesterolemia; 14% were current smokers. Plasma homocyst(e)line level was 14.5 ± 10.8 nmol/ml. The mean creatinine was 1.1 ± 0.4 mg/dl. The mean proximal coronary score was 2.5 ± 2.0 , indicating on average significant single vessel disease. The mean Friesinger score was 6.8 ± 4.5 , suggesting that the average patient had a moderate degree of diffuse CAD. The average left ventricular ejection fraction was $50 \pm 15\%$, which is low normal. The mean hematocrit was $39.7 \pm 4.8\%$, and the mean serum vitamin B-12 and folate levels were 384 ± 232 pmol/l and 24.5 ± 12.9 nmol/l, respectively.

The mean homocyst(e)line level by proximal coronary disease score ranged from 14.2 in patients with a

TABLE 1

Mean homocyst(e)line by coronary disease index

	No. of patients	Mean homocyst(e)line
		nmol/ml
Proximal index scores ¹		
1	170	14.2
2	66	13.8
3	37	18.2
4-5	38	12.4
6-7	49	15.2
Friesinger index scores ²		
0-1	68	15.0
2-6	101	13.6
7-11	111	15.3
12-14	87	14.1

¹ P = 0.17.
² P = 0.67.

score of 1, to 15.2 nmol/ml in patients with scores of 6 to 7 (Table 1). There was no significant trend in change of homocyst(e)line as the extent of CAD increased. The mean homocyst(e)line by Friesinger (diffuse) CAD score ranged from 15.0 in patients with scores of 0 to 1, to 14.1 nmol/ml in patients with scores of 12 to 14 (Table 1). There was no significant change in homocyst(e)line as the extent of diffuse coronary artery disease increased.

Spearman correlation coefficients between plasma homocyst(e)line and several variables are shown in Table 2. The data demonstrate a significant inverse correlation between plasma homocyst(e)line and vitamin B-12 and folate and a significant direct correlation between plasma homocyst(e)line and creatinine and age. These correlations are consistent with findings in other studies [Selhub et al. 1993, Ubbink et al. 1993]. We also found a significant inverse correlation between plasma homocyst(e)line and left ventricular ejection fraction. There was no correlation between homocyst(e)line and coronary artery scores.

TABLE 2

Spearman correlation coefficients

	LV ejection fraction	Plasma homocyst(e)line
Serum vitamin B-12	0.03	-0.35 ¹
Serum folate	0.02	-0.33 ¹
LV ejection fraction		-0.11 ²
Proximal score	-0.29 ¹	0.04
Friesinger score	-0.37 ¹	0.03
Creatinine	-0.14 ³	0.45 ¹
Age	-0.01	0.19 ³

¹ P < 0.0001.
² P < 0.05.
³ P < 0.01.

Abbreviation used: LV = left ventricular.

TABLE 3

Relationship between vitamin B-12 deficiency and ejection fraction (EF) categories

	No. of deficient patients	Deficient %	P value
	total no. of patients		
Vitamin B-12 deficiency ¹			
Low EF (<40%)	14/79	17.7	0.014
Moderately low EF (40-49%)	5/54	9.3	
Normal EF (≥50%)	14/216	6.5	
Vitamin B-12 deficiency ²			
Low EF (<40%)	8/29	27.6	0.028
Moderately low EF (40-49%)	5/17	29.0	
Normal EF (≥50%)	5/59	8.0	

¹ Vitamin B-12 < 221 pmol/l and homocyst(e)line > 16 nmol/ml.
² Methylmalonic acid > 271 nmol/l and 2-methylcitric acid (nmol/l) < methylmalonic acid.

Spearman correlation coefficients between left ventricular ejection fraction and several variables are shown in Table 2. As expected, there was a significant inverse correlation between the extent of CAD and left ventricular ejection fraction. Left ventricular ejection fraction did not correlate significantly with serum vitamin B-12, folate or age. Left ventricular ejection fraction did correlate inversely and significantly with serum creatinine.

We considered patients with vitamin B-12 levels <221 pmol/l and homocyst(e)line levels >16 nmol/ml to be vitamin B-12 deficient. Nine percent of the patients were vitamin B-12 deficient. The mean vitamin B-12 level was 160 pmol/l and the range was 39 to 220. The mean homocyst(e)line was 28.7 nmol/ml and the range was 16.2 to 138. The mean hematocrit was 38.1 ± 5.0%. For nonvitamin B-12-deficient patients, the mean hematocrit was 40.4 ± 4.8. Folate deficiency, defined as folate <10.4 nmol/l and homocyst(e)line >16 nmol/ml, occurred in 3.3% of the patients.

TABLE 4

Crude and multivariate adjusted relationships between vitamin B-12 deficiency¹ and low vs. normal ejection fractions²

Model	Odds ratio	95% CI
Unadjusted	3.1	1.4-6.9
Adjusted for Age, Sex	3.0	1.3-6.7
Multivariate adjusted ³	4.0	1.4-11.3

¹ Vitamin B-12 < 221 pmol/l and homocyst(e)line > 16 nmol/ml.
² Low EF (<40%) compared with normal EF (≥50%).
³ Adjusted for age, sex, creatinine, Friesinger index, history of hypercholesterolemia, diabetes mellitus and hypertension.
Abbreviation used: CI = confidence interval.

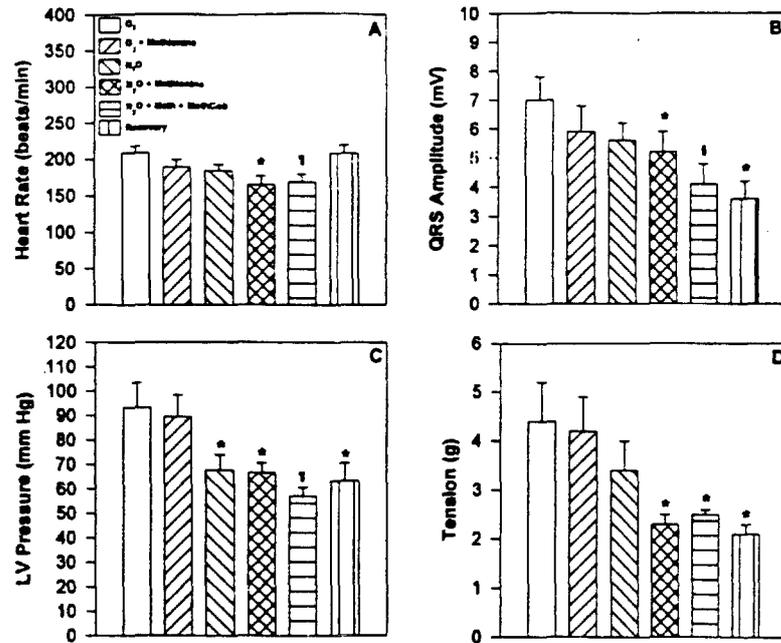


FIGURE 1 Effect of L-Methionine (200 μM), nitrous oxide (N₂O, 50%) and methylcobalamin (100 μM) on heart rate (A), QRS amplitude (B), left ventricular systolic pressure (C) and tension (D) responses of the isolated rat heart. Each bar represents the mean ± SE obtained from eight experiments. Significance of difference from control (O₂) was calculated by Student's *t* test for paired variates and is indicated as **P* < 0.05, †*P* < 0.01, and ‡*P* < 0.001.

The relationship between vitamin B-12 deficiency and ejection fraction categories is shown in Table 3. Fourteen of 79 patients (17.7%) with low ejection fractions were vitamin B-12 deficient. Five of 54 patients (9.3%) with moderately low ejection fractions were vitamin B-12 deficient. Fourteen of 216 patients (6.5%) with normal left ventricular ejection fractions were vitamin B-12 deficient. The trend between left ventricular ejection fraction and percent of patients with vitamin B-12 deficiency was significant (*P* = 0.014). Of 105 selected serum samples assayed for methylmalonic acid and 2-methylcitric acid, 18 were determined to be vitamin B-12 deficient and 4 were folate deficient. Eight of 29 patients (27.6%) with low ejection fractions were vitamin B-12 deficient, 5 of 17 patients (29.0%) with moderately low ejection fractions were vitamin B-12 deficient and 5 of 59 patients (8.0%) with normal ejection fractions were vitamin B-12 deficient (Table 3). The trend between left ventricular ejection fraction and percent of patients who were vitamin B-12 deficient was significant (*P* = 0.028).

The crude and multivariate adjusted relationships between vitamin B-12 deficiency and low vs. normal ejection fractions are shown in Table 4. The odds of a vitamin B-12-deficient patient having low vs. normal ejection fraction are 3.1 [95% confidence interval (CI) 1.4–6.9]. When adjustment is made for age and sex, the odds ratio equals 3 [CI 1.3–6.7]. When the data are adjusted for multiple factors, the odds ratio equals 4.0 [CI 1.4–11.3].

The data in Figure 1 were obtained from rat experiments. After perfusion with normal salt solution, mean heart rate was 208 beats per minute, the QRS amplitude was 7 mV, the left ventricular systolic pressure was 93 mm Hg and the peak tension was 4.5 g. After 30 min perfusion with solution containing 200 μmol/l methionine, there were small reductions in heart responses. After perfusion with solution equilibrated with a gas mixture of 50% nitrous oxide, 45% oxygen and 5% carbon dioxide with 200 μmol/l methionine, the heart rate decreased by 21% (*P* < 0.05), the left ventricular systolic pressure by 28% (*P* < 0.05) and the tension by 43% (*P* < 0.05). These reductions were not reversed by perfusion with 100 μmol/l methylcobalamin. On wash with normal solution, the depression in heart rate was reversed, but the other parameters did not recover. In a separate experiment, perfusion with nitrous oxide (50%), oxygen (45%) and carbon dioxide (5%), significantly reduced the left ventricular systolic pressure by 27% (*P* < 0.05), but not the other parameters of the heart function. These studies indicate that nitrous oxide, an inhibitor of methionine synthase [Ermens et al. 1991], in the presence of methionine, reduced contractility of the heart.

DISCUSSION

Homocyst(e)line is a vasculotoxic and thrombogenic amino acid that has been associated with premature atherosclerosis.

EXHIBIT M

EFFECT OF CALCIUM AND VITAMIN D SUPPLEMENTATION ON BONE DENSITY IN MEN AND WOMEN 65 YEARS OF AGE OR OLDER

BESS DAWSON-HUGHES, M.D., SUSAN S. HARRIS, D.Sc., ELIZABETH A. KRALL, Ph.D., AND GERARD E. DALLAL, Ph.D.

ABSTRACT

Background Inadequate dietary intake of calcium and vitamin D may contribute to the high prevalence of osteoporosis among older persons.

Methods We studied the effects of three years of dietary supplementation with calcium and vitamin D on bone mineral density, biochemical measures of bone metabolism, and the incidence of nonvertebral fractures in 176 men and 213 women 65 years of age or older who were living at home. They received either 500 mg of calcium plus 700 IU of vitamin D₃ (cholecalciferol) per day or placebo. Bone mineral density was measured by dual-energy x-ray absorptiometry, blood and urine were analyzed every six months, and cases of nonvertebral fracture were ascertained by means of interviews and verified with use of hospital records.

Results The mean (\pm SD) changes in bone mineral density in the calcium-vitamin D and placebo groups were as follows: femoral neck, $+0.50\pm 4.80$ and -0.70 ± 5.03 percent, respectively ($P=0.02$); spine, $+2.12\pm 4.06$ and $+1.22\pm 4.25$ percent ($P=0.04$); and total body, $+0.06\pm 1.83$ and -1.09 ± 1.71 percent ($P<0.001$). The difference between the calcium-vitamin D and placebo groups was significant at all skeletal sites after one year, but it was significant only for total-body bone mineral density in the second and third years. Of 37 subjects who had nonvertebral fractures, 26 were in the placebo group and 11 were in the calcium-vitamin D group ($P=0.02$).

Conclusions In men and women 65 years of age or older who are living in the community, dietary supplementation with calcium and vitamin D moderately reduced bone loss measured in the femoral neck, spine, and total body over the three-year study period and reduced the incidence of nonvertebral fractures. (N Engl J Med 1997;337:670-6.)

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INADEQUATE intake of calcium and vitamin D leads to reduced calcium absorption, increased serum parathyroid hormone concentrations, and bone loss. Low bone mass is a strong predictor of fracture.¹ Supplemental calcium reduces bone loss in middle-aged, postmenopausal women²⁻⁸ and lowers rates of vertebral fracture in women with previous vertebral fractures.⁹ Supplementation with vitamin D alone reduced bone loss from the femoral neck in postmenopausal women,^{10,11} but it did not reduce the rate of hip fracture among elderly Dutch men and women.¹² Annual intramuscular injections

of vitamin D did, however, reduce rates of arm fracture among elderly Finnish subjects.¹³

There is a rationale for supplementing the diets of elderly subjects with a combination of calcium and vitamin D. Absorption of calcium¹⁴ and possibly of vitamin D¹⁵ and production of vitamin D¹⁶ by the skin decline with aging. Diets that are deficient in calcium tend also to be deficient in vitamin D because a single food, milk, is the principal dietary source of both these nutrients. Combined calcium and vitamin D supplementation has reduced rates of nonvertebral fracture among elderly women living in retirement homes.¹⁷ In the one available study of men (mean age, 58 years) who lived at home, calcium and vitamin D together did not reduce bone loss.¹⁸ The role of combined supplements in elderly men and women living at home is unknown. We examined the effects of combined calcium and vitamin D supplementation on bone loss, biochemical measures of bone metabolism, and the incidence of nonvertebral fractures in men and women 65 years of age or older who were living in the community.

METHODS

Subjects

We studied only healthy, ambulatory men and women 65 years of age or older who were recruited through direct mailings and presentations in the community. The criteria for exclusion included current cancer or hyperparathyroidism; a kidney stone in the past five years; renal disease; bilateral hip surgery; therapy with a bisphosphonate, calcitonin, estrogen, tamoxifen, or testosterone in the past six months or fluoride in the past two years; femoral-neck bone mineral density more than 2 SD below the mean for subjects of the same age and sex; dietary calcium intake exceeding 1500 mg per day; and laboratory evidence of kidney or liver disease.

We prescreened 848 subjects by means of a questionnaire and invited 545 for screening. Of these, 51 were found to be ineligible, 49 were potentially eligible but were not enrolled, and 445 (199 men and 246 women) were enrolled. There were 430 whites, 11 blacks, and 4 Asians. The protocol was approved by the Human Investigation Review Committee at Tufts University, and written informed consent was obtained from each subject.

Study Design and Supplements

In this three-year, double-blind, placebo-controlled trial, the subjects were randomly assigned to either the placebo or the calcium-vitamin D group with stratification according to sex, race, and decade of age. At study entry, we performed physical examinations and assessed the subjects' medical history, diet, and phys-

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ical-activity level; analyzed blood and urine; and measured bone mineral density. The subjects were advised to maintain their usual diets and to avoid taking supplemental calcium and vitamin D on their own for two months before and throughout the study. At bedtime, the subjects took separate pills containing 500 mg of elemental calcium in the form of calcium citrate malate¹⁹ and 700 IU of cholecalciferol or separate placebo tablets containing microcrystalline cellulose.

Calcium citrate malate (Procter & Gamble, Cincinnati) was prepared in two batches; assays confirmed that the contents were as expected. The vitamin D tablets used initially contained 707 IU; two years later, the tablets were found to contain 563 IU (80 percent) of the planned dose of 700 IU; a second lot initially containing 768 IU was used during the second half of the study. The tablets were stored in opaque bottles at room temperature.

Status of Subjects and Compliance

During the trial, 127 subjects discontinued treatment; 4 died, 40 stopped for personal reasons (e.g., they lost interest or moved away), 46 withdrew because of illness, 17 started estrogen or glucocorticoid therapy, and 20 withdrew because of problems with the medication. The majority of subjects who discontinued treatment did so in the first year. These subjects were encouraged to return for all subsequent follow-up evaluations. At the last visit, 389 subjects (87 percent of the 445 enrolled) were evaluated and were included in the main intention-to-treat analyses. The 318 subjects who remained in the two study groups (i.e., those who took the supplements throughout the study period) were included in the analyses of subjects who completed the study according to the protocol.

The mean (\pm SD) rate of compliance with treatment, assessed on the basis of pill counts, was 92 ± 10 percent for the calcium or placebo tablets and 93 ± 10 percent for the vitamin D or placebo tablets among the 318 subjects who completed the study.

Measurements

The subjects came to the center every six months for measurements of bone mineral density, biochemical assays, and other measurements. Their calcium and vitamin D intake was estimated on the basis of a food-frequency questionnaire.²⁰ During the study, 44 of the subjects who completed the study treatment (23 in the placebo group and 21 in the calcium-vitamin D group) reported taking products that contained some calcium or vitamin D. They were asked to stop taking these products, and the intake from supplements was added to their dietary intake during the relevant period. Leisure, household, and occupational activity was estimated with use of the Physical Activity Scale for the Elderly questionnaire.²¹ Tobacco use was determined by questionnaire. Height was measured with a stadiometer, and weight with a digital scale.

The subjects were asked to send in a postcard after any fall. When such a postcard was received, a staff member called the subject to verify the circumstances. Subjects reported any additional falls at each follow-up visit. Nonvertebral fractures were identified during interviews at the same visits. The principal investigator, who was unaware of the subjects' study-group assignments, classified the fractures as nonosteoporotic (resulting from severe trauma) or osteoporotic (resulting from moderate-to-minor trauma — i.e., a fall from standing height or less). All but one nonvertebral fracture (a presumed toe fracture that was not treated) were verified by review of x-ray reports or hospital records.

Analytic Methods

Bone mineral density in the hip, spine, and total body was measured by dual-energy x-ray absorptiometry with use of a DPX-L scanner (Lunar Radiation, Madison, Wis.). Scanner software versions 1.2 and 1.3y were used for data acquisition and analysis, respectively. The coefficients of variation for the measurements were 2.0 percent (femoral neck), 1.0 percent (spine), and 0.6 percent (total body). The scans of the hip were performed in duplicate,

with repositioning between scans, and the values were averaged. A phantom consisting of bone ash embedded in a 12-cm block was scanned every other week as a control; the bone mineral density of the phantom was stable throughout the study.

Blood was drawn between 7:00 and 9:30 a.m. after the subjects had fasted for at least eight hours. Urine measurements were made in 24-hour collections. Plasma 25-hydroxyvitamin D was measured by the method of Preece et al.,²² plasma 1,25-dihydroxyvitamin D by a competitive protein-binding method,²³ serum parathyroid hormone by immunometric assay (Nichols Institute, San Juan Capistrano, Calif.), serum osteocalcin by immunoradiometric assay (Nichols Institute), urinary *N*-telopeptide cross-links by enzyme-linked immunosorbent assay (Ostex International, Seattle), and serum ionized calcium and urinary calcium and creatinine as reported previously.²⁰ The coefficients of variation for these assays ranged from 5.6 percent to 7.7 percent. Analyses were performed as the samples were collected, except for the plasma 1,25-dihydroxyvitamin D and urinary *N*-telopeptide assays, for which initial and final samples were analyzed at the same time.

Statistical Analysis

Comparisons between the study groups were made with two-sample *t*-tests and, when adjustments were required, with analysis of covariance. Terms for the interaction of sex and study group in analysis-of-variance models of the change in bone mineral density were statistically significant only at the femoral neck in the subjects in the intention-to-treat analysis; this term did not remain significant after adjustment for the duration of treatment. The relative risks of fracture among the subjects in the calcium-vitamin D and placebo groups were compared by means of the chi-square test. Analyses were conducted with SPSS (SPSS Inc., Chicago) and SAS (SAS Institute, Cary, N.C.) software. All *P* values are two-sided. Intention-to-treat analyses were conducted according to the principles described by Newell²⁴; selected secondary analyses were restricted to subjects who completed the study.

RESULTS

The base-line characteristics of the 389 subjects are shown in Table 1. As compared with placebo, supplementation with calcium and vitamin D had a significant positive effect on the change over three years in bone mineral density measured at the femoral neck, spine, and total body in all subjects together and in the men (Table 2). The women in the calcium-vitamin D group had significantly less total-body bone loss than those in the placebo group; the differences in the changes at the femoral neck and spine were smaller and not statistically significant. Adjustment for differences between the study groups in base-line bone mineral density and calcium intake did not alter the results.

The time course of the response to treatment was examined in the 318 subjects who completed the study. Their clinical characteristics and bone mineral density at base line did not differ significantly from those of subjects who discontinued the study treatment, except that smoking was more prevalent in the latter group (10 percent, as compared with 4 percent among those who completed the study; $P = 0.02$). During the first year there was significantly less bone loss at the hip, spine, and total body in the calcium-vitamin D group; during the second and third years, however, there was significantly less loss only in the total body (Table 3).

TABLE 1. BASE-LINE CHARACTERISTICS OF THE 389 STUDY SUBJECTS.*

CHARACTERISTIC	MEN		WOMEN	
	PLACEBO GROUP (N = 90)	CALCIUM-VITAMIN D GROUP (N = 86)	PLACEBO GROUP (N = 112)	CALCIUM-VITAMIN D GROUP (N = 101)
Age (yr)	71±5	70±4	72±5	71±4
Height (cm)	173.8±6.9	174.3±6.2	159.5±6.6	159.2±6.4
Weight (kg)	81.5±12.8	82.4±11.3	68.1±12.4	67.6±12.1
Dietary calcium intake (mg/day)	673±349	748±391	798±366	689±286
Dietary vitamin D intake (IU/day)	197±117	202±104	184±110	174±90
Smoker (%)	4.4	7.0	5.4	5.9
Physical-activity score	127±56 (89)	124±60 (85)	108±54	105±48
Bone mineral density (g/cm ²)				
Femoral neck	0.95±0.12	0.99±0.14	0.81±0.11	0.80±0.11
Spine	1.27±0.20 (89)	1.32±0.21	1.05±0.20 (109)	1.03±0.18 (97)
Total body	1.19±0.09 (89)	1.22±0.09	1.02±0.09	1.02±0.10

*Plus-minus values are means ±SD. When there were missing data, the number of subjects for whom data were available is shown in parentheses.

TABLE 2. CHANGE IN BONE MINERAL DENSITY OVER THREE YEARS IN ALL SUBJECTS AND IN SUBJECTS WHO COMPLETED THE STUDY.*

SUBJECTS AND SITE	ALL SUBJECTS (N = 389)			SUBJECTS COMPLETING STUDY (N = 318)		
	PLACEBO GROUP (N = 202)	CALCIUM-VITAMIN D GROUP (N = 187)	P VALUE	PLACEBO GROUP (N = 170)	CALCIUM-VITAMIN D GROUP (N = 148)	P VALUE
	percent change			percent change		
All subjects						
Femoral neck	-0.70±5.03 (201)	+0.50±4.80 (185)	0.02	-0.45±5.07 (170)	+0.81±4.44 (148)	0.02
Spine (L2-L4)	+1.22±4.25 (197)	+2.12±4.06 (180)	0.04	+1.27±4.31 (166)	+2.56±3.93 (145)	0.006
Total body	-1.09±1.71 (199)	+0.06±1.83 (186)	<0.001	-1.04±1.71 (168)	+0.30±1.58 (148)	<0.001
Men						
Femoral neck	-1.35±4.70 (90)	+0.95±4.07 (85)	<0.001	-0.88±4.59 (77)	+0.91±3.92 (71)	0.01
Spine (L2-L4)	+1.74±3.85 (89)	+2.93±3.42 (84)	0.03	+2.03±3.69 (76)	+3.34±3.33 (70)	0.03
Total body	-0.85±1.53 (88)	+0.34±1.40 (86)	<0.001	-0.67±1.47 (75)	+0.48±1.34 (71)	<0.001
Women						
Femoral neck	-0.17±5.25 (111)	+0.11±5.34 (100)	0.70	-0.09±5.43 (93)	+0.71±4.90 (77)	0.31
Spine (L2-L4)	+0.78±4.54 (108)	+1.41±4.45 (96)	0.32	+0.63±4.71 (90)	+1.85±4.32 (75)	0.09
Total body	-1.29±1.82 (111)	-0.17±2.11 (100)	<0.001	-1.34±1.84 (93)	+0.14±1.76 (77)	<0.001

*Plus-minus values are means ±SD. The number of subjects for whom data were available is shown in parentheses. An interaction of sex with study group was statistically significant only at the femoral neck in all subjects (P=0.05); the P value for this interaction in subjects who completed the study was 0.36.

Among the 318 subjects who completed the study, those treated with calcium and vitamin D had significantly greater changes in a number of biochemical measures of bone metabolism (Table 4). Serum osteocalcin concentrations and urinary excretion of N-telopeptide were significantly lower in the men than in the women throughout the study (P = 0.005).

Among the 389 study subjects, 37 (5 men and 32 women) had at least one nonvertebral fracture during the study period. The cumulative incidence of a first fracture at three years was 5.9 percent in the cal-

cium-vitamin D group and 12.9 percent in the placebo group (relative risk, 0.5; 95 percent confidence interval, 0.2 to 0.9; P = 0.02) (Table 5 and Fig. 1). Among the women in the placebo group, the incidence of fractures at three years was 19.6 percent. Twenty-eight subjects (76 percent) had fractures classified as osteoporotic; the three-year cumulative incidence of a first osteoporotic fracture in the calcium-vitamin D group was lower than that in the placebo group (relative risk, 0.4; 95 percent confidence interval, 0.2 to 0.8; P = 0.01). Only two men, both

EFFECT OF CALCIUM AND VITAMIN D SUPPLEMENTATION ON BONE DENSITY IN OLDER PERSONS

TABLE 3. RATES OF CHANGE IN BONE MINERAL DENSITY IN 318 SUBJECTS WHO COMPLETED THE STUDY, ACCORDING TO THE DURATION OF TREATMENT.*

SUBJECTS AND SITE	YEAR 1			YEARS 2 AND 3		
	PLACEBO GROUP	CALCIUM-VITAMIN D GROUP	P VALUE	PLACEBO GROUP	CALCIUM-VITAMIN D GROUP	P VALUE
	percent change/year			percent change/year		
All						
Femoral neck	-0.22±3.65 (168)	+0.64±3.96 (145)	0.05	-0.08±2.42 (168)	+0.18±1.90 (145)	0.30
Spine (L2-L4)	-0.29±2.92 (165)	+1.09±2.59 (145)	<0.001	+0.79±1.90 (166)	+0.73±1.50 (144)	0.75
Total body	-0.76±1.28 (168)	-0.16±1.11 (146)	<0.001	-0.14±0.68 (168)	+0.23±0.70 (146)	<0.001
Men						
Femoral neck	-0.55±3.61 (76)	+0.56±3.36 (69)	0.06	-0.12±2.22 (76)	+0.36±1.72 (69)	0.15
Spine (L2-L4)	+0.31±2.83 (76)	+1.29±1.95 (71)	0.02	+0.87±1.59 (76)	+1.00±1.54 (70)	0.61
Total body	-0.33±1.11 (76)	-0.10±1.14 (70)	0.22	-0.17±0.65 (76)	+0.30±0.59 (70)	<0.001
Women						
Femoral neck	+0.05±3.68 (92)	+0.72±4.46 (76)	0.30	-0.04±2.60 (92)	+0.01±2.04 (76)	0.88
Spine (L2-L4)	-0.80±2.91 (89)	+0.90±3.08 (74)	<0.001	+0.72±2.13 (90)	+0.46±1.43 (74)	0.36
Total body	-1.11±1.30 (92)	-0.22±1.08 (76)	<0.001	-0.11±0.71 (92)	+0.18±0.79 (76)	0.02

*Plus-minus values are means ±SD. The number of subjects for whom data were available is shown in parentheses.

TABLE 4. INITIAL LABORATORY VALUES AND CHANGES AT THREE YEARS IN 313 SUBJECTS WHO COMPLETED THE STUDY, ACCORDING TO STUDY GROUP.*

INDEX AND STUDY GROUP	MEN (N = 146)		WOMEN (N = 167)	
	INITIAL VALUE	CHANGE	INITIAL VALUE	CHANGE
Serum ionized calcium (mg/dl)				
Placebo	5.0±0.2	+0.0±0.1	5.0±0.2	+0.0±0.2
Calcium-vitamin D	5.0±0.2	+0.1±0.2†	5.1±0.2	+0.1±0.1
Plasma 25-hydroxyvitamin D (ng/ml)				
Placebo	33.6±12.7	-2.68±10.2	24.5±10.3	+0.7±8.1
Calcium-vitamin D	33.0±16.3	+11.8±11.6†	28.7±13.3‡	+16.1±14.3‡
Plasma 1,25-dihydroxyvitamin D (pg/ml)§				
Placebo	33.3±6.7	-4.8±8.7	37.3±8.0	-6.7±8.7
Calcium-vitamin D	33.6±7.0	-6.3±11.0	36.5±7.3	-5.8±9.5
Serum parathyroid hormone (pg/ml)				
Placebo	34.8±13.6	+6.2±11.2	42.6±18.9	+5.7±15.0
Calcium-vitamin D	38.0±19.1	-7.0±12.9†	37.4±15.3‡	-5.5±13.2†
Serum osteocalcin (ng/ml)				
Placebo	5.7±1.9	+0.2±1.6	7.0±2.4	+0.0±2.1
Calcium-vitamin D	5.3±1.3	-0.5±1.4†	6.9±2.5	-0.9±1.9†
24-hr urinary calcium:creatinine ratio (mg/g)				
Placebo	98±46	-4±44	119±55	+9±62
Calcium-vitamin D	98±50	+35±51†	113±64	+67±64†
24-hr urinary N-telopeptide:creatinine ratio (nmol/mmol)				
Placebo	32±16	+1±10	48±30	-2±32
Calcium-vitamin D	29±9	-2±12	45±17	-2±16

*Plus-minus values are means ±SD. To convert values for calcium to millimoles per liter, multiply by 0.25; to convert values for 25-hydroxyvitamin D to nanomoles per liter, multiply by 2.50; to convert values for 1,25-dihydroxyvitamin D to picomoles per liter, multiply by 2.40; to convert values for parathyroid hormone to picomoles per liter, multiply by 0.106; to convert values for osteocalcin to nanomoles per liter, multiply by 0.172; to convert values for the 24-hour urinary calcium:creatinine ratio to millimoles per mole, multiply by 2.82. Initial or final laboratory values were missing for five subjects.

†P<0.005 for the comparison between the study groups.

‡P=0.05 for the comparison between the study groups.

§Final measurements were made at 18 months.

TABLE 5. NUMBER OF FIRST NONVERTEBRAL FRACTURES AMONG ALL SUBJECTS, ACCORDING TO SKELETAL SITE.

SITE OF FRACTURE	PLACEBO GROUP (N=202)	CALCIUM-VITAMIN D GROUP (N=187)
Face	1	1
Shoulder, humerus, or clavicle	4	3
Radius or ulna	5	1
Hand	1	1
Ribs	2	2
Pelvis	2	0
Hip	1	0
Tibia or fibula	1	1
Ankle or foot	7	2
Multiple sites	2	0
Total	26	11

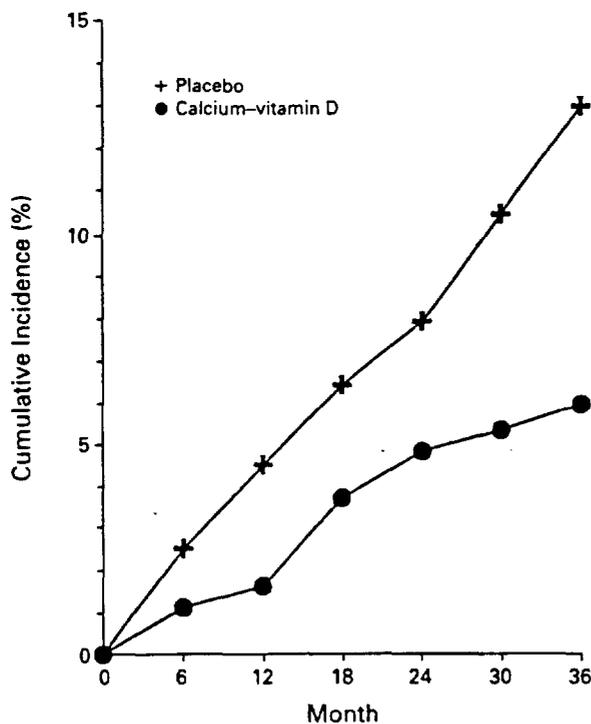


Figure 1. Cumulative Percentage of All 389 Subjects with a First Nonvertebral Fracture, According to Study Group. By 36 months, 26 of 202 subjects in the placebo group and 11 of 187 subjects in the calcium-vitamin D group had had a fracture (P=0.02).

in the placebo group, had osteoporotic fractures, and the best predictor of osteoporotic fracture was female sex (P<0.001). Among the 318 subjects who completed the study, the relative risk of any first nonvertebral fracture in the calcium-vitamin D group as compared with the placebo group was 0.4 (95 percent confidence interval, 0.2 to 1.0; P=0.03), and that for fractures classified as osteoporotic was 0.4 (95 percent confidence interval, 0.2 to 1.1; P=0.06). There was no significant difference between the treatment groups in the percentage of subjects who fell; among women, the number of falls per subject who fell was somewhat higher in the calcium-vitamin D group than in the placebo group (data not shown). Two women (one in each study group) had a second osteoporotic fracture during the study.

The supplements were generally well tolerated, but 11 subjects discontinued treatment because of difficulty swallowing the pills and 9 discontinued because of other side effects (in the placebo group: 2 because of epigastric distress and 1 because of flank pain; in the calcium-vitamin D group: 3 because of constipation, 1 because of epigastric distress, 1 because of sweating, and 1 because of hypercalciuria).

DISCUSSION

In this study, dietary supplementation with calcium and vitamin D reduced bone loss moderately in men and women 65 years of age or older who were living in the community. Among the men, there was a significant effect of treatment at the hip, spine, and total body. In an earlier study by Orwoll et al., a similar regimen of calcium and vitamin D had no effect, perhaps because the men in that study were younger and had a higher mean calcium intake than the men we studied (1160 vs. about 700 mg per day).¹⁸ The reduction in total-body bone loss in women in this study was similar to that in other trials of calcium supplementation alone.^{3,4} The estimated differences in bone mineral density at the femoral neck and spine among the women in the two study groups were similar to those found in other studies,^{2,4,6,10,11,25} although the differences did not reach statistical significance in our study. The effect of supplementation in all subjects was similar to that in the subjects who completed the study, as would be expected, given the high degree of overlap between the two groups. Treatment caused few symptoms or side effects.

In both men and women, calcium-vitamin D supplementation reduced total-body bone loss not only in the first year (an effect that could be ascribed to the closure of bone-remodeling space²⁶), but also in the second and third years, suggesting long-term effectiveness of supplementation in terms of the skeleton as a whole. The initial effects of supplementation at the hip and spine during year 1 were maintained but not increased during the ensuing two years of the

study. Others have reported a cumulative benefit in terms of total-body^{3,4} and femoral-neck³ bone density with the use of higher doses of calcium in younger postmenopausal women. Spinal bone mineral density increased in both study groups, probably because of increases in osteoarthritis and aortic calcification.^{27,28}

After three years of calcium-vitamin D supplementation, serum osteocalcin concentrations were 9 percent lower in the men and 14 percent lower in the women than at base line, indicating that supplementation led to a sustained reduction in the rate of bone remodeling. The lack of change in urinary N-telopeptide excretion may reflect the variability of this measurement. Our study confirms previous observations that the rate of bone turnover, as measured by urinary excretion of pyridinoline cross-links²⁹ and serum osteocalcin concentrations,³⁰ is lower in men than in women.

The reduction in the incidence of nonvertebral fractures in the calcium-vitamin D group should be interpreted with some caution, because of the small number of study subjects. Nonetheless, the magnitude of the reduction in the risk of fracture was similar to that reported in a study of more than 3400 elderly French women treated with 1200 mg of calcium plus 800 IU of vitamin D or placebo each day.¹⁷ In a study of 2600 elderly Dutch men and women, there was no reduction in the incidence of fractures among those given 400 IU of vitamin D daily (without calcium), as compared with those given placebo.¹² Our results differ from those of the Dutch study, possibly by chance (we studied fewer subjects) or because the treatments differed. When comparing the three-year rates of nonvertebral fractures among women assigned to placebo in several recent trials, we found that the 19.6 percent rate in this study was intermediate between the 9 percent reported for women who were, on average, 7 years younger than our subjects³¹ and the 27 percent reported for women who were 13 years older.¹⁷ We do not know the individual contributions of calcium or vitamin D to the results in our study.

The limited effect of calcium and vitamin D on bone mineral density, which was evident primarily in year 1, seems unlikely to account for the constant decline in the rate of nonvertebral fractures during the three-year study. A treatment-induced reduction in the incidence of falls does not appear to account for the reduction in the rate of fractures, since the number of falls was similar in the two groups. The reduction in the rate of bone turnover may have influenced the fracture rate by reducing the potential for trabecular perforation and reducing cortical porosity.

In conclusion, calcium and vitamin D supplementation leads to a moderate reduction in bone loss and may substantially reduce the risk of nonvertebral fractures among men and women 65 years of age or older who live in the community.

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This article does not necessarily reflect the views or policies of the U.S. Department of Agriculture, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government.

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Symposium: Nutritional Advances in Human Bone Metabolism

Calcium and Vitamin D Nutritional Needs of Elderly Women^{1,2}

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ABSTRACT Because osteoporosis is irreversible, the effective approach to reduce morbidity and mortality from this disease is to maximize peak bone mass and minimize bone loss. This presentation reviews the evidence that calcium and vitamin D influence rates of bone loss in postmenopausal women. In the first five years after menopause, women lose bone very rapidly. During this period, high dose calcium supplementation modestly reduces cortical loss from long bones but has minimal effect on more trabecular sites such as the spine. In addition, vitamin D appears to increase the effectiveness of supplemental calcium. Postmenopausal women are generally more responsive to added calcium, and those with the lowest calcium intakes benefit the most. In calcium-deficient women, supplementation with vitamin D reduces bone loss and fracture incidence. Available evidence indicates that postmenopausal women should consume 1000–1500 mg of calcium and 400 to 800 IU of vitamin D per day to minimize bone loss. *J. Nutr.* 126: 1165S–1167S, 1996.

INDEXING KEY WORDS:

calcium • vitamin D • bone density
osteoporosis • postmenopausal women

Increasing peak bone mass and retarding age-related bone loss will have a favorable impact on osteoporotic fracture rates. With the loss of estrogen at menopause, women lose bone mineral very rapidly (3% per year) about 5 years and then lose more slowly (1% per year) thereafter. Many factors affect the rate of bone loss including heredity, physical activity, smoking and calcium and vitamin D nutritional status. Here I will discuss changes in calcium homeostasis that occur with aging and how they appear to influence the calcium and vitamin D intakes needed to minimize bone loss in postmenopausal women. Most of the informa-

tion on the bone response to changes in dietary calcium and vitamin D comes from intervention studies in which change in bone mineral density (BMD), not fracture incidence, is the end point. Although antifracture efficacy is of primary interest, change in BMD is widely used in clinical trials because BMD is a strong predictor of fracture risk, and with its use, a far smaller sample size (or study duration) is required.

Calcium absorption, estrogen and aging

At low to moderate calcium intakes, intestinal calcium absorption occurs largely by active transport, a process mediated by 1,25-dihydroxyvitamin D [$1,25\text{(OH)}_2\text{D}$]. As intake increases >500 mg/d, passive diffusion accounts for an increasing proportion of calcium absorbed (Ireland and Fordtran 1973). Calcium absorption efficiency declines at menopause (Heaney et al. 1989) and also with aging (Bullamore et al. 1970). Estrogen enhances calcium absorption indirectly by enhancing $1,25\text{(OH)}_2\text{D}$ production in the kidney. It also preserves a normal intestinal responsiveness to $1,25\text{(OH)}_2\text{D}$ (Gennari et al. 1990), perhaps by modulating the function of the intestinal vitamin D receptor (VDR). The age-related decline in calcium absorption efficiency may be related to increasing intestinal VDR resistance to the action of $1,25\text{(OH)}_2\text{D}$ (Ebeling et al.

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² The contents of this publication do not necessarily reflect the views or policies of the U.S. Department of Agriculture, nor does mention of tradenames, commercial products or organizations imply endorsement by the U.S. government.

1992) and also to reduced $1,25(\text{OH})_2\text{D}$ production capacity (Slovik et al. 1981). Because the loss of estrogen and aging affect the active transport mechanism, it is likely (though never demonstrated) that the decline in intestinal calcium absorption after menopause and with continued aging would be most pronounced in subjects with low calcium intakes.

Calcium intake and bone loss

Loss of estrogen triggers bone loss that in turn provides skeletal calcium to the extracellular fluid. This subtle increase in circulating ionized calcium suppresses PTH secretion, lowers $1,25(\text{OH})_2\text{D}$ levels and reduces calcium absorption. Accordingly, one might anticipate that supplemental calcium would have a relatively modest effect on the skeleton in early menopause. This in fact has been the finding in a number of controlled calcium trials in early menopausal women (Dawson-Hughes et al. 1990, Elders et al. 1991), although the skeletal responsiveness to calcium does appear to vary some by skeletal site. A modest cumulative benefit from calcium supplementation is seen at the radius but not at the spine. Fewer data are available for the hip but in an important 3-y study, Aloia et al. (1994) found that combined calcium (1700 mg/d) and vitamin D (400 IU/d) supplementation retarded bone loss from the femoral neck.

A role for calcium in reducing bone loss in late postmenopausal women is apparent from controlled trials (Dawson-Hughes et al. 1990, Reid et al. 1993). For women in this age group, calcium reduces bone loss from the hip and radius, and the benefit is greater in those with lowest dietary calcium intakes. As with the early menopausal women, cortical sites are more responsive than trabecular sites to calcium. A recent NIH Consensus Conference on Calcium considered optimal intakes of calcium to be 1000 mg/d for postmenopausal women taking estrogen and 1500 mg/d for women not taking estrogen (Optimal Calcium Intake 1994). Women in the United States typically consume far less than is recommended, ~500–600 mg/d.

Vitamin D metabolism and aging

Vitamin D is central to the issue of preserving skeleton mass because its active metabolite, $1,25(\text{OH})_2\text{D}$, stimulates calcium absorption. By the same regulatory system described above, vitamin D insufficiency causes reduced calcium absorption, a subtle fall in blood ionized calcium, a rise in circulating PTH and increased bone resorption. The serum concentration of 25-hydroxyvitamin D ($25(\text{OH})\text{D}$) is the best clinical index of vitamin D status because it reflects contributions from dietary and skin sources of the vitamin and because its serum concentration fluctuates the least after an acute change in sun exposure or dietary intake. With aging,

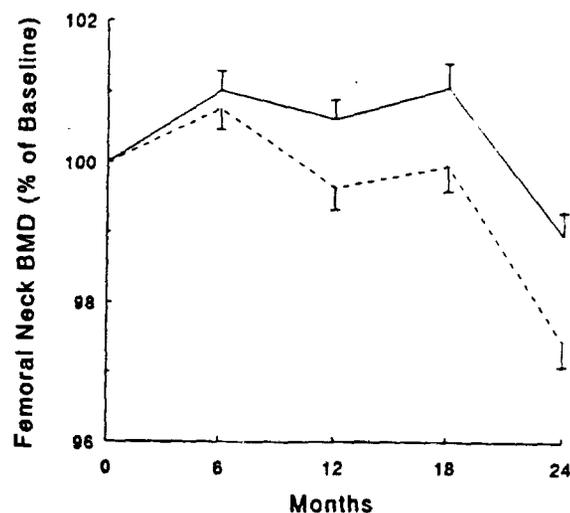


FIGURE 1 Change in femoral neck bone mineral density (BMD) over 2 years in 243 women treated with 700 IU (17.5 µg, —) and 100 IU (2.5 µg, ----) of vitamin D per day. All women received 500 mg of supplemental calcium per day. Baseline measurements in all women were made in June and July so that the first 6-mo interval of each study year was summer-fall and the second was winter-spring. Overall percent change in BMD differed significantly by treatment group ($P = 0.003$). [Reprinted with permission from Dawson-Hughes et al. 1995].

serum $25(\text{OH})\text{D}$ declines as a result of less efficient skin synthesis of vitamin D, less efficient intestinal absorption and possibly also reduced sun exposure and intake of the vitamin.

In much of the heavily populated temperate zone, season influences $25(\text{OH})\text{D}$ levels (Webb et al. 1988). For example, in Boston, latitude 42°N , skin synthesis of vitamin D does not occur between October and March because the ultraviolet B rays that photoconvert 7-dehydrocholesterol to previtamin D do not reach the earth's surface. During the winter and early spring, therefore, people at this latitude rely entirely on dietary sources of vitamin D. Individuals who have limited sun exposure or who use sunscreens depend on dietary vitamin D year round. In 1991 we found that a 400-IU vitamin D supplement prevented wintertime declines in serum $25(\text{OH})\text{D}$ and rises in serum PTH concentrations in healthy ambulatory postmenopausal women consuming ~100 IU/d of vitamin D in their diets (Dawson-Hughes et al. 1991).

Vitamin D, bone loss and fractures

In the 1-y study just cited (Dawson-Hughes et al. 1991), the 400-IU vitamin D supplement reduced wintertime bone loss from the spine and provided a net benefit at this site. To test other vitamin D doses, we enrolled 247 healthy women, mean age 64 y, in a 2-y trial. Volunteers were randomized to treatment with

100 IU or 700 U of vitamin D/d. The women consumed ~100 IU in their diets so that total vitamin D intakes of ~200 IU [the current Recommended Dietary Allowance (RDA)] and 800 IU were being compared. All women received supplemental calcium, which brought their mean intakes to just over 1000 mg/d. At the spine, change in BMD was similar in the two groups. At the femoral neck, however, loss was greater in the low vitamin D supplement group (see Fig. 1). Seasonal fluctuation in BMD is also apparent in Figure 1. From this study, we conclude that the RDA of 200 IU/d is not sufficient to minimize bone loss from the hip in healthy postmenopausal women residing at latitude 42°N.

Two recent studies assessed the effect of vitamin D, with and without calcium, on fracture rates in the very elderly. Chapuy et al. (1994) found that combined calcium (1200 mg) and vitamin D (800 IU) supplementation reduced hip and other nonvertebral fracture rates in French nursing home residents. Supplementation with 400 IU of vitamin D alone did not alter hip fracture rates significantly in free-living, elderly Dutch women (Lips et al. 1994). Collectively, these studies seem to place the vitamin D intake requirement at 400–800 IU/d in postmenopausal women who have adequate calcium intakes.

In conclusion, supplemental calcium can reduce bone loss in postmenopausal women. Added calcium is more effective at cortical than at trabecular skeletal sites, and women with the lowest dietary calcium intakes benefit the most from supplementation. Intakes of 1000 mg/d for estrogen-replete and 1500 mg/d for estrogen-deficient women are considered optimal. Adequate vitamin D is needed to achieve the full benefit from calcium. An intake of 400–800 IU/d appears to be needed for healthy postmenopausal healthy women who have optimal calcium intakes and limited skin synthesis of vitamin D. Skin synthesis is limited in the wintertime in women who reside in the temperate zone and is limited year round for those who wear sunscreens and for those who are home or institution bound or for other reasons avoid sun exposure.

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Exhibit N



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*Osteoporosis, Postmenopausal / dt [Drug Therapy]

Abstract

A systematic review of the literature was conducted to assess the effectiveness of calcium supplements and/or **dietary** calcium for the prevention of osteoporotic **fractures** in postmenopausal women. Studies were identified by conducting a Medline search using the text words "fracture" and "calcium" for the period 1966 to March 1997 and by reviewing articles known to the authors. Only studies with fracture outcomes were eligible. There were 14 studies of calcium supplements (including 4 randomized trials), 18 studies of **dietary** calcium and hip fracture (no randomized trials), and 5 studies of **dietary** calcium and other fracture sites (no randomized trials). The 4 randomized trials of calcium supplements (mean calcium dose: 1050 mg) found relative risk (RR) reductions between 25% and 70%. Meta-analytic techniques for dose-response data were used to investigate and pool the findings of 16 observational studies of **dietary** calcium and hip fracture. These hip fracture studies were not consistent and heterogeneity of study findings ($p = 0.02$) was not easily explained by subject characteristics or study design. Pooling study results gave an odds ratio (OR) of 0.96 (95% confidence interval, (CI) 0.93-0.99) per 300 mg/day increase in calcium intake (the equivalent of one glass of milk). This is likely to be an underestimate of calcium's true effect because of inaccurate measurement of **dietary** calcium in observational studies. This review supports the current clinical and public health policy of recommending increased calcium intake among older women for fracture prevention.

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EXHIBIT O

Elevated Intakes of Supplemental Chromium Improve Glucose and Insulin Variables in Individuals With Type 2 Diabetes

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Chromium is an essential nutrient involved in normal carbohydrate and lipid metabolism. The chromium requirement is postulated to increase with increased glucose intolerance and diabetes. The objective of this study was to test the hypothesis that the elevated intake of supplemental chromium is involved in the control of type 2 diabetes. Individuals being treated for type 2 diabetes (180 men and women) were divided randomly into three groups and supplemented with: 1) placebo, 2) 1.92 μmol (100 μg) Cr as chromium picolinate two times per day, or 3) 9.6 μmol (500 μg) Cr two times per day. Subjects continued to take their normal medications and were instructed not to change their normal eating and living habits. HbA_{1c} values improved significantly after 2 months in the group receiving 19.2 μmol (1,000 μg) Cr per day and was lower in both chromium groups after 4 months (placebo, $8.5 \pm 0.2\%$; 3.85 μmol Cr, $7.5 \pm 0.2\%$; 19.2 μmol Cr, $6.6 \pm 0.1\%$). Fasting glucose was lower in the 19.2- μmol group after 2 and 4 months (4-month values: placebo, 8.8 ± 0.3 mmol/l; 19.2 μmol Cr, 7.1 ± 0.2 mmol/l). Two-hour glucose values were also significantly lower for the subjects consuming 19.2 μmol supplemental Cr after both 2 and 4 months (4-month values: placebo, 12.3 ± 0.4 mmol/l; 19.2 μmol Cr, 10.5 ± 0.2 mmol/l). Fasting and 2-h insulin values decreased significantly in both groups receiving supplemental chromium after 2 and 4 months. Plasma total cholesterol also decreased after 4 months in the subjects receiving 19.2 $\mu\text{mol/day}$ Cr. These data demonstrate that supplemental chromium had significant beneficial effects on HbA_{1c} , glucose, insulin, and cholesterol variables in subjects with type 2 diabetes. The beneficial effects of chromium in individuals with diabetes were observed at levels higher than the upper limit of the Estimated Safe and Adequate Daily Dietary Intake. *Diabetes* 46:1786-1791, 1997

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Conclusive evidence of the role of trivalent chromium in human nutrition was reported in 1977 (1) when the severe diabetic symptoms of a female patient on total parenteral nutrition were alleviated by supplemental chromium. Diabetic symptoms, in addition to elevated blood glucose, included unexpected weight loss, impaired nerve conduction, and abnormal respiratory quotient that were refractory to exogenous insulin. Upon the daily addition of 4.81 μmol supplemental Cr to her total parenteral nutrition solution for 2 weeks, the diabetic symptoms were alleviated and the exogenous insulin requirement dropped from 45 U/day to zero. This work has been verified on many occasions and documented in the scientific literature on three occasions (2-4). Chromium is now routinely added to total parenteral nutrition solutions (5). However, the chromium concentrations in total parenteral nutrition solutions may not be adequate, since the normalization of nerve conduction occurred in a patient on home parenteral nutrition after the administration of supplemental chromium (6).

Signs of chromium deficiency in humans are not limited to subjects on total parenteral nutrition. Improvements in glucose and/or lipid concentrations have been reported in children with protein calorie malnutrition (7,8); the elderly (9); and individuals with type 1 and type 2 diabetes (10-13), hypoglycemia (14,15), and marginally impaired glucose tolerance (16,17).

Individuals with diabetes have altered chromium metabolism, compared with nondiabetic control subjects, with higher chromium absorption but also greater chromium excretion (18). Hair and tissue chromium levels of individuals with diabetes are lower than those of nondiabetic control subjects. Depending on the stage of diabetes, individuals with diabetes tend to lose the ability to convert chromium to a useable form (18). Diabetic mice also lose the ability to convert inorganic chromium to a useable form that potentiates insulin (19).

We conducted a double-blind placebo-controlled study involving 180 people with type 2 diabetes to determine the role of supplemental chromium in the control of diabetes. Our hypothesis was that the elevated intake of supplemental chromium is involved in the control of type 2 diabetes. The study was conducted in China to obtain a relatively homogeneous study group free of nutrient supplementation.

RESEARCH DESIGN AND METHODS

Subjects. A total of 303 individuals being treated for diabetes at two hospitals in Beijing, China, were screened to obtain 180 subjects meeting the selection criteria. To be eligible for the study, subjects had to be free of disease other than type

2 diabetes and 35–65 years of age and have a fasting blood glucose concentration of 7.2–15.5 mmol/l, a 2-h blood glucose concentration of 9.4–16.7 mmol/l, and an HbA_{1c} level of 5.0–12.0%. Subjects were informed of the purpose of the study; that there were no known risks associated with the study other than the minimal risks associated with blood drawing, and that they were free to drop out of the study with no effect on their present health care. Subjects were not reimbursed for their participation. Subjects were motivated to participate because of the possible benefits of the study. Compliance appeared to be very good and was assessed by personal communication and pill count. The study was approved by the Beijing Medical Review Committee with concurrence from the U.S. Department of Agriculture Human Studies Review Board.

A total of 180 individuals with diabetes were randomly divided into three groups. Sixty subjects received placebo, 60 received 1.92 µmol Cr as chromium picolinate (furnished by Nutrition 21, San Diego, CA) 2 times per day, and the remainder received 9.6 µmol Cr as chromium picolinate twice per day. Subjects were instructed to take one tablet in the morning and one in the evening between meals. Subjects were also urged to maintain their normal eating and exercise habits. Subjects continued their normal visits to monitor their diabetes. A fasting blood sample and a blood sample after a 2-h glucose challenge (75 g glucose) were obtained at the beginning of the study and after 2 and 4 months. Subjects were middle-aged healthy subjects of normal height, weight, and BMI with diabetes for <10 years (Table 1). Nineteen subjects did not complete all three testing dates, and six subjects had missing values for at least one variable; their results were not included in the final analyses. Data from these subjects were omitted to maintain a complete homogeneous data set with all subjects represented during each study period. Data for all subjects who completed all phases of the study were included in all of the respective analyses, and there were no samples omitted. Of the 155 subjects who were included in the final analyses, most of the subjects (92) were taking sulfonylurea drugs (i.e., glibenclamide, gliclazid, glipizide). Sixty-nine were on phenformin, 38 were on traditional Chinese medicines, 22 were on no medication, and nine were on insulin. Several subjects were taking more than one medication. Medications were constant during the study.

Study design was double blind and placebo controlled. Placebo tablets were indistinguishable from those containing either level of chromium. Measured chromium content of the placebo capsules was 0.01 ± 0.001 µmol and was 2.04 ± 0.16 and 11.0 ± 1.2 µmol for the 1.92- and 9.6-µmol capsules, respectively. Data are means ± SD for six capsules from each batch. A crossover study design was discarded because of the possible carryover effects of 1,000 µg Cr/day.

Glucose was analyzed by glucose oxidase method (20), and insulin was analyzed by radioimmunoassay (21). HbA_{1c} values were measured using BioRad

HbA_{1c} columns (BioRad, Richmond, CA). Total cholesterol was determined by chemical hydrolysis (22), HDL cholesterol by phosphotungstate-Mg precipitation (23), and triglycerides by direct enzymic measurement (24). Blood urea nitrogen was determined by a direct method (25). Analyses presented were completed in China. Several dozen samples were exchanged between the U.S. and China laboratories to ensure accuracy and reproducibility of the data.

The variables HbA_{1c}, total cholesterol, blood urea nitrogen, HDL cholesterol, triglycerides, fasting and 2-h glucose, and insulin were analyzed as three-factor repeated-measures mixed linear models, using PROC MIXED (SAS Institute, Cary, NC). Since the variables were measured at 0, 2, and 4 months for each subject, repeated measures analyses were used. Several covariance structures were modeled, and the unstructured model was found to fit best, except for triglycerides and total cholesterol, where the compound symmetry model was best. For HbA_{1c}, cholesterol, and triglycerides, the log₁₀ transformed values fit the model better and were used in the analyses. Data in the table and figures are means ± SE for the nontransformed data.

RESULTS

Fasting blood glucose concentrations were significantly lower in the group receiving 19.2 µmol Cr daily after both 2 and 4 months (Fig. 1). Similar results were observed for blood glucose concentrations 2 h after the ingestion of 75 g glucose (Fig. 2). Fasting and 2-h glucose concentrations of the subjects in the placebo group also decreased, but the decreases in the subjects receiving 19.2 µmol supplemental Cr were much larger. The chromium × time interaction was significant at $P < 0.0001$.

Fasting insulin concentrations were significantly lower in the group receiving 3.85 µmol Cr daily with a mean fasting insulin concentration of 95 ± 2 pmol/l after 4 months, which was identical to that of the group receiving the higher level of chromium, compared with 118 ± 3 pmol/l in the placebo group (Fig. 3). Fasting insulin concentrations were also significantly lower after 2 months in both of the groups receiving supplemental chromium. Similar results were observed for the insulin 2 h after a glucose challenge (Fig. 4). The fasting

TABLE 1
Characteristics of control and chromium-supplemented subjects at the beginning of the study

	Supplemental chromium (µmol/day)		
	0	3.85	19.2
Height (meters)			
All	1.67 ± 0.01 (50)	1.67 ± 0.01 (53)	1.65 ± 0.01 (52)
Women	1.61 ± 0.01 (17)	1.60 ± 0.01 (20)	1.59 ± 0.01 (26)
Men	1.70 ± 0.01 (33)	1.71 ± 0.01 (33)	1.70 ± 0.01 (26)
Weight (kg)			
All	69.1 ± 1.3	69.0 ± 1.5	67.8 ± 1.4
Women	66.4 ± 2.5	63.4 ± 2.5	63.4 ± 1.6
Men	70.5 ± 1.4	72.6 ± 1.5	72.0 ± 1.8
BMI (kg/m ²)			
All	24.8 ± 0.5	25.0 ± 0.5	24.8 ± 0.4
Women	25.8 ± 1.1	25.0 ± 0.9	25.0 ± 0.6
Men	24.3 ± 0.5	25.0 ± 0.5	24.6 ± 0.6
Duration of diabetes (years)			
All	5.4 ± 0.7 [†]	8.0 ± 1.0*	5.3 ± 0.7 [†]
Women	5.6 ± 1.0*	8.4 ± 1.6*	6.8 ± 1.1*
Men	5.2 ± 0.9* [†]	7.8 ± 1.2*	3.7 ± 0.7 [†]
Age (years)			
All	55.5 ± 1.2	55.7 ± 1.2	54.6 ± 1.4
Women	56.4 ± 1.8	53.8 ± 1.8	54.1 ± 2.3
Men	55.1 ± 1.5	56.8 ± 1.7	55.2 ± 1.8

Number in parentheses denotes number of subjects who completed all phases of the study and had no missing experimental analyses. *+Values in the same row with different superscripts are significantly different at $P < 0.05$.

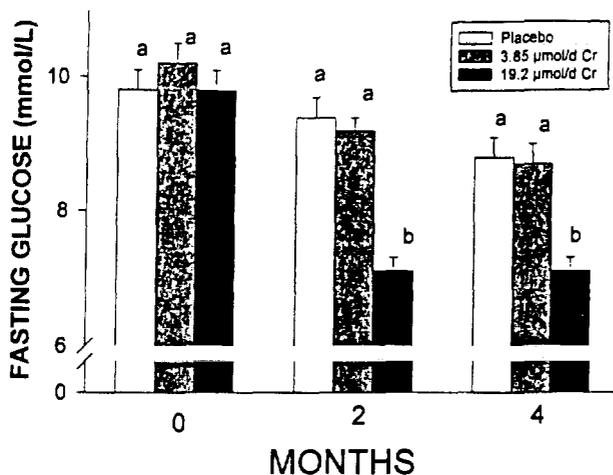


FIG. 1. Supplemental chromium effects on fasting serum glucose. Chromium was taken in two doses between meals. There were 50 subjects in placebo group, 53 in the 3.85-µmol group, and 52 in the 19.2-µmol group. Bars with different letters are significantly different from other groups for the same time period at $P < 0.05$.

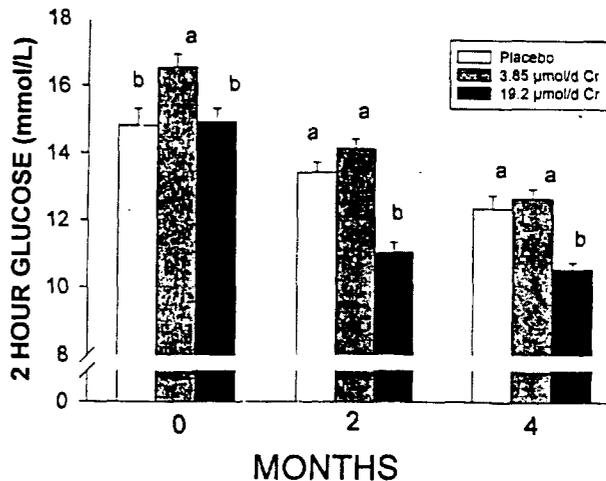


FIG. 2. Supplemental chromium effects on 2-h glucose concentrations. Subjects were given a 75-g glucose challenge at time 0, and blood was drawn 2 h later. Conditions are described in Fig. 1.

and 2-h insulin values of the placebo subjects also decreased over the duration of the study, but the decreases in the chromium groups were much larger. The chromium \times time interaction was also significant at $P < 0.0001$.

Decreases in blood glucose and insulin concentrations due to supplemental chromium (Figs. 1–4) were reflected by decreases in HbA_{1c} values, with significant effects of chromium in both chromium groups after 4 months and in the 19.2-µmol group after 2 months (Fig. 5).

Supplemental chromium at 19.2 µmol/day also led to decreased total cholesterol (Fig. 6). The total cholesterol of the men was higher than that of the women, and both sexes responded to supplemental chromium similarly. There were no chromium \times sex or time \times sex interactions. The chromium \times time interaction was significant at $P < 0.02$. There were no significant effects of supplemental chromium on HDL cholesterol, triglycerides, blood urea nitrogen, weight, or BMI (data not shown).

DISCUSSION

These data demonstrate significant effects both statistically and clinically of supplemental chromium at 3.85 and 19.2 µmol/day on glucose and insulin variables in individuals with type 2 diabetes. Improvements in fasting glucose and insulin concentrations as well as those after a glucose challenge document the role of elevated intakes of supplemental chromium in the control of type 2 diabetes. The improvements due to chromium are not due to changes in body weight, since weight did not change significantly over the duration of the study.

The chromium intake of these subjects is not known, but total dietary chromium intake does not accurately reflect chromium status since other factors affect chromium requirements. For example, different forms of stress including diet, exercise, and diabetes all increase chromium requirements (26). Increased intake of simple sugars also increases chromium losses (27). Urinary chromium losses are correlated with the stress hormone cortisol (28), and

chromium's effects on morbidity and immune function are only observed in stressed animals (29).

There are no methods to predict chromium status. The only method is to measure glucose, insulin, and lipid variables before and after chromium supplementation. Chromium concentrations in blood, hair, urine, and other tissues or body fluids have not been shown to reflect chromium status.

There have been several studies involving chromium supplementation of people with diabetes. The results of these studies are varied, but in retrospect may be consistent (10–13,30–35). The majority of the studies involving daily chromium supplementation with 4.81 µmol Cr as chromium chloride or less to individuals with diabetes reported no significant consistent improvements (31–33). Improved glucose tolerance and blood cholesterol were reported in roughly half the subjects supplemented daily with 2.89–4.81 µmol Cr as chromium chloride (9–10). Mossop (12) reported significant improvements in fasting blood glucose in 13 people being treated for diabetes. Fasting blood glucose concentrations decreased from 14.4 to 6.6 mmol/l after 2 to 4 months of 11.5 µmol supplemental Cr as chromium chloride daily. Fasting blood glucose, glycosylated hemoglobin, total cholesterol, and LDL cholesterol all improved significantly in 11 individuals with type 2 diabetes who consumed 3.85 µmol/day Cr as chromium picolinate for 6 weeks (35). Ravina et al. (13) also reported improved glucose control in 162 individuals with diabetes after daily chromium supplementation with 200 µg Cr as chromium picolinate.

The reasons for the discrepancy in the response to supplemental chromium appear to be due to the amount and form of chromium consumed. In this study, we used chromium as chromium picolinate, which is utilized more efficiently than chromium chloride (36), used chromium twice per day, and used higher levels than most previous studies. The beneficial effects of 19.2 µmol/day Cr, compared with 3.85 µmol, demonstrate that 3.85 µmol Cr is not sufficient to elicit maximal significant improvements in diabetic subjects.

Chromium picolinate is a convenient form of chromium that is used more efficiently than some other forms of chromium. The active compound is chromium, not picolinate,

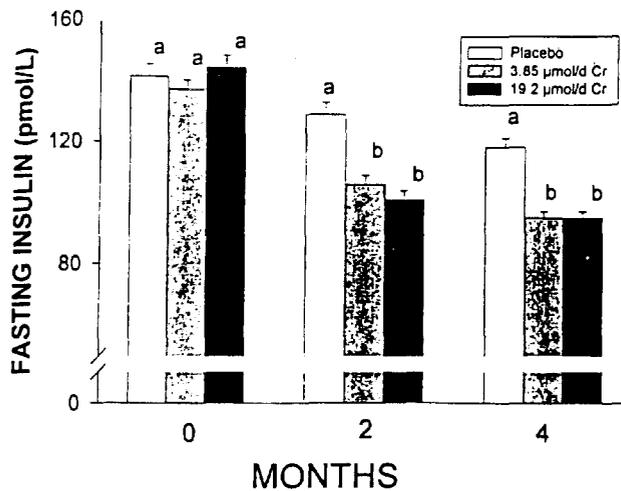


FIG. 3. Supplemental chromium effects on fasting insulin concentrations. Conditions are described in Fig. 1.

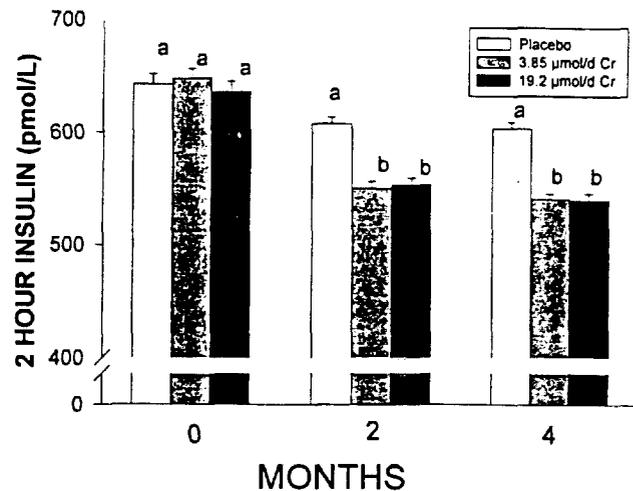


FIG. 4. Supplemental chromium effects on 2-h insulin concentrations. Conditions are described in Fig. 2.

since other studies have shown beneficial effects of chromium as chromium chloride. Chromium chloride is usually the least available of the chromium compounds tested (36). Patients on total parenteral nutrition and individuals with glucose intolerance, hypoglycemia, and diabetes have all been shown to respond to chromium as CrCl_3 . Several different forms of chromium are likely to elicit similar effects but at different intakes due to the varying absorption, transport, and utilization of the different chromium compounds.

The measurement of glycosylated proteins, such as HbA_{1c} , is the most reliable method of assessing long-term glycemic control in individuals with diabetes (37-42). HbA_{1c} values were originally postulated to reflect the simple mean plasma glucose level over a certain period, and considering the erythrocyte life span, glycosylated hemoglobin was thought to be uniformly accumulated in erythrocytes over 120 days. However, theoretical and experimental evidence demonstrates that following a consistent drop in blood glucose, HbA_{1c} values change rapidly in the first 1 to 2 months, followed by a steady-state level after 4 months (41,42). Half of the HbA_{1c}

level is determined by the plasma glucose values during the preceding 1-month period and an additional 25% of the HbA_{1c} level in the preceding month (42). Therefore, 75% of the HbA_{1c} level is proportional to the changes in blood glucose over the preceding 2 months. In our study, we saw a rapid drop in HbA_{1c} values in the first 2 months with HbA_{1c} values of $7.4 \pm 0.2\%$ for individuals receiving $19.2 \mu\text{mol Cr}$ daily, compared with $8.6 \pm 0.2\%$ for those receiving placebo. The drop in HbA_{1c} value in the group receiving $3.85 \mu\text{mol Cr}$ daily after 4 months was accompanied by a decrease in both fasting and postprandial insulin, but differences in blood glucose for the corresponding subjects were not significant. However, there were significant drops from the glucose concentrations determined at the onset of the study. Similar results were observed in both male and female subjects.

Changes in serum lipids in this study are consistent with those observed in our previous studies (14,16,17), namely that the effects of supplemental chromium are greater for glucose and insulin than for lipid concentrations. The delayed response of supplemental chromium on blood lipids is con-

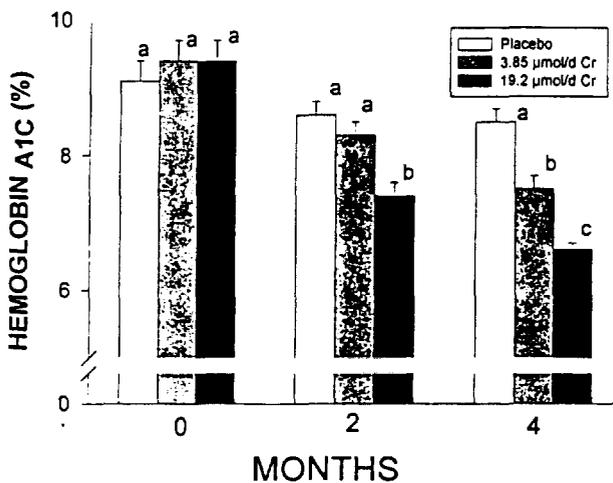


FIG. 5. Supplemental chromium effects on HbA_{1c} values. Conditions are described in Fig. 1.

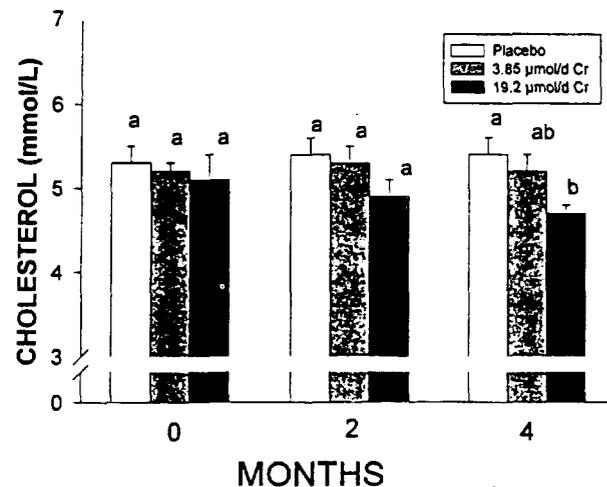


FIG. 6. Supplemental chromium effects on fasting serum cholesterol concentrations. Conditions are described in Fig. 1.

sistent with the study of Abraham et al. (30) that reported no significant effects of supplemental chromium on blood lipids after 3 months but significant decreases in triglycerides and increases in HDL cholesterol after 7–16 months. Similar chromium effects were observed in nondiabetic control subjects and individuals with diabetes.

We did not detect an effect of drug therapy for the control of diabetes and response to supplemental chromium. Diabetic therapy included, in addition to hypoglycemic drugs, traditional Chinese medicines, insulin, and diet alone. Ravina et al. (13) also did not observe an effect of insulin, sulfonylurea, or metformin on improvements in glucose control in diabetic patients receiving 3.85 μmol Cr as chromium picolinate. Supplemental chromium (600 $\mu\text{g}/\text{day}$) was also shown to increase the HDL cholesterol of men taking β -blockers (43). In a separate study, there was a larger effect of chromium on blood lipids of subjects not taking thiazides (44). Martinez et al. (45) also reported no clear effects of 200 μg Cr as chromium chloride daily in women taking medications that affect glucose tolerance but significant effects in 2-h blood glucose concentrations in nonmedicated subjects.

The mechanism of action of chromium on the control of blood glucose concentrations is the potentiation of insulin action. In the presence of chromium in a useable form, much lower levels of insulin are required. In the epididymal fat cell assay, near maximal insulin response can be achieved by adding chromium in a form that potentiates insulin (46). Inorganic chromium is without effect in the epididymal fat cell assay. Supplemental chromium leads to increased insulin binding to cells due to increased insulin receptor number (14). A direct binding of chromium to insulin is postulated (47), and a direct binding of an insulin potentiating form of chromium to insulin has been observed (48). Chromium was also shown to affect β -cell sensitivity measured in euglycemic clamp studies (49). The overall effect of chromium is to increase insulin sensitivity, which is associated with decreased glucose intolerance, decreased risk factors associated with cardiovascular diseases, improved immunity, and increased life span (50).

Trivalent chromium, the form of chromium found in foods and nutrient supplements, is considered one of the least toxic nutrients. The reference dose established by the U.S. Environmental Protection Agency for chromium is 350 times the upper limit of the Estimated Safe and Adequate Daily Dietary Intake of 3.85 μmol (200 $\mu\text{g}/\text{day}$). The reference dose is defined as "an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population, including sensitive subgroups, that is likely to be without an appreciable risk of deleterious effects over a lifetime" (51). This conservative estimate of safe intake has a much larger safety factor for trivalent chromium than almost any other nutrient. The ratio of the reference dose to the Estimated Safe and Adequate Daily Dietary Intake or the Recommended Daily Allowance is 350 for chromium, compared to <2 for zinc, roughly 2 for manganese, and 5–7 for selenium (51). Anderson et al. (52) demonstrated a lack of toxicity of chromium chloride and chromium picolinate in rats at levels several thousand times the upper limit of the estimated safe and adequate daily dietary intake for humans (based on body weight). There was no evidence of toxicity in this study, and there have not been any reported toxic effects in any of the human studies involving supplemental chromium.

In summary, supplemental chromium was shown to have pronounced effects on glucose and insulin variables in individuals with type 2 diabetes. A total of 200 μg Cr daily (3.85 μmol) did not appear to be sufficient for the reversal of diabetic symptoms over the 4-month duration of the study, since larger consistent effects were observed in subjects receiving 1,000 μg (19.2 μmol) supplemental Cr daily. Additional studies are needed to establish the form and amount of supplemental chromium required to elicit maximal responses in individuals with diabetes and in the prevention of diabetes.

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Parts of this study were presented in abstract form at the 56th annual meeting of the American Diabetes Association, San Francisco, California, 8–11 June 1996.

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EXHIBIT P



For Immediate Release

USDA STUDY DEMONSTRATES SAFETY OF CHROMIUM SUPPLEMENTS

New York, NY, March 18, 1996 -- A new study conducted by Dr. Richard Anderson, the lead scientist for trace minerals at the U.S. Department of Agriculture's Human Nutrition Research Center, Beltsville, Md., found no harmful effects in animals supplemented with two widely used forms of dietary chromium: chromium chloride and chromium picolinate. This study adds to the large body of data supporting the safety of nutritional chromium. Further, these results help consumers place into proper perspective the results of a study published in December 1995, which attempted to link chromium picolinate with chromosomal damage in cultured hamster cancer cells.

Dr. Anderson's research team fed rats a stock diet to which was added 0, 5, 25, 50 or 100 micrograms (mcg) of chromium per gram of feed for six months. The supplements were added to the feed in the form of chromium chloride or chromium picolinate, with the highest supplemented level measuring approximately 1500 mcg/day per kilogram of body weight. In human terms, a 150 lb (70 kg) individual would have to consume 1.05 million micrograms, or more than 5,000 capsules containing 200 mcg of chromium, each day for six months to equal the rat intake.

Presented on March 14, 1996 in Anaheim, CA, at the Society of Toxicology's annual meeting, the results of Dr. Anderson's study showed no statistical differences in any measured parameter among animals fed none, high or low doses of supplemental chromium. For example, both body weight and blood chemistries were comparable between the groups when examined at 11, 17 and 24 weeks into the study.

At the end of the study, microscopic evaluation of the liver and kidneys of the rats fed the highest doses of chromium chloride or chromium picolinate did not show any detectable differences from the control or low-dose groups. The tissue concentrations of chromium for both compounds increased in a proportional manner, with higher concentrations associated

with chromium picolinate. Chromium picolinate appeared to be more biologically useful than chromium chloride.

"These results are significant in that we started supplementing these rats with a range of chromium doses at an early age. When we continued supplementation for about one quarter of their natural lifespans, we found no toxicity associated with either chromium compound -- even at extremely high dosages," said Dr. Anderson. "The results reaffirm the extraordinary safety of this essential trace mineral."

This safety study comes at a time when researchers are beginning to understand how chromium enhances the activity of insulin and helps the body improve its metabolic efficiency. "We are now able to pinpoint some of the enzyme systems that affect the action of insulin and are beginning to see how chromium is involved at the molecular level," Dr. Anderson added.

"The biologic defect that leads to the most common form of diabetes also appears to play a key role in coronary artery disease risk. This defect, known as insulin resistance, refers to a type of blockage that prevents insulin from working normally," says Barry Mennen, M.D., Executive Director of the Chromium Information Bureau. "Aside from high blood sugar levels and diabetes, insulin resistance can lead to abnormal blood cholesterol and triglyceride levels and perhaps high blood pressure -- all risk factors for coronary artery disease."

"Conversely, there is evidence that lowering insulin resistance can reduce the risk not only of diabetes, but of coronary artery disease as well. There are three proven ways to lower insulin resistance: prudent dietary choices to achieve or maintain ideal weight, regular exercise and correcting chromium insufficiency," Dr. Mennen adds.

A recent study reported at the annual meeting of the American College of Nutrition last fall demonstrated the clinical efficacy and safety of chromium picolinate in pregnant women. These women suffered from gestational diabetes, a common manifestation of increased insulin resistance during pregnancy. The double-blind, placebo-controlled study found that as little as 4 mcg/kg/day of chromium picolinate (300 mcg/day for a 154 lb pregnant woman) significantly improved blood sugar measurements. The study was conducted by diabetes researcher Lois Jovanovic-Peterson, M.D. in Santa Barbara, CA.

Dr. Anderson will be reporting the results of his study on chromium

supplementation in patients with diabetes at the American Diabetes Association meeting in June.

This clinical trial was done jointly with the USDA and a medical team from China. "Some of the earlier clinical trials with chromium had too few patients or suffered from design flaws," Dr. Anderson noted.

"We expect that these recently completed trials will provide more definitive data on the therapeutic benefits of chromium supplementation." The Chromium Information Bureau, which is headquartered in New York City and staffed by physicians, nutritionists and registered dietitians, is an industry-supported organization. It was created to educate consumers, the media and health professionals on the actions of dietary chromium and provide a clearinghouse for the latest research findings and educational material on this essential trace element.

To contact the CIB, please address inquiries to Chromium Information Bureau, PO Box 189, 1562 First Avenue, New York, NY 10028, or send E-mail to editor@chromium.edu.



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Chromium Information Bureau

Chromium May Reduce Diabetes Risk

NEW YORK, NY, 6/27/97- A new study presented at the 57th Annual Scientific Session of the American Diabetes Association Meeting in Boston, MA, on June 23 demonstrates that daily supplementation with 1,000 micrograms of chromium picolinate significantly enhanced the action of insulin. The study enrolled moderately obese people with a high risk of developing type II (adult-onset) diabetes.

The study was a randomized, double-blind, placebo-controlled clinical trial directed by William Cefalu, M.D., Director of the Diabetes Comprehensive Care and Research Program at Bowman Gray School of Medicine, Wake Forest University. The study enrolled 29 overweight individuals with a family history of diabetes. The patients received either a placebo or 1,000 micrograms (1 mg) of chromium per day.

After four months of treatment, insulin resistance among chromium recipients was reduced a statistically significant 40%. This improvement was maintained at the end of eight months, Cefalu reported.

The researchers also evaluated the patients' abdominal body fat before and after treatment using a sophisticated imaging technique. The placebo group showed a gain of six percent, compared with a gain of one percent in the chromium group. This difference was not statistically significant.

"Even though only a small number of subjects were studied, the improvement in insulin sensitivity in chromium-supplemented subjects was quite significant and impressive," noted Dr. Cefalu.

"This is a potentially important finding in light of the fact that insulin

resistance often precedes type II diabetes. Chromium picolinate is a nutritional supplement that can reduce risk factors for the development of diabetes."

Richard Anderson, Ph.D., Senior Scientist at the USDA's Human Nutrition Laboratory in Beltsville, Md, and a leading authority of dietary chromium added, "Dr. Cefalu's findings are exciting and could prove to be of great importance if replicated in future studies. They are certainly consistent with our findings which were reported at last year's ADA meeting: We found improved blood sugar control in a group of Chinese patients with type II diabetes who were supplemented with chromium"

Insulin is the master metabolic hormone in the body, regulating blood sugar. Chromium is an essential trace mineral required by humans in order for insulin to work properly. In persons with the most common form of diabetes, the effects of insulin on lowering blood sugar are reduced. This decrease is referred to as insulin resistance and is now believed to be the primary defect in type II diabetes. Years before any signs or symptoms of diabetes are seen in at-risk individuals (including those with a family history of diabetes), insulin resistance begins to take its toll on blood sugar control.

Related Links

- [Cefalu Abstract](#)
- [American Diabetes Association](#)
- [USDA China Study](#)
- [Backgrounder](#)



The CIB is a non-profit, industry supported organization created to provide information to nutrition researchers, health care providers, the media and consumers on the functions, actions and clinical effects of dietary chromium.

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Chromium Information Bureau

Chromium May Reduce Diabetes Risk

Washington, DC (November 1, 1997)- Chromium supplements appear beneficial in the treatment of type II diabetes, suggest the results of a placebo-controlled clinical trial conducted in China. The final results of a study appear in the current issue of the medical journal **Diabetes**.

The USDA sponsored study was designed to determine whether supplemental chromium could help in the control of type II (non-insulin dependent) diabetes. One-hundred and eighty men and women in China who were already being treated for type II diabetes received either placebo, 100 mcg (micrograms) of chromium picolinate two times per day, or 500 mcg of chromium two times per day. The volunteers continued to take their normal medications and were asked not to change their normal eating and living habits.

Glycosylated hemoglobin (HbA1c) values improved significantly after 2 months in the group receiving 1,000 mcg/day of chromium. The test is a measure of how well glucose is metabolized, and is considered a gold standard in medicine. These values were lower in both chromium groups after 4 months. Fasting glucose levels were lower in the high-dose group after 2 and 4 months. Two-hour glucose values were also significantly lower for the high dose chromium group after both 2 and 4 months. In addition, fasting and two-hour insulin values decreased significantly in both groups receiving supplemental chromium after 2 and 4 months. Moreover, plasma total cholesterol also decreased after 4 months in the subjects receiving the high dose of chromium.

"These data demonstrate that supplemental chromium had significant beneficial effects on HbA1c, glucose, insulin, and cholesterol

variables in subjects with type 2 diabetes. The beneficial effects of chromium in individuals with diabetes were observed at levels higher than the upper limit of the Estimated Safe and Adequate Daily Dietary Intake," the researchers note

Both the high- and low-dose chromium groups had a significant drop in plasma insulin just two months after beginning the supplements and a further drop at four months. People with type II, or maturity-onset, diabetes produce more insulin than normal in the early stages of the disease, because the insulin is less efficient at clearing glucose from the blood. Chromium apparently makes the hormone more efficient.

The study was a collaboration between USDA investigator Dr. Richard Anderson and Dr. Nanzheng Cheng. Cheng--a former visiting scientist in Anderson's laboratory--and her sister Nanping Cheng, a physician in Beijing. The study participants were recruited at three Beijing hospitals.

In the U.S., it's possible that people with diabetes would need higher levels of chromium to realize similar improvements because Americans are larger than the Chinese and eat more fat and sugar. All of these factors raise the requirement for chromium. No other studies have seen consistent improvements with 200 mcg.

Anderson also said he has maintained rats on daily doses of chromium picolinate or an inorganic form of the mineral several thousand times above the highest suggested intake for humans with no adverse effects on the sensitive organs.

There is no Recommended Dietary Allowance for chromium. The estimated safe and adequate dietary intake is between 50 and 200 mcg daily. Most Americans consume less than 50 mcg, Anderson said. He has analyzed well-balanced diets prepared by dietitians and found them to contain only about 33 mcg per day.

The study results appear in *Diabetes*, November 1997, Volume 46, Number 11 *For further details contact Richard A. Anderson, nutritional biochemist, Beltsville Human Nutrition Research Center, USDA, Agricultural Research Service, Beltsville, Md. 20705; telephone (301) 504-8091.*

Related Links

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- [Diabetes Journal abstract](#)
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EXHIBIT Q

Table 3. Comparison of Follow-up Reports from Calcium Supplementation Trials in Children

Follow-up Report	Original Trial	Time Between End of Trial and Follow-up (months)	Differences in BMD Between Supplemented Subjects and Controls
Lee et al. ¹¹	Lee et al. ⁵	18	NS*
Siemenda et al. ¹⁰	Johnston et al. ²	24, 36	NS
Lee et al. ¹²	Lee et al. ⁴	18	NS
Bonjour et al. ⁸	Same	12	Mean BMD lower in controls ($p \leq 0.05$)

*No significant differences.

mentation is discontinued. Unfortunately, no effect of calcium on linear growth has been shown in other clinical trials, even when baseline calcium intake was low (e.g., Lee et al.⁴).

Although calcium-enriched foods appeared to improve bone mineral accretion in girls with spontaneous calcium intakes below 900 mg/day, further work is needed to determine the long-term effects of increased calcium intake on bone. As well, more evidence is required to confirm an effect of calcium on bone modeling and whether any positive effect would be translated into decreased risk of osteoporosis later in life.

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Confirmation: Chromium Levels in Serum, Hair, and Sweat Decline with Age

A study of tissue chromium concentrations measured in 40,872 subjects in the United Kingdom with modern, validated analytical techniques confirms previous reports of the continuous decline in chromium concentrations with age.

This review was prepared by Walter Mertz, M.D., former Director of the U.S. Department of Agriculture Human Nutrition Research Center at Beltsville, MD, USA. Reprint requests should be addressed to *Nutrition Reviews*, 711 Washington Street, Boston, MA 02111, USA.

When the essential role of trivalent chromium in maintaining glucose tolerance and its close interaction with insulin were established in the late 1950s and early 1960s,^{1,2} atomic absorption spectrometry had just begun to replace older colorimetric methods for determining trace elements in biologic materials. Moreover, it took approximately 20 years of analytical developments for a continuous decline in reported chromium levels in blood and urine to finally stabilize at one one-thousandth of values earlier considered "normal."³ Although this development affected the accuracy of analyses in the range below 1 ng/gram in serum and urine much more than in tissues and foods with more than 10 times higher concentrations, it created con-

siderable doubt as to the validity of all chromium analytical data published during those 20 years.

Among those potentially questionable data was the report by Schroeder et al.⁴ of chromium concentrations in 1018 human tissue samples obtained in the United States at autopsy and analyzed by emission spectrography. Chromium levels, reported on the basis of ash weight, declined markedly during the first 10 years of life in all tissues analyzed (kidney, liver, lung, aorta, heart, pancreas, spleen, testes), followed by a slower but continuous rate of decline during the following years, except for the lungs, which, in middle age, began to accumulate the element. This unexpected finding set chromium apart not only from the "toxic" elements that tend to accumulate with age in the organism, but also from other essential elements that maintain their tissue concentrations after an initial decline following birth.

These data, together with the first reports of beneficial effects of chromium supplementation in subjects with impaired glucose tolerance,^{5,7} raised challenging questions as to the adequacy of habitual chromium intake, but remained unconfirmed for many years. More recently, impressive technical improvements in instruments, the use of "absolute" analytical methods such as isotope dilution spectrometry,⁸ and most of all, the availability and use of standard reference materials⁹ have provided a solid, reliable basis for chromium analysis in biologic material. These developments, however, require special training of the analyst in all procedures (from sample collection and preparation to final analysis), expensive instrumentation, and work areas of clean room quality. For these reasons, chromium analysis is not a routine procedure. The fact that it is reliably performed in only a few laboratories worldwide accounts for the dearth of analytical data for blood and urine in large numbers of subjects.

The recent report by Davis et al.¹⁰ has changed that situation and met an urgent need for data. These authors collected approximately 22,000, 18,000, and 12,000 samples of hair, sweat, and serum, respectively, from almost 41,000 human subjects, ranging in age from 1 to more than 75, who were referred by their physicians for a wide spectrum of complaints. Diagnostic details of complaints are not provided, but "the data presented in this report are derived from a broad, ambulatory, nonhospitalized population, and, on the basis of the referral patterns to this unit, it is extremely unlikely that a significant proportion of the subjects were moribund or malnourished."¹⁰ Thus, the data do not describe an ideal, healthy population but a more realistic mixture of people who sought medical advice for prevention or therapy. Sample collection, preparation, and analysis were well controlled, according to the authors, and their results are well within the ranges obtained by other modern methods and are considered accurate.

The authors divided their subjects into 16 age groups

from 1 to 4 years to 75 and older and found a highly significant negative correlation between age and chromium concentration in all three tissues studied. Levels in serum declined from 0.51 ng/gram in those 1-4 years old ($n=49$) to 0.29 ng/gram in those 75 years old and older ($n=289$). The corresponding values for hair were 980 ng/gram ($n=553$) and 500 ng/gram ($n=292$), and those for sweat were 3.86 ng/gram ($n=424$) and 2.04 ng/g ($n=256$). The decline continued in all tissues into old age without any noticeable break. Middle-aged females had slightly but significantly higher chromium concentrations than males, but these differences disappeared after menopause. Chromium concentrations in the three tissues were highly correlated with each other.

This is the first study using reliable analytical methods in a very large number of subjects to demonstrate a marked, continuous decline of chromium in three tissues. A decline in the internal organs with age has been demonstrated in the past,⁴ but questions of analytical accuracy have tempered the impact of these findings. Similar questions have been raised with regard to the validity of important early studies of hair chromium in children^{11,12} and women.¹³ All of these early results and several more recent ones are validated, directly or indirectly, by Davis et al.'s study. Their demonstration of a significant decline of chromium in a very large number of subjects in the United Kingdom emphasizes the need to investigate the cause(s) for that decline. The authors speculate, as others have, that poor nutrition, especially excessive consumption of refined carbohydrate, may be to blame, and this opinion is consistent with several studies of chromium metabolism.¹⁴ There may be additional causes, because it appears unlikely that a large population group from a country without severe malnutrition would uniformly develop a deficiency entirely because of inadequate intake of a micronutrient. The individual variation of dietary habits would afford protection to at least some individuals, as we know from past experience in the goiter belt of North America and the Keshan area of China. Comparing chromium intakes of populations with national or international recommended intakes is of little help, because the latter are no more than estimates.

An answer to this very important question could come from a well-controlled study of a population that could be shown to maintain its tissue chromium concentrations throughout the life span. Thailand or South Africa may be a promising area, according to Schroeder's early autopsy data.⁴ If a study could provide such evidence, it would suggest an enormous public health problem in the United Kingdom, the United States, and probably other Western countries. Lacking such evidence, the question raised almost three decades ago—whether the decline is a normal physiologic development or due to poor nutrition¹⁵—is still unanswered.

The data of Davis et al. provide a valuable and accurate yardstick of what is considered normal in children, men, and women at different ages in the United Kingdom and most likely in other countries with similar nutritional habits. They also demonstrate a close correlation between the values in the three tissues studied, rehabilitating to a degree hair analysis that, in the past, has been misused.¹⁶ There is no doubt the data can and should be used as standards with which to compare data pertaining to groups of subjects. Comparisons between groups can provide valuable information by identifying risks of chromium deficiency in certain populations. The difference in hair chromium concentrations in children from the United Kingdom and Turkey,¹² where chromium deficiency has been proved by supplementation trials,¹⁷ is one example.

The remaining question of whether analysis in serum, hair, or sweat individually is capable of diagnosing the chromium status of individuals is more difficult to answer. The only reliable test at this time is the diagnostic supplementation trial, in which an individual with impaired glucose tolerance and normal or elevated insulin levels does or does not respond to a chromium supplement with improvement. Although deviations of individual levels from the standards established by Davis et al. do not prove chromium deficiency, they may indicate a risk that should be followed up.

Future progress in speciation analysis may well increase the power of data from individuals. We know of the large differences among chromium compounds in their in vitro activity on epididymal fat tissue or cells, which have led to the theory that dietary chromium must be converted in the organism into its biologically active form, called glucose tolerance factor. If that species could be isolated and analyzed in serum and other tissues, the results would have much greater diagnostic significance than the determination of total chromium. It would be similar to the power of vitamin B-12 analysis compared with that of total cobalt. Notwithstanding these future needs, Dr. Davis and his associates deserve thanks for the great effort and care dedicated to their study. It will have a substantial impact on the field of chromium research.

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EXHIBIT R

Normal Subjects Consuming Physiological Levels of 18:3(n-3) and 20:5(n-3) from Flaxseed or Fish Oils Have Characteristic Differences in Plasma Lipid and Lipoprotein Fatty Acid Levels^{1,2}

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ABSTRACT The study assessed the effect of low doses of fatty acids from fish or flaxseed oil on plasma lipid concentrations in normal humans consuming diets with either high (0.87, $n = 11$) or low (0.48, $n = 15$) dietary polyunsaturated/saturated fatty acid (P/S) ratios. The dose of (n-3) fatty acids reflected an (n-3) intake that could easily be attained by selection of foods in a normal diet. The individuals were initially supplemented with olive oil [35 mg 18:1/(kg body weight · d)], and then were randomly assigned to either flaxseed or fish oil [35 mg 18:3(n-3) or 35 mg 20:5(n-3) + 22:6(n-3)/(kg body weight · d), respectively] treatments. Participants consumed each oil supplement for 3 mo. Blood samples were drawn for analysis at the end of each 3-mo period. Plasma triacylglycerol, total, LDL and HDL cholesterol concentrations, and lipoprotein fatty acid concentrations are shown. Fish oil reduced plasma triacylglycerol and increased lipoprotein levels of 20:5(n-3) and 22:6(n-3). The flaxseed oil did not alter plasma triacylglycerol level and produced small changes in 20:5(n-3) and 22:6(n-3) concentrations. Total, LDL and HDL cholesterol levels were not affected by either (n-3) fatty acid. Significant differences in plasma triacylglycerol concentrations and total and LDL cholesterol levels were found between the two dietary P/S groups after all oil treatment periods. Levels of 18:3(n-3), 20:4(n-6), 20:5(n-3), and 22:6(n-3) in LDL were also different in high vs. low dietary P/S groups for all oil treatments and in the VLDL for the olive oil and fish oil supplementation. This study indicates that low intake of purified fish oil induces changes in plasma triacylglycerol, 20:5(n-3) levels in VLDL, LDL, and HDL, and 22:6(n-3) levels in LDL and HDL that are apparent after 3 mo and which might influence atherogenicity of lipoprotein particles in normal free-living individuals. *J. Nutr.* 126: 2130-2140, 1996.

INDEXING KEY WORDS:

- dietary fatty acids • plasma lipoprotein
- phospholipid • cholesterol • humans

Epidemiological evidence indicates that Eskimo populations consuming high levels of (n-3) fatty acids display a reduced incidence of coronary heart disease regardless of total fat (Dyerburg et al. 1975) and cholesterol intake (Bang et al. 1976). Inuit adults consume ~4.0 g/d of eicosapentaenoic acid, whereas the average Canadian ingests less than 0.1 g/d (Holub 1988). This finding has prompted both animal and human studies designed to assess the effects of (n-3) fatty acids. It would be typical of intakes possible when consuming normal foods [reviewed by Schmidt et al. 1993, Scarpicini and Valli 1994]. The (n-3) fatty acids affect metabolic variables, including plasma triglyceride and VLDL concentrations (Saynor et al. 1988), cellular fatty acid content, 20:4(n-6) utilization, eicosanoid balance, thromboxane A₂ synthesis, platelet aggregation (Weinreb and Leaf 1991), and cell-lipoprotein interactions (Rustan and DiCorelto 1988, Hostmark et al. 1988, Shimokata and Vanhoutte 1989).

Generally, normal individuals fed marine (n-3) fatty acids demonstrate a reduction in plasma triacylglycerol and VLDL levels. These effects are dose dependent (Moms et al. 1985) and reversible (Goodnight et al. 1982). A variety of mechanisms have been suggested to explain the hypotriacylglyceridemic effect of (n-3) fatty acids (Harris 1989, Lang and Davis 1990, Rustan and Drevon 1989, Rustan et al. 1988, Sanders 1991).

Although the effect of 18:3(n-3), 20:5(n-3), and 22:6(n-3) on plasma cholesterol levels is debatable,

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² The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereinafter marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

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Individuals fed large amounts of marine fish display reductions in LDL cholesterol (Connor 1988). These reductions are due to changes in diet and increases in the unsaturated/saturated fatty acid (P/S) ratio, but the results fail to accurately reflect the specific effects of (n-3) fatty acids on serum lipid levels.

Animal studies indicate that the efficacy of (n-3) fatty acids in lowering plasma triacylglycerol, cholesterol levels and arachidonate levels depends on the relative amounts of linoleic acid and saturated fatty acids in the diet (Garg et al. 1988, 1989a and 1989b). Because the attributes of (n-3) fatty acids are limited to the longer-chain forms, it is important that conversion of 18:3(n-3) to 20:5(n-3) and 22:6(n-3) occur. In rats, the conversion of 18:3(n-3) to 20:5(n-3) and the inhibition of conversion of 18:2(n-6) to 20:4(n-6) is maximized by partial replacement of dietary 18:2(n-6) with saturated fatty acids (Garg et al. 1989a). This suggests that (n-3) fatty acid supplementation for humans may be more effective when the dietary P/S ratio is low. This hypothesis deserves investigation because many Canadians normally consume diets with a lower P/S ratio.

To date, the effects of (n-3) fatty acids have been assessed in a variety of short-term placebo-controlled and long-term uncontrolled studies (reviewed by Schmidt et al. 1993). However, the effect of low doses of (n-3) fatty acids on quantitative pool sizes of plasma lipoprotein fatty acids has not been assessed in a longer-term (>30 d), randomized, double-blind controlled crossover design. The objective of this study is to determine the long-term effects of purified (n-3) fatty acids on plasma triacylglycerol, cholesterol and lipoprotein fatty acid content in normal individuals. The effect of 18:3(n-3) vs. 20:5(n-3) and 22:6(n-3) was assessed by providing a purified flaxseed or fish oil supplement to individuals who normally consume a low P/S ratio diet compared with individuals who normally consume a higher P/S ratio diet.

MATERIALS AND METHODS

Purified oils. Chromatographically pure triglycerides of olive oil, flaxseed oil and fish oil were provided by Lipidteknik, Stockholm, Sweden. These oils were prepared by commercial-scale physical and chromatographic fractionation procedures to prepare a triglyceride fraction free of other polar or nonpolar lipids, peroxides and hydroperoxides that characterize many commercially available fish oils. Major fatty acid constituents of the purified oils were analyzed and are illustrated (Table 1). Artificial vanilla extract was added at a concentration of 0.05 g/100 g of olive oil and 1.2 g/100 g of flaxseed and fish oils to blind participants to the type of oil treatment. Flavored oils were encapsulated in 0.5-g capsules under nitrogen by Banner Gelatin (Olds, Alberta, Canada) and stored in nitrogen-

TABLE 1

Major fatty acid composition of purified oils^{1,2}

Fatty Acid	Olive	Fish	Flaxseed
g/100 g fatty acid			
14:0	0.2	9.6	0.2
16:0	16.1	16.6	8.6
16:1 (n-7)	0.1	8.9	—
16:1 (n-5)	1.5	0.2	0.1
18:0	2.6	2.3	3.3
18:1	62.7	14.2	5.8
18:2 (n-6)	13.0	1.6	14.2
18:3 (n-3)	0.9	1.3	57.5
20:0	0.1	—	—
20:2 (n-6)	—	0.2	—
20:3 (n-9)	—	0.2	—
20:4 (n-6)	—	1.9	—
20:5 (n-3)	—	20.8	—
22:4 (n-6)	—	0.1	—
22:5 (n-6)	—	0.2	—
22:5 (n-3)	—	1.6	—
22:6 (n-3)	—	12.6	—

¹ Oils were flavored with artificial vanilla extract and encapsulated in 0.5-g capsules.

² The fatty acid composition was determined by gas liquid chromatography using fused silica capillary columns.

flushed brown bottles at -20°C. Participants were instructed to store oil capsules in the freezer. Peroxide analysis was performed every 1-2 mo to ensure that there was no increase in oil peroxide level over the course of the study (Turini et al. 1991).

Participants and experimental design. The study protocol was approved by the Human Ethics Review Committees of the Faculty of Home Economics and the Faculty of Medicine, University of Alberta, Canada. Participants were recruited through advertisements posted at the University of Alberta campus. An initial visit was arranged to outline the project, review participant responsibilities and obtain informed written consent. Free-living individuals were instructed to document all food and drink consumed on a daily basis for a period of 7 d. This record was taken over 1 wk to establish normal dietary patterns and was repeated at 3-mo intervals (total of five dietary records of 7 d each). Dietary intakes were calculated using a nutritional database derived from the nutrient data bank set up within the computer system at the University of Alberta. The values used were based on Nutritional Values of Some Common Foods (1988) and revised to include an updated fatty acid composition of foods. Participants were grouped into higher or lower P/S groups based on this analysis.

The physical, dietary and clinical characteristics of participants in the low and high P/S groups are illustrated (Table 2). Percentage of ideal body mass was calculated using Metropolitan Life Insurance Company tables (1983) and fell within normal ranges for all parti-

TABLE 2
Descriptive data for subjects on entry^{1,2}

Dietary P/S group:	Low (n = 15)	High (n = 11)
Physical data		
Age, y	33.7 ± 10.1	27.1 ± 3.9
% Ideal body mass	104.3 ± 1.6	97.7 ± 7.8
Dietary data		
Energy, kJ	8245.0 ± 2773.0	7717.9 ± 3008.5
Protein, % energy	14.8 ± 3.4	14.1 ± 1.4
Carbohydrate, % energy	48.7 ± 7.5 ^b	54.7 ± 7.3 ^a
Fat, % energy	34.3 ± 7.6	30.2 ± 6.7
Saturated	13.0 ± 3.5 ^a	9.5 ± 3.0 ^b
Oleic acid	9.7 ± 2.0	8.4 ± 1.5
Linoleic acid	5.3 ± 1.4	6.4 ± 2.3
Cholesterol, mg	257.8 ± 84.9	190.6 ± 100.0
P/S ³	0.48 ± 0.15 ^b	0.87 ± 0.38 ^a
Clinical data ⁴		
Triglyceride (0.6–2.3 mmol/L)	1.02 ± 0.46	0.82 ± 0.23
Total cholesterol (3.2–5.2 mmol/L)	4.44 ± 0.84	3.86 ± 0.76
LDL cholesterol (1.7–3.4 mmol/L)	2.49 ± 0.77 ^a	1.86 ± 0.59 ^b
HDL cholesterol (0.9–2.20 mmol/L)	1.49 ± 0.35	1.62 ± 0.27
Glucose (3.5–6.4 mmol/L)	4.78 ± 0.31	4.88 ± 0.36
HbA1c ⁵ (0.040–0.063 % Hb)	0.042 ± 0.01	0.043 ± 0.01
Insulin (5–20 mU/L)	6.07 ± 2.66	5.55 ± 2.21
C-peptide (0.5–3.0 mg/L)	0.70 ± 0.39	0.71 ± 0.37
Glucagon (>60 ng/L)	38.73 ± 8.71	38.27 ± 8.49

¹ Values are means ± SD of base-line measurements.

² Within a row, values without a common superscript are significantly different ($P < 0.05$). Significant differences between high and low P/S subjects were determined by a two tailed t test.

³ Polyunsaturated/saturated fatty acid ratio.

⁴ Normal ranges for clinical data according to University of Alberta Hospitals standards are given in parentheses.

⁵ Glycosylated hemoglobin.

Participants throughout the study. The participants were free from any known metabolic diseases and were not on any drug therapy. Initially, 32 individuals were recruited into the study, with 16 participants in each dietary group. The low P/S group consisted of individuals with a dietary P/S ratio of 0.74 or less (range of 0.25 to 0.74), while the high P/S participants had a minimum P/S ratio of 0.43 when assessed by the two dietary records (range of 0.43 to 1.52).

Final analyses were based on results from 15 participants consuming low P/S diets and 11 participants consuming high P/S diets. These 26 subjects exhibited consistent intakes of fat, protein and carbohydrate expressed on a % energy basis (not significantly different for the five dietary records collected). Of the initial 32 participants, one individual was unable to comply with the oil supplement protocol, and five of the remaining participants were not included in the final data analysis because they altered their fat intake and did not remain within their initial dietary P/S group. Participant compliance was based on regular contact and on counts of

returned oil capsules. Compliance was also evident in plasma fatty acid changes. Initial fasting blood samples were drawn at the Metabolic Day Care Centre, University of Alberta Hospitals, to ensure that participants had normal lipid profiles and were free of metabolic complications. Participants were counseled at the time and throughout the study to keep within their usual fat intake level and P/S grouping. A follow-up phone call was made approximately once a month to reinforce advice, assess compliance and answer questions.

A controlled, randomized, double-blind crossover study design was used in this study. All participants were initially supplemented with olive oil (placebo) for a period of 3 mo and were instructed to consume the same number of olive oil capsules that they would consume during the flaxseed oil treatment period. The dose of olive oil during this period was ~35 mg 18:1/(kg body weight · d). Blood samples and diet records were collected and analyzed at 0, 3, 6 and 9 mo. Following the placebo phase, participants were randomly assigned to either flaxseed oil or fish oil for 3 mo and finally crossed over to the alternative (n-3) fatty acid treatment for the last 3 mo. Throughout the experiment, treatment refers to either the fish oil or flaxseed oil [(n-3) fatty acid] treatment. Flaxseed oil was supplemented at a dose of 35 mg of 18:3(n-3)/(kg body weight · d) and fish oil at a dose of 35 mg of 20:5(n-3) and 22:6(n-3) (total combined)/(kg body weight · d). For example, for a 70-kg subject, the oil supplementation level would be ~4.3 g/d for flaxseed and olive oils and ~7 g/d for fish oil. This (n-3) intake could be achieved while consuming a normal diet by selection of appropriate foods.

Plasma and biochemical analysis. All plasma and biochemical analyses were done by investigators who were unaware of the treatment group. Blood samples were drawn into tubes containing disodium EDTA after participants fasted for 12–14 h. Cholesterol and triacylglycerol concentrations were determined using triglycerides and cholesterol determination kits manufactured by BMC (Boehringer Mannheim, Laval, Québec, Canada) on a Hitachi 911 Analyzer (Boehringer Mannheim, Laval, Québec, Canada). The enzymatic methods used for the assays are as described in the literature (Allain et al. 1974, Bucolo and David 1973). Plasma lipoprotein fractions (VLDL, LDL and HDL) were separated by sequential ultracentrifugation at 356, 160 × g and 20°C in a Beckman TLA-100.2 angle head rotor (Beckman Instruments, Palo Alto, CA) (Clandinin et al. 1995). VLDL, the upper layer with a density range of 0.96–1.006 kg/L after ultracentrifugation for 3 h, was removed by micropipetting. To the remaining sample, 1.3199 kg/L solution was added at a volume ratio of 1:5, and the solution was mixed well. This was followed by layering on top with 1.063 kg/L NaBr solution. The sample was ultracentrifuged for 4 h, and the top LDL layer was removed. HDL isolation followed a similar process, with the exception that 7.593 mol/L NaBr so-

so evident. A solution (v/v 1:2) was added to adjust the density of the food sample solution, and the mix was layered with 2.973 mol/L n-tre, Unisolv NaBr solution. The samples were ultracentrifuged for 6 h. LDL and HDL samples were placed in dialysis tubing into 0.196 mol/L NaCl overnight at 4°C to remove any extraneous density gradient that may have been taken up with the sample. The purity of the lipoprotein fractions separated was checked by electrophoresis to ensure that fractions were not cross-contaminated (Nobel 1968).

Lipoprotein lipids were extracted by a modified Folch method (Folch et al. 1957) using chloroform/methanol (2:1 v/v) solution containing 0.05% (v/v) butylated hydroxyquin as an antioxidant. Phospholipid, triacylglycerol and cholesteryl ester fractions were separated on silica G plates (Analtech, Newark, DE) using a solvent system of petroleum ether/diethyl ether/glacial acetic acid 80:20:1 (v/v/v). Triacylglycerol and cholesteryl ester fractions were saponified with 0.5 mol/L methanolic KOH in a 110°C sand bath for 60 and 90 min, respectively. Following saponification, samples were methylated with 14 g/100 g boron trifluoride in methanol and hexane (Morrison and Smith 1964). Triacylglyceride supplements were methylated with boron trifluoride. A known amount of standard (tripentadecanoin and cholesteryl pentadecanoate) was added to the lipid extract prior to thin layer chromatography for quantification of the fatty acid content of the triacylglycerol and cholesteryl ester fractions. Heptadecanoic acid was added prior to methylation for quantification of the fatty acid content of the phospholipid fraction. All samples were stored at -70°C.

Fatty acid methyl esters were separated and quantitated by automated gas liquid chromatography using a BP20 capillary column (Supelco Canada, Mississauga, Ontario) (Hargreaves and Clandinin 1987). Peaks for fatty acid methyl esters were identified by comparison with authentic compounds and standards (PUFA 1 and 2, Bacterial Methester MIX CP, GLC MIXES 10 to 100 and methylester MIX-14) purchased from Supelco Canada and Sigma Chemical (St. Louis, MO) and with known samples of biological source (MaxEPA oil) as described earlier (Hargreaves and Clandinin 1987). These GLC operating conditions separate methyl esters of all saturated, *cis*-monounsaturated, and *cis*-polyunsaturated fatty acids from 14 to 24 carbons in chain length.

Statistical analysis. Food intake records were compared by repeated measures ANOVA (SAS Inc., Cary, NC). The effects of (n-3) fatty acids on plasma triacylglycerol, total, LDL and HDL cholesterol levels, and lipoprotein fatty acid levels were assessed by repeated measures ANOVA procedures. Significant differences between treatments were determined by Duncan's multiple range test (Steel and Torrie 1980). Statistical significance was set at $P < 0.05$. Significant overall group effects were tested for plasma triacylglycerol and total, LDL and HDL cholesterol by two-way ANOVA

(diet P/S group, oil treatment) and in plasma lipoprotein fatty acids by three-way ANOVA (diet P/S group, oil treatment, lipoprotein fraction). To test if (n-3) fatty acid treatments lowered plasma triacylglycerol, cholesterol and 20:4(n-6) levels, and increased 20:5(n-3) and 22:6(n-3) levels to a greater extent in participants consuming low P/S diets than in participants consuming high P/S diets, changes in these parameters were compared by ANOVA. In this analysis, the order of treatment and time elapsed from the placebo period was also considered. Changes in plasma triacylglycerol, cholesterol and lipoprotein fatty acids were also calculated and compared by subtracting placebo (olive oil) values from (n-3) fatty acid treatment values. Significant differences in lipid changes between high P/S participants and low P/S participants were determined by two-way ANOVA (diet P/S group, oil treatment) for plasma triacylglycerol and total, LDL and HDL cholesterol, and three-way ANOVA (diet P/S group, oil treatment, lipoprotein fraction) for plasma lipoprotein fatty acids.

RESULTS

Participants' weights and dietary intakes did not differ significantly among the three oil treatment phases and were therefore considered to be consistent throughout the study. Significant dietary differences between the high and low P/S groups were evident in carbohydrate and saturated fatty acid intakes only (Table 2). Initial blood samples indicated that plasma triacylglycerol, total, LDL, and HDL cholesterol, glucose, glycosylated hemoglobin, insulin, C-peptide, and glucagon fell within normal ranges for all participants and remained normal during (n-3) fatty acid supplementation. Base-line LDL cholesterol concentration, the only clinical variable that differed significantly between the two dietary groups, was lower in the high P/S group.

Effect of (n-3) fatty acids on plasma triacylglycerol and lipoprotein cholesterol levels. Plasma triacylglycerides were significantly reduced by the fish oil treatment in the low P/S group (Table 3). The flaxseed oil treatment did not reduce triacylglycerol levels in either dietary group. Plasma total, LDL and HDL cholesterol were not significantly affected by dietary supplementation with fish or flaxseed oils in either P/S group.

Plasma lipoprotein fatty acid concentrations. Triacylglycerol. Fish oil significantly increased 20:5(n-3) and 22:6(n-3) in VLDL and LDL fractions for both P/S groups (Table 4). In the HDL fraction, 20:5(n-3) and 22:6(n-3) levels were also elevated in the HDL fraction of participants with high dietary P/S intakes. In the low dietary P/S group, only 20:5(n-3) HDL was increased (Table 4). The level of 20:4(n-6) was elevated only in LDL particles for participants consuming a low P/S diet. Participants consuming high P/S diets dis-

TABLE 3

Plasma triacylglycerol and lipoprotein cholesterol levels in subjects consuming high or low P/S diets after olive, fish or flaxseed oil treatments¹

	Diet P/S ²	Oil treatment		
		Olive	Fish	Flaxseed
		mmol/L		
TG	Low	0.99 ± 0.08 ^a	0.72 ± 0.04 ^b	0.99 ± 0.10 ^a
	High	0.87 ± 0.10	0.66 ± 0.03	0.93 ± 0.14
TC	Low	4.79 ± 0.23	4.85 ± 0.26	4.81 ± 0.18
	High	4.25 ± 0.24	4.30 ± 0.19	4.29 ± 0.25
LDL cholesterol	Low	2.83 ± 0.18	3.01 ± 0.20	2.87 ± 0.15
	High	2.20 ± 0.19	2.35 ± 0.17	2.31 ± 0.19
HDL cholesterol	Low	1.50 ± 0.09	1.48 ± 0.10	1.47 ± 0.09
	High	1.60 ± 0.08	1.64 ± 0.09	1.55 ± 0.09

¹ Values are means ± SE, $n = 15$ and $n = 11$ for low and high P/S groups, respectively. Significant oil treatment effects were determined by repeated measures ANOVA. Within a row values without a common superscript are significantly different ($P < 0.05$).

² Abbreviations used: P/S, polyunsaturated/saturated fatty acid ratio; TG, triacylglycerol; TC, total cholesterol.

played no change in 20:4(n-6) levels, reduced 18:1 and elevated 18:3(n-3) levels in LDL. Fish oil supplementation lowered 16:0, 18:0 and 18:1 in the HDL of the high P/S group and 16:0 and 18:0 in the HDL of the low P/S group. Flaxseed oil supplementation significantly increased 20:5(n-3) levels in the HDL triacylglycerol of participants consuming high P/S ratio diets (Table 4). The flaxseed oil treatment did not affect 20:5(n-3) or 22:6(n-3) levels; however, 20:4(n-6) was increased significantly in the HDL triacylglycerol fraction of participants consuming a low P/S diet. Flaxseed oil supplementation increased triacylglycerol 18:3(n-3) levels in all participant lipoproteins, with the exception of the HDL triacylglycerol fraction for participants consuming a high P/S diet. The low P/S group displayed an elevation in VLDL 14:0 and a reduction in HDL 18:0.

Cholesteryl ester. The fish oil treatment increased 20:5(n-3) levels in all lipoproteins of both dietary P/S groups (Table 5). Substantial incorporation of 20:5(n-3) into the cholesteryl ester fraction confirmed participant compliance to fish oil supplements (Glatz et al. 1989). Lipoprotein 22:6(n-3) levels were elevated in the LDL and HDL fractions of both dietary groups (Table 5). Fish oil supplementation did not significantly alter the 20:4(n-6) concentration of any lipoprotein. The level of 18:3(n-3) was elevated in the LDL fraction of participants in the low P/S group and the HDL fraction of participants in the high P/S group. Fish oil reduced 14:0, 16:0, 18:0 and 18:1 in the VLDL of participants consuming a low P/S diet. Participants consuming high P/S diets displayed reduced VLDL 14:0 and 18:0 and elevated HDL 18:0.

Flaxseed oil supplementation increased 20:5(n-3) levels only in the VLDL cholesteryl ester fraction of participants consuming high P/S diets (Table 5). Levels of 22:6(n-3) and 20:4(n-6) were not significantly affected. Participants supplemented with flaxseed oil dis-

played increased 18:3(n-3) levels in the VLDL particles of participants consuming a high P/S diet and in the LDL particles of participants in both dietary P/S groups. In low P/S participants, flaxseed oil reduced the VLDL 14:0 and decreased 18:0 in all lipoproteins.

Phospholipid. In both dietary P/S groups, fish oil increased 20:5(n-3) in all lipoproteins (Table 6). The level of 22:6(n-3) was also elevated in the LDL and HDL of both P/S groups (Table 6), and 18:3(n-3) was elevated in the LDL fraction of participants consuming a high P/S diet and the HDL fraction of participants consuming a low P/S diet. In both dietary P/S groups, LDL 14:0 and 18:0 levels increased, and VLDL 18:1 level was reduced.

Participants consuming high P/S ratio diets supplemented with flaxseed oil displayed increased 20:5(n-3) levels only in the VLDL fraction (Table 6). Levels of 22:6(n-3) and 20:4(n-6) were not significantly affected. The flaxseed oil treatment elevated 18:3(n-3) in all lipoproteins and both dietary P/S groups.

DISCUSSION

Low doses of fish oil significantly reduced plasma triacylglycerol concentrations in participants with low intakes of polyunsaturated to saturated fatty acids. This suggests that, in normal free-living individuals consuming a typical North American diet, the hypotriacylglyceridemic effects of marine (n-3) fatty acids may be sustained for 3 mo, particularly in those consuming a diet relatively high in saturated fats, e.g., dairy fats. Short-term studies (Bronsggeest-Schoute et al. 1981, Sanders and Roshanai 1983, Sztern and Harris 1991) confirm this finding. Participants in the present study demonstrated 24–27% reductions in triacylglycerol levels after 3 mo of fish oil treatment, and this finding

TABLE 4

Fatty acid concentration of plasma triacylglycerol fraction of VLDL, LDL and HDL from subjects consuming high or low polyunsaturated/saturated fatty acid (P/S) diets after olive, fish or flaxseed oil treatments¹

Fatty acid	Oil treatment	Composition					
		Olive		Fish		Linseed	
		Low P/S	High P/S	Low P/S	High P/S	Low P/S	High P/S
	P/S Group	mg fatty acid/L plasma					
VLDL							
14:0		4.02 ± 0.55 ^a	3.85 ± 0.77	3.94 ± 0.55 ^a	3.29 ± 0.48	6.45 ± 1.08 ^b	5.14 ± 1.49
16:0		60.65 ± 7.49 ^{ab}	49.20 ± 7.52 ^{xy}	47.46 ± 6.01 ^a	34.23 ± 3.49 ^x	70.79 ± 10.79 ^b	57.08 ± 12.35 ^y
18:0		7.07 ± 0.84	5.58 ± 0.53	5.75 ± 0.71	4.44 ± 0.50	7.66 ± 1.43	5.99 ± 1.16
18:1		102.24 ± 12.7 ^{ab}	85.52 ± 10.72 ^{xy}	68.14 ± 8.17 ^a	54.07 ± 3.89 ^x	120.56 ± 20.59 ^b	99.07 ± 21.15 ^y
18:2 (n-6)		41.30 ± 6.61	43.66 ± 6.23	32.30 ± 5.77	33.35 ± 2.89	49.17 ± 3.76	34.61 ± 6.15
18:3 (n-3)		1.15 ± 0.32 ^a	0.85 ± 0.32 ^x	2.43 ± 0.35 ^a	2.68 ± 0.37 ^x	6.37 ± 1.50 ^b	5.23 ± 1.29 ^y
20:4 (n-6)		2.01 ± 0.32	1.48 ± 0.19	1.76 ± 0.24	1.15 ± 0.07	2.34 ± 0.43	1.86 ± 0.39
20:5 (n-3)		0.52 ± 0.11 ^a	0.27 ± 0.04 ^x	1.94 ± 0.44 ^b	1.37 ± 0.17 ^y	0.90 ± 0.25 ^a	0.55 ± 0.09 ^x
22:6 (n-3)		1.21 ± 0.27 ^a	0.80 ± 0.11 ^x	3.35 ± 0.71 ^b	2.80 ± 0.28 ^y	1.38 ± 0.25 ^a	1.00 ± 0.15 ^x
LDL							
14:0		1.22 ± 0.15	1.39 ± 0.23	1.60 ± 0.21	1.15 ± 0.08	1.39 ± 0.22	1.17 ± 0.16
16:0		20.36 ± 1.92	19.77 ± 3.27	19.12 ± 1.30	15.68 ± 1.02	20.92 ± 3.38	16.25 ± 1.89
18:0		4.57 ± 0.70	3.92 ± 0.62	3.62 ± 0.20	3.07 ± 0.18	3.42 ± 0.48	2.86 ± 0.30
18:1		37.48 ± 3.43	40.14 ± 6.22 ^x	33.07 ± 2.00	26.46 ± 1.29 ^y	37.09 ± 6.83	30.00 ± 3.22 ^y
18:2 (n-6)		10.92 ± 1.29	15.71 ± 2.32	13.35 ± 1.16	14.99 ± 0.83	13.18 ± 2.31	12.81 ± 1.88
18:3 (n-3)		0.54 ± 0.11 ^a	0.29 ± 0.11 ^x	0.94 ± 0.12 ^a	1.03 ± 0.19 ^y	1.61 ± 0.42 ^b	1.14 ± 0.17 ^y
20:4 (n-6)		1.13 ± 0.12 ^a	1.18 ± 0.21	1.60 ± 0.16 ^b	1.40 ± 0.13	1.45 ± 0.20 ^{ab}	1.06 ± 0.14
20:5 (n-3)		0.28 ± 0.04 ^a	0.19 ± 0.03 ^x	1.69 ± 0.29 ^b	1.54 ± 0.18 ^y	0.45 ± 0.05 ^a	0.33 ± 0.04 ^x
22:6 (n-3)		0.56 ± 0.10 ^a	0.47 ± 0.09 ^x	2.04 ± 0.38 ^b	1.95 ± 0.22 ^y	0.51 ± 0.08 ^a	0.44 ± 0.07 ^x
HDL							
14:0		0.91 ± 0.10	0.94 ± 0.19	0.86 ± 0.12	0.67 ± 0.05	0.96 ± 0.18	0.91 ± 0.13
16:0		10.56 ± 0.86 ^b	12.09 ± 1.55 ^y	7.30 ± 0.82 ^a	7.39 ± 0.55 ^x	10.28 ± 1.52 ^b	8.57 ± 1.34 ^{xy}
18:0		2.27 ± 0.16 ^b	2.07 ± 0.19 ^y	1.67 ± 0.17 ^a	1.48 ± 0.10 ^x	1.66 ± 0.18 ^a	1.98 ± 0.24 ^{xy}
18:1		19.82 ± 1.30	24.11 ± 2.36 ^y	15.50 ± 1.86	13.55 ± 0.95 ^x	18.75 ± 2.26	18.57 ± 2.42 ^{xy}
18:2 (n-6)		5.76 ± 0.45	8.89 ± 1.14	5.37 ± 0.61	7.58 ± 0.56	7.41 ± 1.25	6.52 ± 0.95
18:3 (n-3)		0.27 ± 0.04 ^a	0.36 ± 0.09	0.37 ± 0.09 ^a	0.51 ± 0.08	0.83 ± 0.12 ^b	0.59 ± 0.09
20:4 (n-6)		0.39 ± 0.05 ^a	0.51 ± 0.07	0.47 ± 0.05 ^{ab}	0.50 ± 0.04	0.58 ± 0.06 ^b	0.45 ± 0.06
20:5 (n-3)		0.13 ± 0.02 ^a	0.13 ± 0.02 ^x	0.44 ± 0.09 ^c	0.50 ± 0.06 ^z	0.20 ± 0.02 ^b	0.32 ± 0.06 ^y
22:6 (n-3)		0.31 ± 0.05 ^{ab}	0.25 ± 0.04 ^x	0.45 ± 0.07 ^a	0.61 ± 0.07 ^y	0.27 ± 0.04 ^b	0.21 ± 0.05 ^x

¹ Values are means ± SE, n = 15 for low and n = 11 for high P/S groups of subjects. Significant oil treatment effects were determined by repeated measures ANOVA. abcIndicate oil treatment effects within subjects in the low dietary P/S group (P < 0.05). xyzIndicate oil treatment effects within subjects in the high dietary P/S group (P < 0.05).

is confirmed by other studies (Blonk et al. 1990, Sanders et al. 1989, Schmidt et al. 1993).

The lack of a flaxseed oil effect on triacylglycerol levels has been observed by others (Sanders and Roshanai 1983), and it suggests that 20:5(n-3) and 22:6(n-3) are responsible for the hypotriglyceridemic effect. The results of the present study suggest that either the flaxseed oil intake was not great enough to produce sufficient amounts of long-chain (n-3) fatty acids (Tables 4 and 5) or the conversion of 18:3(n-3) to 20:5(n-3) was limited, even in participants consuming a low P/S diet. This limitation in conversion of 18:3(n-3) to 20:5(n-3) was apparent in the changes in fatty acid concentration of the lipoproteins after each oil treatment (Fig. 1, 2). Although the flaxseed treatment ele-

vated 18:3(n-3) levels of lipoprotein fractions more than the fish oil treatment (Fig. 1), no changes occurred in the 20:5(n-3) levels when consuming flaxseed oil (Fig. 2).

Plasma total and LDL cholesterol levels were slightly (P < 0.1) but not significantly elevated by fish oil and flaxseed oil supplements in participants in both dietary P/S groups. Other investigators (Brown et al. 1990) have reported similar increases in plasma total and LDL cholesterol levels when humans were supplemented with lower doses (1.5–2.0 g/d) of marine (n-3) fatty acids for shorter periods of feeding. In contrast, some researchers (Illingworth et al. 1984) report that ten-fold higher doses (24 g/d) of 20:5(n-3) and 22:6(n-3) reduce total and LDL cholesterol levels. Studies that

TABLE 5

Fatty acid concentration of plasma cholesteryl ester fraction of VLDL, LDL and HDL from subjects consuming high or low polyunsaturated/saturated fatty acid (P/S) diets after olive, fish or flaxseed oil treatments¹

Oil treatment P/S group	Composition						Oil treatment P/S group
	Olive		Fish		Linseed		
	Low P/S	High P/S	Low P/S	High P/S	Low P/S	High P/S	
mg fatty acid/L plasma							
VLDL							
14:0	0.59 ± 0.10 ^b	0.41 ± 0.07 ^y	0.28 ± 0.04 ^a	0.18 ± 0.02 ^x	0.28 ± 0.07 ^a	0.34 ± 0.07 ^y	VLDL 14:0
16:0	4.89 ± 0.61 ^b	3.57 ± 0.53 ^y	3.19 ± 0.29 ^a	2.78 ± 0.22 ^x	3.94 ± 0.58 ^{ab}	4.27 ± 0.90 ^y	16:0
18:0	1.96 ± 0.49 ^b	1.35 ± 0.35 ^y	0.71 ± 0.04 ^a	0.59 ± 0.03 ^x	0.69 ± 0.06 ^a	0.85 ± 0.13 ^y	18:0
18:1	9.34 ± 1.14 ^b	9.76 ± 1.78	6.31 ± 0.65 ^a	4.64 ± 0.39	7.29 ± 1.13 ^{ab}	9.66 ± 2.35 ^y	18:1
18:2 (n-6)	13.71 ± 1.46	11.56 ± 2.20	11.15 ± 1.40	10.58 ± 1.36	15.59 ± 2.80	19.27 ± 5.23 ^y	18:2 (n-6)
18:3 (n-3)	0.29 ± 0.05	0.19 ± 0.07 ^x	0.32 ± 0.06	0.21 ± 0.03 ^x	0.47 ± 0.10	0.62 ± 0.14 ^y	18:3 (n-3)
20:4 (n-6)	1.62 ± 0.28	1.21 ± 0.25	1.26 ± 0.14	0.98 ± 0.14	1.62 ± 0.22	1.76 ± 0.37 ^y	20:4 (n-6)
20:5 (n-3)	0.32 ± 0.08 ^a	0.09 ± 0.03 ^x	0.65 ± 0.10 ^b	0.54 ± 0.08 ^z	0.40 ± 0.07 ^a	0.27 ± 0.04 ^y	20:5 (n-3)
22:6 (n-3)	0.31 ± 0.08	0.33 ± 0.11	0.34 ± 0.08	0.24 ± 0.04	0.18 ± 0.04	0.33 ± 0.18 ^y	22:6 (n-3)
LDL							
14:0	2.17 ± 0.21 ^{ab}	1.44 ± 0.17	2.66 ± 0.20 ^b	1.86 ± 0.23	1.90 ± 0.24 ^a	1.65 ± 0.17 ^y	LDL 14:0
16:0	30.96 ± 2.04 ^{ab}	22.97 ± 2.66 ^x	35.15 ± 2.95 ^b	30.13 ± 2.98 ^y	29.29 ± 1.73 ^a	23.23 ± 2.01 ^y	16:0
18:0	3.25 ± 0.27 ^b	2.61 ± 0.27	2.83 ± 0.17 ^b	2.25 ± 0.10	2.26 ± 0.15 ^a	2.31 ± 0.30 ^y	18:0
18:1	54.95 ± 3.60	42.37 ± 4.85	54.70 ± 4.12	45.07 ± 5.11	49.11 ± 3.08	40.80 ± 3.92 ^y	18:1
18:2 (n-6)	138.39 ± 7.68	113.90 ± 11.47	137.48 ± 9.47	131.08 ± 9.80	133.50 ± 8.51	111.40 ± 10.40 ^y	18:2 (n-6)
18:3 (n-3)	1.53 ± 0.22 ^a	1.13 ± 0.19 ^x	1.96 ± 0.14 ^b	1.66 ± 0.23 ^x	2.80 ± 0.19 ^c	2.61 ± 0.39 ^y	18:3 (n-3)
20:4 (n-6)	16.68 ± 1.48	12.44 ± 2.10	17.19 ± 1.97	13.31 ± 2.04	15.95 ± 1.52	11.05 ± 1.21 ^y	20:4 (n-6)
20:5 (n-3)	2.17 ± 0.53 ^a	0.78 ± 0.10 ^x	10.53 ± 1.43 ^b	8.66 ± 0.81 ^y	2.96 ± 0.36 ^a	1.63 ± 0.30 ^y	20:5 (n-3)
22:6 (n-3)	1.57 ± 0.16 ^a	1.03 ± 0.14 ^x	2.66 ± 0.32 ^b	2.37 ± 0.24 ^y	1.25 ± 0.12 ^a	0.89 ± 0.12 ^y	22:6 (n-3)
HDL							
14:0	1.48 ± 0.16	1.24 ± 0.09	1.66 ± 0.18	1.39 ± 0.25	1.38 ± 0.17	1.42 ± 0.23 ^y	HDL 14:0
16:0	19.20 ± 1.51	20.33 ± 1.60	21.19 ± 2.39	21.68 ± 1.83	17.48 ± 1.87	17.71 ± 2.41 ^y	16:0
18:0	2.27 ± 0.29 ^b	2.09 ± 0.31	1.94 ± 0.16 ^{ab}	1.66 ± 0.13	1.53 ± 0.06 ^a	1.73 ± 0.18 ^y	18:0
18:1	33.23 ± 2.82	36.44 ± 2.37	35.93 ± 3.76	33.57 ± 3.07	31.86 ± 2.65	31.81 ± 4.38 ^y	18:1
18:2 (n-6)	83.72 ± 6.34	102.37 ± 7.44	84.46 ± 7.64	97.70 ± 9.21	78.23 ± 8.50	85.46 ± 11.79 ^y	18:2 (n-6)
18:3 (n-3)	0.94 ± 0.13 ^a	1.02 ± 0.13 ^x	1.70 ± 0.34 ^a	2.01 ± 0.28 ^y	2.06 ± 0.30 ^b	1.77 ± 0.35 ^y	18:3 (n-3)
20:4 (n-6)	10.47 ± 0.96	11.89 ± 1.58	11.53 ± 1.65	10.23 ± 0.91	10.04 ± 1.25	8.46 ± 1.41 ^y	20:4 (n-6)
20:5 (n-3)	1.40 ± 0.34 ^a	0.90 ± 0.17 ^x	6.46 ± 1.07 ^b	6.98 ± 0.70 ^y	1.72 ± 0.22 ^a	1.80 ± 0.57 ^y	20:5 (n-3)
22:6 (n-3)	0.99 ± 0.09 ^a	0.96 ± 0.13 ^x	1.57 ± 0.23 ^b	1.76 ± 0.14 ^y	0.88 ± 0.12 ^a	0.76 ± 0.16 ^y	22:6 (n-3)

¹ Values are means ± SE, n = 15 for low and n = 11 for high P/S groups of subjects. Significant oil treatment effects were determined by repeated measures ANOVA. ^a^b^cIndicate oil treatment effects within subjects in the low dietary P/S group (P < 0.05). ^x^y^zIndicate oil treatment effects within subjects in the high dietary P/S group (P < 0.05).

¹ Values repeated in effects with

used large doses of marine oil supplements may be testing the effect of a change in fat quality and P/S ratio rather than the specific effects of (n-3) fatty acids. The relative amounts of 20:5(n-3) and 22:6(n-3) in fish oil preparations may also influence the net change in plasma lipids and lipoprotein cholesterol levels observed (Childs et al. 1990).

Although the lipid hypothesis focuses on reducing total and LDL cholesterol levels, plasma triacylglycerol may also be a predictor of coronary heart disease (Austin 1991). Therefore, reductions in plasma triacylglycerol may also promote a less atherogenic plasma lipid profile (Assmann et al. 1991). The present study demonstrates that low doses of fish oil, easily within the range of normal intakes, reduce plasma triacylglycerol

levels without significantly affecting total and LDL cholesterol levels.

Fish oil-induced fatty acid changes in plasma lipoproteins may alter in vivo LDL oxidation and reduce atherogenic potential (Simopoulos 1991). In rabbits fed 300 mg/(kg body weight · d) of purified eicosapentaenoate, LDL particles were less susceptible to oxidative modification by Cu²⁺ than the LDL of control rabbits (Saito et al. 1991). The incorporation of 20:5(n-3) and 22:6(n-3) into LDL cholesterol ester may also increase lipoprotein core fluidity and reduce the particle atherogenicity (Harris 1989). These studies suggest ways in which a change in fatty acid composition of the lipoprotein particle may also be important in determining atherogenicity of lipoprotein particles present.

Participated in 20:4(n-3) acid levels significant results and moderate consumption et al. 19 was increased significantly re high level unclear a result for 20:4

TABLE 6

Fatty acid concentration of plasma phospholipid fraction of VLDL, LDL and HDL from subjects consuming high or low polyunsaturated/saturated fatty acid (P/S) diets after olive, fish or flaxseed oil treatments¹

low	Composition						
	Oil treatment	Olive		Fish		Linseed	
		P/S group	Low P/S	High P/S	Low P/S	High P/S	Low P/S
		mg fatty acid/L plasma					
	VLDL						
4 ± 0.07 ^y	14:0	0.16 ± 0.07 ^a	0.11 ± 0.05	0.34 ± 0.06 ^{ab}	0.25 ± 0.04	0.39 ± 0.08 ^b	0.56 ± 0.28
7 ± 0.90 ^x	16:0	20.10 ± 2.80	17.02 ± 3.39 ^{xy}	15.59 ± 2.10	13.82 ± 1.81 ^x	20.93 ± 2.77	22.50 ± 4.24 ^y
5 ± 0.13 ^y	18:0	9.44 ± 1.20	7.77 ± 1.25	7.80 ± 1.12	7.49 ± 1.14	9.72 ± 1.23	10.53 ± 2.42
5 ± 2.35	18:1	14.06 ± 2.14 ^a	13.19 ± 2.69 ^x	9.37 ± 1.18 ^b	6.69 ± 0.77 ^y	11.79 ± 1.63 ^a	12.86 ± 2.39 ^x
7 ± 5.23	18:2 (n-6)	14.17 ± 1.59 ^{ab}	14.31 ± 2.69	10.81 ± 1.66 ^a	11.00 ± 1.63	15.70 ± 2.01 ^b	17.88 ± 4.01
2 ± 0.14 ^y	18:3 (n-3)	0.15 ± 0.05 ^a	0.11 ± 0.05 ^x	0.22 ± 0.05 ^{ab}	0.14 ± 0.04 ^x	0.35 ± 0.04 ^b	0.32 ± 0.07 ^y
5 ± 0.37	20:4 (n-6)	6.43 ± 1.06	5.12 ± 1.04 ^{xy}	4.29 ± 0.56	3.28 ± 0.53 ^x	6.23 ± 0.91	6.14 ± 1.12 ^y
7 ± 0.04 ^y	20:5 (n-3)	0.54 ± 0.19 ^a	0.21 ± 0.06 ^x	1.67 ± 0.26 ^b	1.37 ± 0.18 ^z	0.80 ± 0.14 ^a	0.78 ± 0.14 ^y
3 ± 0.18	22:6 (n-3)	2.35 ± 0.40	1.66 ± 0.23	2.70 ± 0.41	2.29 ± 0.39	2.17 ± 0.37	1.96 ± 0.33
	LDL						
5 ± 0.17	14:0	0.25 ± 0.12 ^a	0.15 ± 0.09 ^x	0.83 ± 0.14 ^b	0.88 ± 0.09 ^y	0.92 ± 0.09 ^b	0.73 ± 0.18 ^y
3 ± 2.01 ^x	16:0	55.12 ± 3.48	46.81 ± 6.08	62.84 ± 4.65	53.91 ± 5.51	59.94 ± 4.11	50.47 ± 4.92
1 ± 0.30	18:0	22.60 ± 1.26 ^a	17.99 ± 1.40 ^x	26.74 ± 1.69 ^b	23.72 ± 1.09 ^y	23.94 ± 1.25 ^{ab}	20.56 ± 1.61 ^{xy}
1 ± 3.92	18:1	25.25 ± 1.89	22.87 ± 2.74	26.75 ± 1.68	22.71 ± 2.32	27.58 ± 1.70	23.04 ± 2.16
1 ± 10.40	18:2 (n-6)	37.51 ± 2.25	35.21 ± 3.82	38.47 ± 2.59	38.84 ± 2.90	42.18 ± 2.50	35.97 ± 4.12
1 ± 0.39 ^y	18:3 (n-3)	0.36 ± 0.05 ^a	0.22 ± 0.05 ^x	0.45 ± 0.06 ^a	0.52 ± 0.08 ^y	0.79 ± 0.08 ^b	0.71 ± 0.07 ^z
5 ± 1.21	20:4 (n-6)	14.65 ± 0.96	12.01 ± 1.84	14.29 ± 1.48	11.28 ± 1.35	15.41 ± 1.28	11.43 ± 1.04
5 ± 0.30 ^x	20:5 (n-3)	1.21 ± 0.25 ^a	0.56 ± 0.06 ^x	5.91 ± 0.65 ^b	5.25 ± 0.48 ^y	1.79 ± 0.18 ^a	1.14 ± 0.18 ^x
2 ± 0.12 ^x	22:6 (n-3)	6.15 ± 0.49 ^a	4.61 ± 0.59 ^x	8.35 ± 1.02 ^b	7.53 ± 0.60 ^y	4.68 ± 0.42 ^a	3.34 ± 0.36 ^x
	HDL						
1 ± 0.23	14:0	1.33 ± 0.11	1.51 ± 0.11	1.40 ± 0.18	1.45 ± 0.16	1.24 ± 0.17	1.57 ± 0.31
1 ± 2.41	16:0	80.47 ± 6.93	95.42 ± 8.36	90.24 ± 8.59	103.24 ± 7.69	89.02 ± 7.73	103.05 ± 14.23
1 ± 0.18	18:0	35.67 ± 2.93	39.54 ± 1.76	40.46 ± 3.39	47.43 ± 3.40	36.58 ± 2.95	42.95 ± 5.47
1 ± 4.38	18:1	37.16 ± 3.10	41.55 ± 3.27	42.74 ± 3.64	45.27 ± 3.41	42.86 ± 3.17	50.40 ± 6.83
1 ± 11.79	18:2 (n-6)	62.73 ± 6.04	78.55 ± 5.38	63.10 ± 5.59	79.46 ± 6.44	67.98 ± 6.24	84.22 ± 12.01
1 ± 0.35 ^y	18:3 (n-3)	0.44 ± 0.09 ^a	0.51 ± 0.09 ^x	0.78 ± 0.11 ^b	0.81 ± 0.16 ^x	1.19 ± 0.17 ^c	1.40 ± 0.26 ^y
5 ± 1.41	20:4 (n-6)	29.98 ± 2.12	33.86 ± 3.30	27.98 ± 2.62	27.65 ± 1.45	30.21 ± 2.72	30.14 ± 4.04
1 ± 0.57 ^x	20:5 (n-3)	2.32 ± 0.41 ^a	1.67 ± 0.27 ^x	11.19 ± 1.77 ^b	13.66 ± 0.85 ^y	3.66 ± 0.43 ^a	4.16 ± 1.40 ^x
1 ± 0.16 ^x	22:6 (n-3)	9.67 ± 0.85 ^a	10.51 ± 1.12 ^x	15.14 ± 1.56 ^b	19.16 ± 0.80 ^y	9.40 ± 1.09 ^a	10.09 ± 1.58 ^x

¹ Values are means ± SE, $n = 15$ for low and $n = 11$ for high P/S groups of subjects. Significant oil treatment effects were determined by repeated measures ANOVA. abcIndicate oil treatment effects within subjects in the low dietary P/S group ($P < 0.05$). xyzIndicate oil treatment effects within subjects in the high dietary P/S group ($P < 0.05$).

Participants consuming a low P/S diet, supplemented with fish oil, displayed a significant increase in 20:4(n-6) levels in LDL triacylglycerol. Arachidonic acid levels in all other lipoprotein lipid classes were not significantly affected by the fish oil treatment. Similar results were reported when low (DeLany et al. 1990) and moderate doses of long-chain (n-3) fatty acids were consumed by normal individuals (Bronsggeest-Schoute et al. 1981). However, when the (n-3) fatty acid dose was increased to 8 g/d, 20:4(n-6) levels were significantly reduced (DeLany et al. 1990). In cases in which high levels of (n-3) fatty acids are supplemented, it is unclear whether the observed reduction in 20:4(n-6) is a result of the dietary substitution of (n-3) fatty acids for 20:4(n-6) or is an effect of the (n-3) fatty acid. In

the present study, 20:4(n-6) levels were in general not significantly altered, indicating that the hypotriacylglyceridemic effect may be specifically attributed to the (n-3) fatty acids supplemented.

Flaxseed oil treatment significantly increased 20:5(n-3) levels in the HDL triacylglycerol, VLDL phospholipid and VLDL cholesteryl ester fractions of participants consuming a high P/S diet, suggesting that some desaturation and elongation of 18:3(n-3) occurred. The dose of flaxseed oil may have been inadequate to compete for $\Delta 6$ -desaturation, even in the low dietary P/S group. Others (Sanders and Roshanai 1983) have failed to demonstrate significant increases in 20:5(n-3) when participants were fed 9 g/d of 18:3(n-3), and they suggest that human conversion of 18:3(n-3) to 20:5(n-

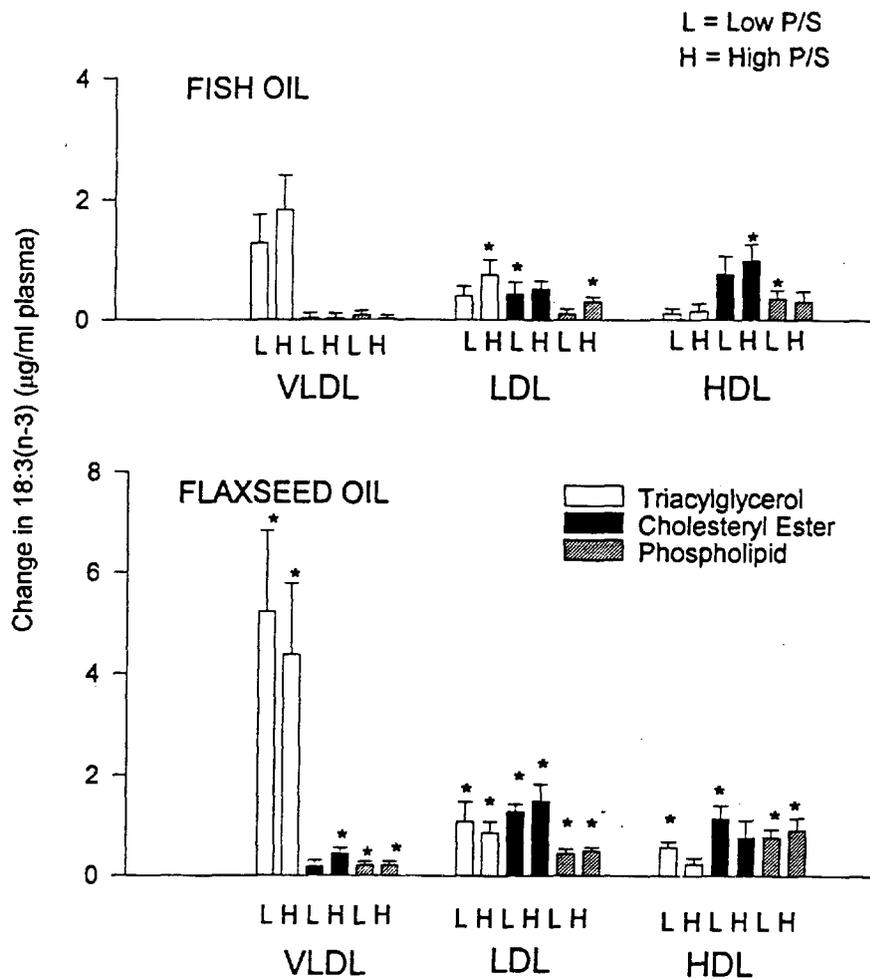


FIGURE 1 Effect of (n-3) fatty acid treatments on change in 18:3(n-3) in plasma lipoproteins of subjects receiving flaxseed oil treatments. Values reported are means \pm SE ($n = 26$) of differences in fatty acid levels between olive oil and fatty acid treatments. *Indicates significant effect of oil treatment ($P < 0.05$). L and H indicate low and high dietary polyunsaturated/saturated fatty acid ratio (P/S) groups, respectively.

3) is limited, whereas the 18:2(n-6) to 20:4(n-6) pathway is more efficient. This theory was recently challenged by Emken et al. (1993), who found that high dietary levels (30 g/d vs. 15 g/d) of 18:2(n-6) did not influence desaturation of 18:3(n-3) or incorporation of 18:3(n-3) into plasma lipids. These researchers found that the conversion of 18:3(n-3) was three to four times greater than the conversion of 18:2(n-6); however, the total amount of 18:3(n-3) metabolites produced from 3.5 g/d of 18:3(n-3) was very low (0.35 g/d), and a significantly larger dose of 18:3(n-3) (21–29 g/d) might be required to provide the equivalent effects of 2–2.5 g/d of marine (n-3) fatty acids.

It was hypothesized that plasma lipid and lipoprotein parameters would be affected more by a (n-3) fatty acid treatment in individuals consuming low P/S ratio diets than in individuals consuming high P/S ratio diets. This hypothesis was based on the rationale that lower levels of the linoleate metabolite, 20:4(n-6), would compete less with fish oil for incorporation into plasma lipid and lipoprotein fractions. In this regard,

plasma triacylglycerol levels were significantly reduced in the low P/S group but not significantly reduced in the high P/S group. Although it was hypothesized that the conversion of 18:3(n-3) to 20:5(n-3) would be more efficacious in the low P/S group, this was not observed.

Presently, many human studies rely on dietary micronutrient analysis but do not account for specific dietary fatty acids, for example, arachidonate. This may be important when fish oil is supplemented in low doses because small changes in dietary arachidonate may influence the (n-3) fatty acid effect, particularly when the subject's intake of (n-3) fatty acids from marine sources is low.

It is apparent that modest intake of marine (n-3) fatty acids promotes change in lipoprotein fatty acid content, which may affect a variety of physiological events associated with atherosclerosis. Cellular events such as reductions in fibrinogen (Hostmark et al. 1988), platelet activating factor (Sperling et al. 1987), platelet-derived growth factor (Fox and DiCorelto 1988), and increase

L = Low P/S
H = High P/S

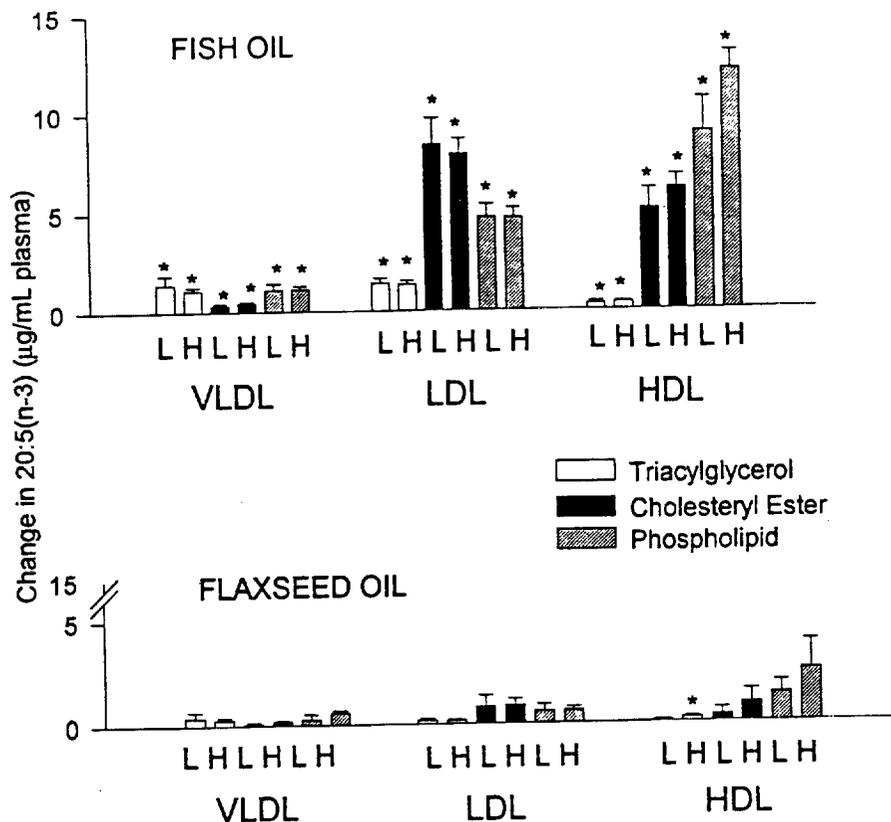


FIGURE 2 Effect of (n-3) fatty acid treatments on change in 20:5(n-3) in plasma lipoproteins of subjects receiving fish or flaxseed oil treatments. Values reported are means ± SE of differences in fatty acid levels between olive oil and (n-3) fatty acid treatments. *Indicates significant effect of oil treatment (P < 0.05). L and H indicate low and high dietary polyunsaturated/saturated fatty acid ratio (P/S) groups, respectively.

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In tissue plasminogen activator and endothelial-derived relaxation factor (Shimokawa and Vanhoutte [1989]) have been induced by fish oil supplementation and may augment the cardiovascular benefits of 20:5(n-3) and 22:6(n-3). It is important for future studies to assess the effects of (n-3) fatty acids with regard to lipoprotein composition, eicosanoid production, platelet aggregation, lipid-dependent enzymes, endothelial and smooth muscle cell interactions, and other factors involved in the atherogenic process in relation to the intake of specific dietary saturated fats currently perceived to influence the atherosclerotic process.

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EXHIBIT S

Effect of garlic and fish-oil supplementation on serum lipid and lipoprotein concentrations in hypercholesterolemic men¹⁻³

Adam J Adler and Bruce J Holub

See corresponding editorial on page 560.

ABSTRACT This study examined the effects of garlic and fish-oil supplementation (alone and in combination) on fasting serum lipids and lipoproteins in hypercholesterolemic subjects. After an initial run-in phase, 50 male subjects with moderate hypercholesterolemia were randomly assigned for 12 wk to one of four groups: 1) 900 mg garlic placebo/d + 12 g oil placebo/d; 2) 900 mg garlic/d + 12 g oil placebo/d; 3) 900 mg garlic placebo/d + 12 g fish oil/d, providing 3.6 g n-3 fatty acids/d; and 4) 900 mg garlic/d + 12 g fish oil/d. In the placebo group, mean serum total cholesterol, low-density-lipoprotein cholesterol (LDL-C), and triacylglycerols were not significantly changed in relation to baseline. Mean group total cholesterol concentrations were significantly lower with garlic + fish oil (-12.2%) and with garlic (-11.5%) after 12 wk but not with fish oil alone. Mean LDL-C concentrations were reduced with garlic + fish oil (-9.5%) and with garlic (-14.2%) but were raised with fish oil (+8.5%). Mean triacylglycerol concentrations were reduced with garlic + fish oil (-34.3%) and fish oil alone (-37.3%). The garlic groups (with and without fish oil) had significantly lower ratios of total cholesterol to high-density-lipoprotein cholesterol (HDL-C) and LDL-C to HDL-C. In summary, garlic supplementation significantly decreased both total cholesterol and LDL-C whereas fish-oil supplementation significantly decreased triacylglycerol concentrations and increased LDL-C concentrations in hypercholesterolemic men. The combination of garlic and fish oil reversed the moderate fish-oil-induced rise in LDL-C. Coadministration of garlic with fish oil was well-tolerated and had a beneficial effect on serum lipid and lipoprotein concentrations by providing a combined lowering of total cholesterol, LDL-C, and triacylglycerol concentrations as well as the ratios of total cholesterol to HDL-C and LDL-C to HDL-C. *Am J Clin Nutr* 1997;65:445-50.

KEY WORDS Garlic, fish oil, total cholesterol, low-density-lipoprotein cholesterol, high-density-lipoprotein cholesterol, triacylglycerol

INTRODUCTION

The role of elevated serum total cholesterol and low-density-lipoprotein cholesterol (LDL-C) as well as reduced high-density-lipoprotein cholesterol (HDL-C) in the development of coronary artery disease is well-established (1). Patients with accompanying elevated serum triacylglycerol concentrations

are at increased risk of developing atherosclerosis and coronary artery disease (2). Both the PROCAM study (3) and the Helsinki Heart Study (4) suggested that hypertriglyceridemia is a powerful additional risk factor of coronary artery disease, particularly when excessive triacylglycerol concentrations coincide with a high ratio (> 5.0) of LDL-C to HDL-C. In addition, recent studies have shown that triacylglycerol is independently related to coronary artery disease risk (5) and to the extent of coronary atherosclerosis (6).

Effective and safe treatment to reduce the simultaneous elevation of total cholesterol and triacylglycerol concentrations is limited (7). The use of nutritional supplements either alone (8) or in combination with a drug (7, 9) has been shown to be effective in lowering total cholesterol and triacylglycerol concentrations in hyperlipidemic subjects. Recently, Contacos et al (7) showed that pravastatin and fish oil, in combination only, effectively lowered both total cholesterol and triacylglycerol concentrations in subjects with both elevated cholesterol and triacylglycerol.

Fish oils rich in the n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to effectively reduce elevated triacylglycerol concentrations (10). Fish oils may also raise HDL-C to a modest extent (10). However, supplementation with fish oil has been found to increase serum LDL-C concentrations (10, 11). To provide a more complete management of hyperlipidemia with fish oil, it may be beneficial to use an additional nutritional supplement to simultaneously lower LDL-C concentrations. Supplementation with garlic alone has been found to significantly reduce total cholesterol concentrations (8, 12). The purpose of the present study was to determine whether garlic powder, when given in combination with fish oil, could provide an effective and well-tolerated nutritional regimen for simultaneously reducing

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elevated total cholesterol, LDL-C, and triacylglycerol concentrations in hypercholesterolemic men.

SUBJECTS AND METHODS

Subjects

Of 100 men screened for their total serum cholesterol concentration with a compact total cholesterol analyzer (Chemtrak 228; Accumeter, Sunnyvale, CA), 50 took part in the study, with each giving written informed consent. The principal criterion for entry was a total cholesterol concentration > 5.2 mmol/L (200 mg/dL). Subjects taking lipid-altering or blood pressure-altering medications or supplements within 4 wk of the beginning of the study were excluded. Other medications were allowed if they were not initiated within 4 wk of the beginning of the study. Subjects taking part in the study did not have diagnosed diabetes mellitus or cardiovascular disease.

The study conformed with the ethical guidelines of the University of Guelph.

Materials and supplements

Garlic pills (Kwai; Lichtwer Pharma, Berlin) were given as one 300-mg sugar-coated pill three times daily with meals. Garlic placebo pills (also from Lichtwer Pharma) were identical in appearance but contained no garlic powder. Fish oil containing *n*-3 fatty acids (Nupulse; Nu-Life, Markham, Canada) was given as four 1-g capsules (each containing 180 mg EPA and 120 mg DHA) three times daily with meals. Evening primrose oil (Bioriginal; PGE Canada, Saskatoon, Canada) containing *n*-6 fatty acids was used as a control oil supplement; it was given as three 1.3-g capsules three times daily with meals. Garlic pills were administered in a double-blind fashion. Fish oil and oil placebo were given in a single-blind fashion.

Study design

This randomized, placebo-controlled trial began with a 3-wk run-in phase in which baseline total cholesterol concentrations were confirmed by standard enzymatic techniques (procedure no. 352; Sigma Diagnostics, St Louis). At baseline (week 0), subjects with serum total cholesterol concentrations > 5.2 mmol/L (200 mg/dL) were randomly assigned to one of four treatment groups for 12 wk (week 0 to week 12). The four study regimens were as follows: 1) 900 mg garlic placebo/d + 12 g oil placebo/d; 2) 900 mg garlic/d + 12 g oil placebo/d; 3) 900 mg garlic placebo/d + 12 g fish oil/d, providing 3.6 g *n*-3 fatty acids/d; and 4) 900 mg garlic/d + 12 g fish oil/d.

Subjects provided overnight (12–14 h) fasting blood samples at weeks 0, 3, 6, 9, and 12. Sitting blood pressure and resting heart rate were taken at each visit by a digital blood pressure monitor. Compliance was assessed by capsule or pill count and serum phospholipid analyses (*see* below). Blinding to the supplements and their respective placebos was checked at the end of the study by a simple questionnaire. Side effects were monitored throughout the study period. Two 3-d estimated dietary records (two weekdays, one weekend day) were obtained from each subject: one during the 3-wk period before baseline (week 0), the other between week 9 and week 12 of the study. Dietary analysis was performed on the CanWest Diet Analysis Plus program (West Publishing Co, St Paul). Fasting

blood samples were analyzed for total cholesterol, LDL-C, HDL-C, and triacylglycerol concentrations at all time points between week 0 and week 12. Additionally, fatty acid compositions of total serum phospholipid were analyzed at week 0 and week 12.

Laboratory analyses

Blood was drawn by venipuncture into evacuated tubes (Vacutainer; Becton Dickinson, Rutherford, NJ), allowed to sit for 1 h, and then centrifuged (2500 rpm, $1000 \times g$) for 15 min at 30 °C to recover serum. Serum fractions were divided into aliquots and stored at -20 °C until assayed in one batch. Total cholesterol and triacylglycerol were determined by standard enzymatic techniques (procedure nos. 352 and 339; Sigma Diagnostics). HDL-C was determined in serum after precipitation of apolipoprotein B-containing lipoproteins (procedure no. 352–3; Sigma Diagnostics). LDL-C was calculated with the Friedewald equation (13) except if triacylglycerols were > 4.6 mmol/L (400 mg/dL) at one or more time point in the study, in which case LDL-C was determined directly (procedure no. 353-A; Sigma Diagnostics) at all time points for that subject. Fatty acid analyses were performed by capillary gas-liquid chromatography of the isolated phospholipid (by thin-layer chromatography) after transmethylation using procedures similar to those cited elsewhere (14).

Statistical analyses

Statistical tests were performed with the SAS computer program (SAS Institute Inc, Cary, NC). After logarithmic transformation of the data, efficacy parameters were analyzed by analysis of covariance. Pair-wise comparisons between treatment groups were performed with protected least-significant-difference techniques. Paired *t* tests were used for within-group effects on difference scores from baseline. All statistical analyses were two-tailed, with $\alpha = 0.05$.

RESULTS

Of the 50 people originally randomly assigned to treatment, 4 withdrew (2 from the garlic placebo + fish oil group, 1 from the garlic + oil placebo group, and 1 from the garlic placebo + oil placebo group) for personal reasons or because of unrelated medical conditions. The baseline characteristics of the 46 remaining subjects are listed in **Table 1**. There were no significant differences between the baseline characteristics of the original 50 subjects and the remaining 46 subjects (data not shown).

Dietary intakes and body weights at entry and after nutritional supplementation for all subjects are shown in **Table 2**. No significant differences ($P > 0.05$) were found in dietary composition or body weight between treatment groups either at baseline or after 12 wk of treatment (data not shown).

In the placebo group, mean serum total cholesterol, LDL-C, triacylglycerol, and HDL-C were not significantly changed in relation to baseline. Mean group total cholesterol concentrations were significantly lower with garlic + fish oil (-12.2%) and with garlic alone (-11.5%) by week 12 (**Table 3**). Significant reductions in total cholesterol with garlic and garlic + fish oil were seen starting at week 9. In the group taking fish oil

TABLE 1
Baseline demographic characteristics by supplementation group¹

Characteristic	Placebo (n = 11)	Garlic (n = 12)	Fish oil (n = 10)	Garlic + fish oil (n = 13)	Total (n = 46)
Age (y)	45.4 ± 9.8	45.9 ± 12.6	45.4 ± 9.3	46.7 ± 13.1	45.9 ± 11.1
Weight (kg)	85.0 ± 11.3	85.1 ± 8.6	84.2 ± 21.7	84.6 ± 11.9	84.8 ± 13.4
BMI (kg/m ²)	26.4 ± 3.5	27.2 ± 3.0	26.8 ± 6.4	26.9 ± 3.5	26.8 ± 4.1
Total cholesterol (mmol/L)	6.46 ± 0.85	6.54 ± 0.88	6.54 ± 1.06	6.51 ± 1.06	6.51 ± 0.93
LDL cholesterol (mmol/L)	4.32 ± 0.83	4.39 ± 0.62	4.42 ± 0.88	4.47 ± 0.96	4.39 ± 0.80
HDL cholesterol (mmol/L)	1.20 ± 0.34	1.26 ± 0.22 ²	1.12 ± 0.34	1.13 ± 0.19	1.18 ± 0.27
Triacylglycerol (mmol/L)	1.95 ± 1.63	1.98 ± 1.10	2.34 ± 1.76	2.17 ± 1.54	2.10 ± 1.47
Total cholesterol/HDL cholesterol	5.80 ± 1.82 ²	5.38 ± 1.29 ²	6.38 ± 2.18	5.94 ± 1.56	5.85 ± 1.69
LDL cholesterol/HDL cholesterol	3.83 ± 1.18 ²	3.64 ± 0.90 ²	4.34 ± 1.60	4.08 ± 1.12	3.96 ± 1.19

¹ $\bar{x} \pm SD$.

² Significantly different from fish oil and garlic + fish oil, $P < 0.05$.

³ Significantly different from fish oil, $P < 0.05$.

alone, mean group total cholesterol concentrations were not significantly changed.

Mean group LDL-C concentrations were significantly reduced with garlic + fish oil (-9.5%) and with garlic (-14.2%) by week 12 (Table 4). In the fish-oil group, mean LDL-C concentrations were significantly raised (+8.5%) by week 3 and persisted until week 12. Significant LDL-C reductions in both groups taking garlic (garlic and garlic + fish oil) were achieved by week 9. There was no significant difference in reductions of LDL-C between the garlic + fish oil and garlic alone groups although the garlic alone group did show a consistent trend toward larger reductions in LDL-C throughout the study.

Mean group fasting serum triacylglycerol concentrations were significantly decreased with garlic + fish oil (-34.3%) and with fish oil alone (-37.3%) (Table 5). Reductions in triacylglycerol with fish oil (garlic + fish oil and fish oil alone) were noted by week 3. There was no significant difference in the triacylglycerol reduction seen between fish oil and garlic +

fish oil. In the garlic group, mean triacylglycerol concentrations were not significantly changed.

Although the fish-oil and garlic + fish oil groups showed significant increases in HDL-C in relation to baseline, there were no significant differences between the four groups at week 12 (Table 6). However, there was a consistent trend of higher HDL-C increases in both fish-oil groups (fish oil alone and garlic + fish oil) compared with both non-fish-oil groups (placebo and garlic alone).

Compared with placebo, for which no significant change was found (by week 12) in the ratios of total cholesterol to HDL-C and LDL-C to HDL-C, the ratio of total cholesterol to HDL-C was significantly reduced in the garlic group (-12.5%) and in the garlic + fish-oil group (-16.2%) but not in the fish-oil (alone) group. The reduction in the ratio of total cholesterol to HDL-C in the garlic + fish-oil group was significantly greater than in the garlic only group ($P < 0.05$). The ratio of LDL-C to HDL-C was significantly decreased in the garlic group (-15.3%) and in the garlic + fish oil group (-19.0%) with no change ($P > 0.05$) in the other two groups.

Mean systolic, diastolic, and arterial blood pressures for all subjects at entry were 120.1, 80.9, and 94.0 mm Hg, respectively. By week 12, reductions of 2.4-4.2% ($P < 0.05$ or $P <$

TABLE 2
Daily dietary intakes and body weight at entry and after supplementation¹

Variable	Entry (week 0)	Supplementation (week 12)
Protein		
(% of energy)	17.3 ± 0.6	16.9 ± 0.5
(g)	103 ± 4	104 ± 4
Carbohydrate		
(% of energy)	50.0 ± 1.5	50.2 ± 1.3
(g)	298 ± 14	307 ± 13
Fat		
(% of energy)	30.4 ± 1.2	30.9 ± 1.3
(g)	86.9 ± 5.3	89.5 ± 5.7
Saturated fat (g)	29.9 ± 2.4	31.2 ± 2.3
Monounsaturated fat (g)	32.4 ± 2.0	33.2 ± 2.2
Polyunsaturated fat (g)	16.5 ± 1.1	17.3 ± 1.1
Dietary fiber (g)	23.6 ± 1.5	24.7 ± 1.5
Cholesterol (mg)	337 ± 25	348 ± 24
Body weight (kg)	84.8 ± 2.0	84.9 ± 2.0
Alcohol (% of energy)	2.46 ± 0.65	2.00 ± 0.52
Total intake (kJ)	9905 ± 383	10196 ± 358

¹ $\bar{x} \pm SEM$; $n = 39$ for dietary variables, $n = 46$ for body weight.

TABLE 3
Effect of nutritional supplementation on total serum cholesterol concentrations¹

Time	Supplementation group			
	Placebo	Garlic	Fish oil	Garlic + fish oil
	<i>mmol/L</i>			
Week 0	6.46 ± 0.26	6.54 ± 0.25	6.54 ± 0.34	6.51 ± 0.29
Week 3	6.41 ± 0.31	6.62 ± 0.29	6.59 ± 0.32	6.46 ± 0.26
Week 6	6.33 ± 0.28 ^{ab}	6.33 ± 0.27 ^{ab}	6.49 ± 0.29 ^a	6.15 ± 0.25 ^{b2}
Week 9	6.43 ± 0.33 ^a	6.23 ± 0.30 ^{b3}	6.67 ± 0.37 ^{ab}	5.81 ± 0.22 ^{c4}
Week 12	6.49 ± 0.31 ^a	5.79 ± 0.23 ^{b4}	6.51 ± 0.38 ^{ab}	5.71 ± 0.20 ^{b4}
	(0.5 ± 1.8) ⁵	(-11.5 ± 2.1) ^{5,6}	(-0.5 ± 2.1)	(-12.2 ± 1.3) ^{5,6}

¹ $\bar{x} \pm SE$. Values with different superscript letters are significantly different from other groups at the corresponding time, $P < 0.05$.

²⁻⁴ Significantly different from baseline: ² $P < 0.01$, ³ $P < 0.05$, ⁴ $P < 0.001$.

⁵ Percentage change from week 0 in parentheses.

⁶ Significantly different from placebo and fish oil, $P < 0.01$.

TABLE 4
Effect of nutritional supplementation on serum LDL-cholesterol concentrations¹

Time	Supplement group			
	Placebo	Garlic	Fish oil	Garlic + fish oil
	<i>mmol/L</i>			
Week 0	4.32 ± 0.25	4.39 ± 0.18	4.42 ± 0.27	4.47 ± 0.27
Week 3	4.19 ± 0.25 ^a	4.26 ± 0.23 ^a	4.75 ± 0.32 ^{b2}	4.68 ± 0.29 ^b
Week 6	4.16 ± 0.27 ^a	4.26 ± 0.27 ^a	4.78 ± 0.41 ^{b2}	4.47 ± 0.28 ^a
Week 9	4.19 ± 0.28 ^a	3.90 ± 0.28 ^{b3}	4.94 ± 0.43 ^{c3}	4.19 ± 0.28 ^{a2}
Week 12	4.26 ± 0.31 ^a	3.77 ± 0.24 ^{b3}	4.81 ± 0.40 ^{c2}	4.06 ± 0.24 ^{b3}
	(-1.1 ± 2.8) ^d	(-14.2 ± 2.6) ^{2,6}	(8.5 ± 5.6) ^{2,7}	(-9.5 ± 3.2) ^{2,6}

¹ $\bar{x} \pm SE$. Values with different superscript letters are significantly different from other groups at the corresponding time, $P < 0.05$.

^{2,3,5} Significantly different from baseline: ² $P < 0.05$, ³ $P < 0.001$, ⁵ $P < 0.01$.

⁴ Percentage change from week 0 in parentheses.

⁶ Significantly different from placebo and fish oil, $P < 0.01$.

⁷ Significantly different from placebo, garlic, and fish oil, $P < 0.05$.

0.005) in mean systolic, diastolic, and arterial pressures were found for all three treatment groups (fish oil alone, garlic alone, and garlic + fish oil) relative to the placebo group.

According to capsule or pill counts at the end of the study, 100% of subjects who completed the study took > 80% of the supplements given. Blinding to the supplements was checked by the use of a questionnaire at the end of the study. Garlic pills and garlic placebo pills were identified correctly by 76% and 67% of the subjects, respectively. Fish oil and oil placebo were identified correctly by 100% and 61% of the subjects, respectively. As a measure of compliance with the fish-oil supplementation, serum phospholipid fatty acid profiles were measured at entry and after 12 wk of supplementation (Table 7). The results are characteristic of other studies in which fish-oil supplementation was at similar levels (14). The ratio of EPA to arachidonic acid increased significantly ($P < 0.001$) in the fish-oil groups (fish oil alone and garlic + fish oil) but did not change significantly ($P > 0.05$) in the groups taking no fish oil (placebo and garlic only groups) (Table 7).

All supplements used in this study had relatively few reported side effects. Odor due to garlic was reported in 20% of

TABLE 5
Effect of nutritional supplementation on serum triacylglycerol concentrations¹

Time	Supplement group			
	Placebo	Garlic	Fish oil	Garlic + fish oil
	<i>mmol/L</i>			
Week 0	1.95 ± 0.49	1.98 ± 0.32	2.34 ± 0.56	2.17 ± 0.43
Week 3	2.15 ± 0.79 ^a	2.07 ± 0.51 ^a	1.64 ± 0.43 ^{b2}	1.53 ± 0.21 ^{b2}
Week 6	1.95 ± 0.51 ^a	1.86 ± 0.31 ^a	1.62 ± 0.26 ^{b2}	1.49 ± 0.19 ^{b2}
Week 9	2.11 ± 0.80 ^a	2.16 ± 0.54 ^a	1.40 ± 0.20 ^{b2}	1.30 ± 0.18 ^{b2}
Week 12	1.94 ± 0.51 ^a	1.85 ± 0.37 ^a	1.46 ± 0.23 ^{b2}	1.36 ± 0.17 ^{b2}
	(-0.6 ± 4.3) ^d	(-6.1 ± 7.6)	(-37.7 ± 4.8) ^{4,5}	(-34.3 ± 4.8) ^{4,5}

¹ $\bar{x} \pm SE$. Values with different superscript letters are significantly different from other groups at the corresponding time, $P < 0.05$.

^{2,4} Significantly different from baseline: ² $P < 0.001$, ⁴ $P < 0.05$.

³ Percentage change from week 0 in parentheses.

⁵ Significantly different from placebo and garlic, $P < 0.001$.

TABLE 6
Effect of nutritional supplementation on serum HDL-cholesterol concentrations¹

Time	Supplement group			
	Placebo	Garlic	Fish oil	Garlic + fish oil
	<i>mmol/L</i>			
Week 0	1.20 ± 0.10	1.26 ± 0.06	1.12 ± 0.27	1.13 ± 0.05
Week 3	1.19 ± 0.10 ^a	1.36 ± 0.11 ^{b2}	1.22 ± 0.09 ^{b3}	1.22 ± 0.08 ^{b3}
Week 6	1.24 ± 0.10 ^a	1.34 ± 0.10 ^{ab2}	1.20 ± 0.14 ^{ab2}	1.27 ± 0.07 ^{b4}
Week 9	1.28 ± 0.11 ^a	1.38 ± 0.09 ^{a3}	1.26 ± 0.12 ^{a4}	1.26 ± 0.08 ^{a4}
Week 12	1.26 ± 0.11 ^{ab}	1.29 ± 0.08 ^a	1.21 ± 0.11 ^{ab2}	1.24 ± 0.06 ^{b3}
	(4.9 ± 1.7) ⁵	(2.6 ± 3.2)	(8.5 ± 4.4) ²	(9.1 ± 3.0) ²

¹ $\bar{x} \pm SE$. Values with different superscript letters are significantly different from other groups at the corresponding time, $P < 0.05$.

²⁻⁴ Significantly different from baseline: ² $P < 0.05$, ³ $P < 0.01$, ⁴ $P < 0.001$.

⁵ Percentage change from week 0 in parentheses.

the subjects taking garlic pills, and in none of the garlic placebo group. One subject reported a slight feeling of nausea with fish oil that did not persist throughout the study. No other serious side effects (other than occasional belching) were reported with either the fish oil or the oil placebo.

DISCUSSION

We evaluated the efficacy of single and combined therapy with garlic and fish oil in subjects with moderate hypercholesterolemia. In our study, treatment with garlic or fish oil significantly decreased serum total cholesterol and triacylglycerol, respectively; the combination of garlic and fish oil significantly reduced both serum lipids.

Garlic supplementation alone has been reported to reduce total cholesterol by 9–12% without a significant effect on HDL-C concentrations (8, 12), and in one study may have moderately lowered triacylglycerol concentrations (8). Although the reduction in total cholesterol (12% by week 12) and LDL-C (14% by week 12) observed with garlic alone in this study is typical for this preparation and dose, the expected decrease in fasting serum triacylglycerol was not seen. The lowering of total cholesterol observed with garlic is believed to be largely due to a reduction in LDL-C (15), which may be due to an inhibition of hepatic cholesterol biosynthesis (possibly via inhibition of hydroxymethylglutaryl-CoA reductase) by allicin and/or other components (16). It should also be noted that certain garlic preparations (eg, garlic oils) do not show the degree of cholesterol lowering seen with specific powdered formulations (17).

Treatment with fish oil in this study showed a reduction in serum fasting triacylglycerol (30–40%) consistent with that in previous reports from hyperlipidemic humans (10). An inhibition of hepatic fatty acid synthesis by EPA and DHA and impaired triacylglycerol synthesis (including very-low-density lipoprotein assembly and secretion) are among some of the mechanisms proposed for the plasma triacylglycerol-lowering effect of dietary fish oil (10, 18). Addition of garlic to fish oil did not significantly change the reduction in triacylglycerol observed with the fish-oil concentrate alone. Although our subjects did not generally have overt hypertriglyceridemia at entry, their mean fasting triacylglycerol values (2.1 mmol/L, or

TABLE 7
Effect of nutritional supplementation on fatty acid composition of serum phospholipid¹

Fatty acid	Baseline (n = 46)	Placebo (n = 11)	Garlic (n = 12)	Fish oil (n = 10)	Garlic + fish oil (n = 13)
	<i>% by wt of total fatty acids</i>				
16:0	28.3 ± 0.2	27.7 ± 0.4	27.6 ± 0.4 ²	27.6 ± 0.4	27.8 ± 0.5
18:0	12.4 ± 0.2	11.8 ± 0.2	12.1 ± 0.5	12.6 ± 0.4 ²	12.9 ± 0.5 ²
18:1	11.8 ± 0.2	10.2 ± 0.4 ²	9.9 ± 0.4 ²	10.0 ± 0.5 ²	10.0 ± 0.3 ²
18:2n-6	20.0 ± 0.4	21.4 ± 0.6	20.8 ± 0.8	15.7 ± 1.1 ^{2,2}	15.5 ± 1.1 ^{2,2}
18:3n-6	0.08 ± 0.01	0.13 ± 0.02	0.14 ± 0.03 ²	0.06 ± 0.01 ²	0.02 ± 0.01 ^{2,2}
20:3n-6	3.1 ± 0.1	3.6 ± 0.2 ²	4.1 ± 0.3 ^{2,2}	1.7 ± 0.2 ^{2,2}	1.8 ± 0.1 ^{2,2}
20:4n-6 (AA)	10.8 ± 0.3	13.0 ± 0.6 ²	13.1 ± 0.7 ²	7.2 ± 0.4 ^{2,2}	7.9 ± 0.4 ^{2,2}
20:5n-3 (EPA)	1.2 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	7.5 ± 0.6 ^{2,2}	7.5 ± 0.6 ^{2,2}
22:6n-3 (DHA)	3.2 ± 0.1	2.9 ± 0.2	3.1 ± 0.2	7.6 ± 0.4 ^{2,2}	7.3 ± 0.4 ^{2,2}
Σ n-6	34.5 ± 0.4	38.5 ± 0.4 ²	38.6 ± 0.5 ²	24.9 ± 1.2 ^{2,2}	23.7 ± 2.0 ^{2,2}
Σ n-3	5.9 ± 0.2	5.2 ± 0.3	5.2 ± 0.3	16.3 ± 1.8 ^{2,2}	16.8 ± 1.0 ^{2,2}
n-3:n-6	0.17 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.74 ± 0.10 ^{2,2}	0.69 ± 0.06 ^{2,2}
EPA:AA	0.11 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	1.08 ± 0.14 ^{2,2}	0.97 ± 0.08 ^{2,2}

¹ $\bar{x} \pm$ SEM for baseline values and after 12 wk of supplementation. The four supplement groups (n = 10–13) are compared with the baseline values (n = 46). AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

² Significantly different from baseline, P < 0.05.

³ Significantly different from placebo, P < 0.05.

⁴ Significantly different from placebo and garlic, P < 0.05.

186 mg/dL) would be considered above desirable based on some recent reports (19).

Fish-oil treatment has often been found to result in an increase in LDL-C concentrations, possibly via an increased conversion of very-low-density lipoprotein to LDL particles (10, 11). A significant rise in LDL-C (+8.5%) was seen in this study in the group taking fish oil alone. The addition of garlic to fish oil not only prevented the moderate LDL-C-raising effect of the fish oil but actually produced a significant lowering (−9.5%).

The rise in serum HDL-C concentrations relative to baseline (by 9%) as observed for the two fish-oil groups (fish oil alone and garlic + fish oil) are of interest as are the apparently favorable shifts (decreases) in the ratios of total cholesterol to HDL-C and LDL-C to HDL-C (including significantly lower ratios at week 12 for the garlic + fish oil combination). The significant reduction in the ratios of total cholesterol to HDL-C and LDL-C to HDL-C with the garlic + fish oil supplement has potential health significance in view of recent emphasis on these ratios as important predictors for coronary artery disease (20).

Blood pressure has been reported to be moderately reduced by both garlic (21) and fish-oil (22) supplements. In this study, there was a small (2–4%) but significant reduction in mean systolic, diastolic, and arterial blood pressures with all treatments (garlic alone, fish oil alone, and garlic + fish oil) when compared with placebo. All treatments produced similar reductions, although this reduction in blood pressure was achieved within 3 wk for the groups taking fish oil (fish oil alone and garlic + fish oil) and by 9 wk for the garlic alone group. The moderate lowering of blood pressure with garlic may be due to increased nitric oxide production and a more vasodilatory state (23), whereas the EPA and DHA in fish oil may operate, at least in part, via altered eicosanoid synthesis (24).

The fatty acid composition of serum phospholipid has been found to reflect intake of dietary n-3 fatty acids well (25).

Along with the capsule or pill count, analysis of serum fatty acids confirmed high compliance with the treatment given.

In conclusion, garlic supplementation significantly decreased both total cholesterol and LDL-C, whereas fish-oil supplementation significantly decreased triacylglycerol concentrations and increased LDL-C concentrations in moderately hypercholesterolemic men. The combination of garlic and fish oil prevented a moderate fish-oil-induced rise in LDL-C. Co-administration of garlic and fish oil was well-tolerated in the short term and had a beneficial effect on serum lipid and lipoprotein concentrations by providing a combined lowering of total cholesterol, LDL-C, and triacylglycerol concentrations along with overall decreases in the ratios of total cholesterol to HDL-C and LDL-C to HDL-C. ■

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EXHIBIT T

Dose-response effects of fish-oil supplementation in healthy volunteers

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ABSTRACT We performed a randomized, controlled study on the dose-response effects of daily supplementation of 1, 5, 3, and 6 g of the marine fatty acids eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) as their ethyl esters for 12 wk in 45 healthy normotriglyceridemic male volunteers. Significant dose-related increases of the n-3 fatty acids 20:5, 22:5, and 22:6 in plasma phospholipids ($p < 0.0001$) were found, corresponding roughly to decreases of the n-6 fatty acids 18:2 and 20:4 ($p < 0.001$). Serum triglycerides and HDL₃-cholesterol concentrations showed a dose-dependent reduction ($p < 0.05$) and HDL₂ cholesterol increased ($p < 0.05$). Results for 3 and 6 g n-3 fatty acids were similar. No dose-dependent effects were observed in the VLDL-, LDL-, and total HDL-cholesterol subfractions; blood pressure; bleeding time; erythrocyte deformability; or capacity of polymorphonuclear leukocytes to kill *Staphylococcus aureus*. This study indicates that 3 g n-3 ethyl ester fatty acids appears to be the appropriate supplementation dose in humans, at least regarding lipid-profile changes and the ability to incorporate such fatty acids in the plasma phospholipids. *Am J Clin Nutr* 1990;52:120-7.

KEY WORDS Fish oil, eicosapentaenoic acid, docosahexaenoic acid, blood pressure, lipid profile, bleeding time, viscosity of erythrocyte suspensions, leukocyte function

Introduction

The observations in the mid-1970s by Bang et al (1) and Dyerberg et al (2) of the low incidence of cardiovascular disease among Greenland Eskimos led to the idea of a possible protective role of their diet, which consists predominantly of marine fish and seal. Marine fatty fish is rich in n-3 polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). Presumably Eskimos ingest ~5-10 g n-3 fatty acids/d (3).

Growing interest in the subject has led to extensive investigations. Beneficial effects of n-3 fatty acids have been attributed to an improved lipid profile (1, 2, 4-7), a reduced platelet aggregability (2, 4, 5, 8, 9), a prolonged bleeding time (10, 11), a reduced blood viscosity (12-14), and a fall in blood pressure (10, 15) as well as to anti-inflammatory and immunological effects (16, 17). However, results from the various studies were inconsistent, especially regarding lipoprotein patterns, as was recently confirmed by Leaf and Weber (18) in a review of the

literature on n-3 PUFAs. This can be explained partly by the lack of proper control groups in many studies, which make correction for time trends, conditions of measurements, and laboratory drift impossible. The majority of the studies show a reduction in serum triglyceride concentrations, with the most striking reductions in hypertriglyceridemic patients (4, 6, 7, 10, 15, 19-21).

Reported changes in concentrations of high-density-lipoprotein (HDL), low-density-lipoprotein (LDL), and very-low-density-lipoprotein (VLDL) cholesterol are not consistent. Cholesterol-lowering effects were reported primarily in studies with extreme doses of n-3 PUFAs of ≤ 30 g/d (4, 6, 21-23). However, the Zutphen study suggests that the consumption of small amounts of fish (a low n-3 PUFA intake) over a long period may reduce cardiovascular risk (24). Whether a high daily intake of n-3 PUFAs is necessary to influence the cardiovascular-risk profile remains open for discussion.

Good data are not available regarding the dose of n-3 fatty acids necessary for producing most of the described beneficial effects. The purpose of the present study was to examine dose-response relationships with blood pressure, lipid and lipoprotein patterns, bleeding time, and erythrocyte and leukocyte function in normotriglyceridemic healthy volunteers to find the appropriate dose.

Subjects and methods

Subjects

Forty-five healthy male volunteers aged 33.7 ± 6.2 y ($\bar{x} \pm$ SD, range 22-48 y) were investigated. Body weight was 77.7 ± 9.3 kg and a body mass index (BMI; wt/ht²) was 23.2 ± 2.0 kg/m². All participants gave informed consent. The study was approved by the Human Research and Ethical Committee of the Free University.

No drugs were taken from ≥ 4 wk before the start until the

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two participants were nonsmokers. The other 15 smoked an average of 7 cigarettes/d (range 1–20 cigarettes/d). The fish consumption before the study never exceeded one fish meal per week. Subjects were excluded from the study when fasting serum triglycerides were > 2.0 mmol/L at baseline. Subjects kept their normal diets during the experiment and a 72-h dietary recall was completed in the first month of the study. The subjects were randomly assigned to one of four groups and took 0, 3, 6, or 12 capsules/d, respectively, of a marine-lipid concentrate (Super EPA, Pharmacaps Inc, Marlow, Buckinghamshire, UK) for 12 wk. Each capsule provided 300 mg EPA and 200 mg DHA as their ethyl esters and 1 mg vitamin E as described in a previous study (25). This implied that daily doses of 0, 1.5, 3, and 6 g n-3 ethyl ester fatty acids (EEFAs) were administered daily.

Measurements were performed before, at 12 wk of, and at 12 wk after supplementation. Subjects fasted overnight from 2100 and venous blood samples were drawn with minimal venous occlusion between 0800 and 1000 the next morning. Before samples were drawn, blood pressure was measured in duplicate with a London School of Hygiene mercury sphygmomanometer by the same observer after the subjects had rested 15 min in supine position and immediately after the subjects stood up. Blood samples were used for measurement of hematologic and biochemical variables, lipid profile, plasma phospholipids, and leukocyte killing capacity. Erythrocyte deformability studies and bleeding-time measurements were performed only before and at the end of the 12-wk period of lipid-concentrate administration.

Methods

The fatty acid composition of plasma phospholipids was assessed by capillary gas chromatography as described previously (26). Plasma triglyceride concentrations were measured enzymatically (GPO-PAP method) after removal of chylomicrons as described by Terpstra (27) as was the total amount of free fatty acids (FFAs; Nefa-C test, Wako Ltd, Tokyo). Lipoprotein fractionation was carried out by density-gradient ultracentrifugation (28). Total plasma cholesterol and cholesterol content of the fractions were analyzed with the CHOD-PAP method (Boehringer Mannheim GmbH, Mannheim, FRG). Bleeding-time measurements were performed as described by Mielke et al (29) with the Simplate II device (General Diagnostics, Turnhout, Belgium). Erythrocyte filterability was studied with the recently introduced St George's Filtrometer (Cassi-Med Ltd, Dorking, UK), which can discriminate between cell deformability and filter occlusion (30–32). An erythrocyte concentration of 10% was used for filtration. The white cell contamination in the erythrocyte suspension was < 0.1×10^9 /L and platelet contamination was not detectable. The erythrocyte suspensions were filtered through a vertical filter (batch 5404 C34, Nucleopore, Pleasanton, CA; nominal pore diameter 5 μ m, filter diameter 13 mm, effective filtration area 0.78 cm²). The filter-to-filter variation was < 5%. All samples were measured in duplicate. Erythrocyte deformability was expressed as erythrocyte transit time.

The killing capacity of polymorphonuclear leukocytes (PMNLs) was measured as described in detail elsewhere (33). *Staphylococcus aureus* was incubated with PMNLs at 2:1 in the

the killing capacity (KC) was defined as

$$KC = \frac{\text{bacteria at 0 min} - \text{bacteria at 30 min (or 60 min)}}{\text{bacteria at 0 min}} \times 100\%$$

Control tubes without PMNLs were included in each experiment. Killing always was found in the presence of PMNLs; no killing was observed without PMNLs.

Statistics

In this paper hypotheses of the form $H_1: \mu_0 \leq \mu_3 \leq \mu_6 \leq \mu_{12}$ or $H_1: \mu_1 \geq \mu_3 \geq \mu_6 \geq \mu_{12}$ (with at least one strict inequality) were investigated, with μ_n denoting the theoretical mean of a population on a diet regimen of n ($n = 0, 3, 6, \text{ or } 12$) capsules of lipid concentrate daily. These hypotheses imply dose-response relations of the treatment on the variables of interest. To test null hypotheses of the form $H_0: \mu_0 = \mu_3 = \mu_6 = \mu_{12}$ against alternative hypotheses (H_1) in which the order is specified, the Jonckheere-Terpstra test was applied to the data (34). This test is a nonparametric test related to the Kruskal-Wallis test. Dose-response relations were considered significant when the two-tailed p value was < 0.05. For the triglycerides the one-tailed Jonckheere-Terpstra test was applied because it is known that triglyceride concentrations decrease with n-3 EEFAs.

Observations at weeks 12 and 24 were compared with the baseline observations. Effects of n-3 EEFAs can be expressed as absolute or as percentage changes. Absolute changes were studied when changes were independent of baseline values. Under such circumstances, the Jonckheere-Terpstra test was applied to the absolute intraindividual changes (eg, week 12 – week 0) to investigate whether sample means increased or decreased with changing doses. By contrast, percentage changes were studied when the changes were dependent on baseline values. In those cases the Jonckheere-Terpstra test was applied to intraindividual quotients [eg, (week 12 – week 0)/week 0].

Although nonparametric tests were used, data for each group were summarized by means and SEMs rather than medians and ranges to allow comparisons with other studies of n-3 EEFAs.

Results

The four groups of subjects were comparable for age, BMI, smoking habits, and alcohol consumption. The amounts of fat, carbohydrate, and protein in the diets of the groups were comparable as shown by the 72-h dietary recalls. The lipid-concentrate capsules were well-tolerated although some subjects described a fishy aftertaste. Body weight did not change during the study period (0.76 ± 0.49 kg, $\bar{x} \pm \text{SD}$). All scheduled visits were completed. The 45 volunteers completed the study successfully with 10, 11, 10, and 14 participants, respectively, in the 0-(control), 3-, 6-, and 12-capsule groups.

Supplementation with n-3 fatty acids for 12 wk resulted in marked changes in fatty acid composition of the plasma phospholipids in all groups except for the control group (Table 1). There was a significant dose-related increase in the proportion of n-3 EEFAs 20:5n-3, 22:5n-3, and 22:6n-3 with the use

TABLE 1
Fatty acid composition of plasma phospholipids after various doses of fish oil*

	0 capsules	3 capsules	6 capsules	12 capsules	<i>p</i> †
<i>mole%</i>					
18:2n-6					
B	22.56 ± 0.77	23.05 ± 0.50	23.55 ± 0.81	21.49 ± 0.58	
F	22.86 ± 0.84 [0.12]	21.23 ± 0.92 [-1.82]	20.21 ± 1.40 [-3.34]	17.78 ± 0.76 [-3.71]	<0.001
W	22.92 ± 0.96 [0.36]	23.47 ± 0.80 [0.41]	23.97 ± 0.78 [0.94]	21.98 ± 0.60 [0.18]	
18:2n-6 + 20:4n-6					
B	29.90 ± 1.10	30.69 ± 0.73	31.76 ± 0.61	29.90 ± 0.53	
F	30.26 ± 1.09 [0.36]	28.19 ± 0.95 [-2.50]	26.80 ± 1.26 [-4.96]	24.27 ± 0.81 [-5.63]	<0.0001
W	29.92 ± 1.09 [0.02]	30.81 ± 0.69 [-0.55]	31.12 ± 0.73 [-0.19]	29.56 ± 0.47 [-0.37]	
20:5n-3					
B	0.67 ± 0.06	0.70 ± 0.06	0.67 ± 0.10	1.03 ± 0.16	
F	0.71 ± 0.06 [0.04]	2.20 ± 0.27 [1.50]	3.65 ± 0.40 [2.98]	4.76 ± 0.46 [3.74]	<0.0001
W	0.79 ± 0.13 [0.12]	0.80 ± 0.11 [0.10]	0.66 ± 0.10 [-0.07]	0.82 ± 0.09 [-0.23]	
22:5n-3					
B	0.82 ± 0.04	0.81 ± 0.04	0.84 ± 0.04	0.82 ± 0.02	
F	0.84 ± 0.03 [0.02]	1.07 ± 0.07 [0.26]	1.13 ± 0.10 [0.29]	1.29 ± 0.06 [0.47]	<0.0001
W	0.85 ± 0.05 [0.03]	0.79 ± [-0.01]	0.84 ± 0.05 [-0.01]	0.90 ± 0.04 [0.07]	
22:6n-3					
B	2.13 ± 0.11	2.82 ± 0.19	2.52 ± 0.23	2.97 ± 0.16	
F	2.17 ± 0.18 [0.04]	4.00 ± 0.28 [1.18]	4.61 ± 0.37 [2.09]	5.02 ± 0.20 [2.05]	<0.001
W	2.24 ± 0.17 [0.11]	2.77 ± 0.22 [-0.11]	2.74 ± 0.16 [0.06]	2.91 ± 0.15 [-0.13]	
Total n-3					
B	3.61 ± 0.16	4.33 ± 0.20	4.04 ± 0.36	4.83 ± 0.31	
F	3.72 ± 0.24 [0.11]	7.25 ± 0.54 [2.92]	9.41 ± 0.80 [5.37]	11.32 ± 0.57 [6.49]	<0.0001
W	3.86 ± 0.29 [0.26]	4.44 ± 0.34 [0.07]	4.23 ± 0.28 [-0.04]	4.68 ± 0.26 [-0.26]	
DBI‡					
B	1.18 ± 0.02	1.21 ± 0.01	1.20 ± 0.02	1.21 ± 0.01	
F	1.16 ± 0.02 [-0.02]	1.29 ± 0.03 [0.08]	1.33 ± 0.04 [0.23]	1.37 ± 0.02 [0.16]	<0.001
W	1.16 ± 0.02 [-0.02]	1.18 ± 0.01 [-0.03]	1.18 ± 0.02 [0.08]	1.19 ± 0.01 [-0.02]	

* $\bar{x} \pm \text{SEM}$; change from baseline given in brackets. B, baseline; F, after 12 wk fish oil; W, 12 wk after withdrawal.

† Dose-response relationship tested two-tailed with the Jonckheere-Terpstra test. The various significant differences within rows for B and F values are not indicated.

‡ Double-bond index, mean number of double bonds per molecule fatty acid.

of ≤ 3 g n-3 EEFAs. However, 6 g n-3 EEFAs/d did not increase the content of these fatty acids much further. The increase in n-3 EEFAs was accompanied by a considerable, dose-related decrease in 18:2n-6 and a minor decrease in 20:4n-6. The dose-related changes in total unsaturation of plasma phospholipids (double-bond index; DBI) also followed closely the supply and withdrawal of dietary n-3 EEFA supplements (Table 1). Because all these effects were independent of initial values, they are expressed as absolute changes.

The influence of n-3 EEFAs on the concentrations of serum triglycerides and total cholesterol and on the distribution of cholesterol in the individual lipoprotein classes before, after 12 wk treatment, and 12 wk after withdrawal of treatment are shown in Table 2. The effects are expressed as percent changes because they were dependent on baseline values. The serum triglycerides fell in a significant, dose-related manner. A significant, dose-related increase was observed in HDL₂ cholesterol. Both triglycerides and HDL₂ cholesterol revealed the most pronounced responses with 6 capsules; with 12 capsules the response was approximately the same as with 6 capsules. The control group showed a higher HDL₃-cholesterol concentration at 12 wk compared with baseline value. This contributed to the dose-dependent decrease of the HDL₃-cholesterol

fraction at the end of the treatment period. No significant changes occurred in plasma cholesterol nor in total HDL-, LDL-, and VLDL-cholesterol concentrations at the end of the treatment period, although there was a tendency for HDL cholesterol to increase. Accordingly, there was no significant dose-response relationship in the ratio of HDL to LDL (HDL:LDL). On the other hand, the 12-wk supplementation of n-3 EEFAs caused marked, dose-dependent increases in HDL₂:LDL and HDL₂:HDL₃ comparable to the dose-response relationship for HDL₂ cholesterol.

Twelve weeks after withdrawal of treatment, triglycerides, total HDL cholesterol, HDL₂ cholesterol, HDL₂:LDL, and HDL₂:HDL₃ returned to baseline in contrast to HDL₃ and VLDL cholesterol, which increased further.

No changes were observed in total plasma FFAs, blood glucose, and screening tests for liver and renal function. The hemoglobin, leukocyte, and platelet counts as well as the hematocrit remained unchanged. Systolic and diastolic blood pressure after 15 min in supine position showed a tendency to decrease in all four groups whereas no consistent alterations were recorded after subjects stood up (Table 3). The bleeding time did not change in any of the groups (Table 3).

Erythrocyte deformability, expressed as erythrocyte transit

TABLE 2
Plasma lipids and lipoproteins after various doses of fish oil*

	0 capsules	3 capsules	6 capsules	12 capsules	<i>p</i>
<i>mmol/L</i>					
Triglyceride					
B	0.95 ± 0.09	1.01 ± 0.14	0.93 ± 0.07	1.00 ± 0.09	
F	0.90 ± 0.10 [1.2]	0.87 ± 0.12 [-11.6]	0.70 ± 0.07 [-23.3]	0.78 ± 0.06 [-18.5]	<0.05†
W	0.88 ± 0.11 [-5.7]	1.00 ± 0.14 [1.6]	0.89 ± 0.15 [-4.5]	1.07 ± 0.13 [7.8]	
Cholesterol					
B	4.67 ± 0.25	5.07 ± 0.33	5.27 ± 0.56	5.59 ± 0.44	
F	4.76 ± 0.23 [2.9]	5.09 ± 0.29 [0.9]	5.19 ± 0.58 [2.8]	5.66 ± 0.39 [1.0]	NS‡
W	4.74 ± 0.22 [2.6]	4.89 ± 0.34 [-3.4]	4.96 ± 0.51 [-5.4]	5.67 ± 0.29 [3.8]	
VLDL cholesterol					
B	0.32 ± 0.03	0.39 ± 0.06	0.37 ± 0.05	0.39 ± 0.06	
F	0.40 ± 0.09 [56.1]	0.41 ± 0.07 [-4.0]	0.38 ± 0.10 [13.1]	0.28 ± 0.04 [-6.2]	NS‡
W	0.44 ± 0.07 [51.7]	0.48 ± 0.06 [36.1]	0.43 ± 0.07 [33.3]	0.75 ± 0.16 [111.3]	
LDL cholesterol					
B	2.99 ± 0.14	3.35 ± 0.31	3.42 ± 0.65	3.94 ± 0.40	
F	2.94 ± 0.14 [-1.0]	3.32 ± 0.25 [-5.0]	3.31 ± 0.60 [3.1]	3.99 ± 0.40 [2.4]	NS‡
W	2.87 ± 0.15 [-1.9]	3.05 ± 0.25 [-10.6]	3.21 ± 0.48 [9.1]	3.35 ± 0.29 [-5.7]	
HDL ₂ cholesterol					
B	0.47 ± 0.07	0.49 ± 0.06	0.41 ± 0.06	0.44 ± 0.06	
F	0.48 ± 0.08 [8.7]	0.65 ± 0.15 [31.5]	0.67 ± 0.09 [56.1]	0.62 ± 0.07 [48.7]	<0.05‡
W	0.42 ± 0.05 [0.5]	0.55 ± 0.14 [7.0]	0.46 ± 0.06 [9.1]	0.48 ± 0.06 [14.4]	
HDL ₃ cholesterol					
B	0.86 ± 0.05	0.78 ± 0.04	0.83 ± 0.05	0.86 ± 0.04	
F	0.90 ± 0.04 [6.8]	0.80 ± 0.05 [4.1]	0.84 ± 0.03 [0.03]	0.79 ± 0.03 [-6.6]	<0.05‡
W	0.93 ± 0.07 [8.9]	0.80 ± 0.03 [4.9]	0.87 ± 0.03 [3.7]	0.90 ± 0.03 [6.2]	
HDL cholesterol					
B	1.32 ± 0.11	1.27 ± 0.07	1.26 ± 0.08	1.30 ± 0.08	
F	1.38 ± 0.10 [6.7]	1.46 ± 0.14 [14.5]	1.51 ± 0.11 [14.8]	1.42 ± 0.07 [9.8]	NS‡
W	1.34 ± 0.11 [3.8]	1.39 ± 0.13 [7.5]	1.32 ± 0.08 [2.2]	1.39 ± 0.07 [7.8]	
HDL ₂ :LDL					
B	0.15 ± 0.02	0.15 ± 0.02	0.17 ± 0.05	0.13 ± 0.02	
F	0.16 ± 0.02 [9.1]	0.23 ± 0.07 [41.7]	0.25 ± 0.04 [64.6]	0.18 ± 0.03 [51.0]	<0.05‡
W	0.15 ± 0.02 [3.6]	0.19 ± 0.05 [17.2]	0.16 ± 0.03 [13.5]	0.15 ± 0.02 [30.5]	
HDL ₂ :HDL ₃					
B	0.52 ± 0.06	0.65 ± 0.08	0.50 ± 0.07	0.51 ± 0.05	
F	0.53 ± 0.09 [2.4]	0.88 ± 0.25 [40.9]	0.79 ± 0.09 [62.5]	0.81 ± 0.11 [68.6]	<0.01‡
W	0.45 ± 0.05 [-2.6]	0.78 ± 0.27 [20.2]	0.52 ± 0.06 [12.7]	0.54 ± 0.06 [12.5]	

* $\bar{x} \pm \text{SEM}$; percent change from baseline given in brackets. B, baseline; F, after 12 wk fish oil; W, 12 wk after withdrawal.

† Dose-response relationship tested one-tailed with the Jonckheere-Terpstra test.

‡ Dose-response relationship tested two-tailed with the Jonckheere-Terpstra test.

time, remained unaltered (Table 3). Duplicate measurements with this method were highly reproducible with a within-assay analytical variation (CV) of 0.7%.

The ability of leukocytes to kill *S aureus* reflected in the percentage bacteria killed in 30 and 60 min, after treatment and 12 wk after withdrawal, is provided in Table 3. Baseline data are not available because of problems that we had initially with the technique. There was no significant dose-response relationship in the killing capacity after 30 min or after 60 min. The within-day CV of duplicate measurements was 21.5% for the killing capacity at 30 min and 17.0% for the killing capacity at 60 min.

Discussion

We demonstrate a significant, dose-dependent increase of the incorporation of n-3 PUFAs into plasma phospholipids

at the expense of 18:2n-6 and to a minor degree of 20:4n-6, comparable with results in other studies (9, 19, 26, 35-38). There was only a relatively small additional increase after the dose was doubled from 3 to 6 g, suggesting that the capacity for incorporation into plasma phospholipids is less efficient in higher doses, at least when EPA and DHA are administered as ethyl esters. An incomplete absorption of n-3 PUFAs from ethyl esters was described by Lawson and Hughes (39). However, in this single-dose study only the incorporation of n-3 PUFAs into plasma triglycerides was measured. The incorporation of n-3 PUFAs into plasma phospholipids that we found with ethyl esters is in agreement with values found after cod liver oil (26). The limited incorporation that we found at higher doses seems in contrast with the results of Harris et al (22) who after a fish diet containing 20-29 g n-3 PUFAs/d, administered as fatty fish for 4 wk, found an increase of n-3 PUFAs from 4% to 33% of total fatty acids in plasma phospholipids. In

TABLE 3

Supine and standing systolic (S) and diastolic (D) blood pressure (BP), bleeding time (BT), erythrocyte transit time (ETT), and killing capacity (KC) of PMNLs after various doses of fish oil*

	0 capsules	3 capsules	6 capsules	12 capsules
Supine SBP (mmHg)				
B	120.3 ± 1.6	119.3 ± 2.6	118.2 ± 2.7	121.1 ± 1.8
F	116.9 ± 2.4 [-2.7]	118.5 ± 3.3 [-0.7]	117.2 ± 2.6 [-0.8]	119.0 ± 1.8 [-1.7]
W	120.9 ± 2.5 [0.5]	119.9 ± 2.0 [0.9]	118.4 ± 2.9 [0.2]	121.4 ± 2.5 [0.3]
Supine DBP (mmHg)				
B	71.3 ± 2.1	73.8 ± 3.0	72.2 ± 1.8	75.6 ± 1.8
F	69.5 ± 2.7 [-2.4]	67.6 ± 4.4 [-8.2]	71.6 ± 2.1 [-0.4]	73.6 ± 1.8 [-2.0]
W	73.4 ± 1.8 [3.4]	70.6 ± 3.7 [-3.8]	72.3 ± 2.4 [0.3]	70.4 ± 1.9 [-6.6]
Standing SBP (mmHg)				
B	118.1 ± 2.7	119.5 ± 2.9	115.5 ± 3.0	120.8 ± 1.9
F	118.1 ± 3.0 [0.2]	122.1 ± 2.6 [2.3]	115.4 ± 2.3 [0.2]	118.2 ± 2.0 [-2.1]
W	118.4 ± 2.8 [0.4]	123.5 ± 1.5 [3.6]	117.1 ± 2.8 [1.7]	121.2 ± 2.7 [0.3]
Standing DBP (mmHg)				
B	82.8 ± 2.1	79.7 ± 2.2	78.7 ± 1.4	84.6 ± 1.9
F	81.1 ± 2.3 [-1.7]	81.6 ± 3.0 [2.4]	80.9 ± 1.1 [3.1]	83.6 ± 1.9 [-0.9]
W	81.9 ± 2.8 [-1.0]	80.6 ± 3.0 [1.3]	81.1 ± 1.3 [3.3]	84.1 ± 2.2 [0.4]
BT (min)				
B	3.8 ± 0.55	4.4 ± 0.38	3.6 ± 0.50	2.9 ± 0.30
F	3.7 ± 0.54 [13.8]	4.1 ± 0.73 [-10.9]	2.7 ± 0.52 [-19.0]	2.7 ± 0.30 [-3.2]
ETT				
B	8.75 ± 0.09	8.57 ± 0.10	8.79 ± 0.11	8.66 ± 0.10
F	8.92 ± 0.11 [2.1]	8.77 ± 0.08 [2.5]	8.95 ± 0.09 [1.9]	8.84 ± 0.13 [2.2]
KC at 30 min (%)				
B	—	—	—	—
F	31.3 ± 5.7 [-14.5]	28.1 ± 5.5 [-14.7]	31.4 ± 4.6 [34.3]	31.3 ± 3.6 [-8.4]
W	33.4 ± 4.1	40.6 ± 5.7	27.3 ± 3.2	35.7 ± 3.0
KC at 60 min (%)				
B	—	—	—	—
F	50.4 ± 6.1 [0.5]	41.9 ± 7.1 [-37.1]	47.9 ± 4.3 [10.3]	47.2 ± 4.9 [-13.8]
W	54.0 ± 5.4	65.0 ± 5.0	48.6 ± 5.3	65.0 ± 5.0

* $\bar{x} \pm \text{SEM}$; percent change from baseline given in brackets. B, baseline; F, after 12 wk fish oil; W, 12 wk after withdrawal.

† Dose-response relationship tested two-tailed with the Jonckheere-Terpstra test.

this study, however, the experimental diets used differed not only in n-3 fatty acid content but also in content of saturated and monounsaturated fatty acids.

The supplementation of the diet with n-3 EEFAs had a favorable influence on plasma lipid and lipoprotein patterns. In agreement with almost every fish-oil study, the plasma triglyceride concentration declined during treatment and appeared to be dose-dependent. Jensen et al (36) in an uncontrolled study found no triglyceride-lowering effect with doses ≤ 2.4 g EPA and DHA but did find a definite effect with 4.9 g EPA and DHA. It was shown that the more marked the triglyceride concentration at baseline, the greater its reduction by n-3 fatty acid supplementation (4, 6, 7, 10, 19-21), which explains the striking response especially in hypertriglyceridemic patients (7, 20). In the studies on healthy volunteers in which the triglycerides did not decrease significantly, the mean initial value was < 1.1 mmol/L (20, 40, 41) or the dose of n-3 PUFAs was very low (4, 15, 19).

We were unable to demonstrate significant, dose-related changes in VLDL cholesterol with the doses of 1.5-6 g n-3 EEFAs that we used. Because the majority of the VLDL particle is composed of triglycerides, it seems evident that only a large decrease in triglyceride concentration will also lead to a decrease in VLDL concentration. Because we found only mod-

erate changes in triglyceride concentration, VLDL-cholesterol concentration was not expected to change much. Thus, reductions in triglyceride and VLDL-cholesterol concentrations were especially striking when found in hypertriglyceridemic patients (7, 42, 43). The exact mechanism by which n-3 EEFAs induce a decline in VLDL-cholesterol and triglyceride concentrations has not been clearly defined. The most likely mechanism is thought to be a reduction in hepatic VLDL synthesis (42) and the secretion of smaller VLDL particles, which are more easily removed in the peripheral tissues or by the liver as VLDL remnants (7). This might explain the increase in LDL-cholesterol concentrations as observed in hypertriglyceridemic patients (7, 20, 43) as well as in patients on continuous ambulatory peritoneal dialysis (CAPD) or hemodialysis therapy (37, 38).

Variable results have been found for the changes in LDL cholesterol in healthy subjects. Most studies, including the present one, found no effect on LDL-cholesterol concentrations (10, 35, 41, 44) whereas others found a fall in LDL cholesterol (21, 22) and some found a rise (6, 42, 45) when very high doses of n-3 PUFAs were used.

In contrast to the effects on total HDL cholesterol, the influence of n-3 PUFAs on HDL-cholesterol subfractions has been examined. We found a dose-dependent increase

HDL₂ cholesterol and a dose-dependent decrease in HDL₃ cholesterol. It is doubtful whether the dose-dependent decrease in HDL₃ cholesterol has any significance at all. This dose-dependent decrease was partly caused by the rise in HDL₃-cholesterol concentrations in the control group. Therefore caution in the interpretation of this result is necessary. The combination of a dose-dependent increase in HDL₂ cholesterol and a dose-dependent decrease in HDL₃ cholesterol explains why there was no significant change in total HDL cholesterol compared with the control group, an observation in agreement with the majority of previous studies in healthy volunteers (19, 22, 40, 41, 46). HDL₂:HDL₃, which reflects the balance between cholesterol uptake in and clearance from cells (47), showed a significant, dose-dependent increase. The dose-dependent rise in HDL₂:LDL shown in this study is indicative of a possible beneficial effect of dietary n-3 EEFA (48).

A slight reduction in mean supine blood pressure after 15 min rest was noticed in the treatment groups as well as in the control group. It was likely to be caused by habituation to the experimental conditions as was also suggested by van Houwelingen et al (11). Note that a blood pressure-lowering effect of n-3 PUFAs has been claimed mainly in studies lacking a proper control group (4, 41, 44, 49), although three studies performed with a proper control group recorded a slight but significant fall in supine (15, 46) or standing blood pressure (10).

The increased bleeding tendency for Greenland Eskimos was affirmed by protracted template bleeding times (8). However, with the doses used by us and others (9, 43, 46), no changes in bleeding time were observed. Only very high doses appear to be able to show definite and consistent changes (50).

Another aspect considered in the prevention of atherosclerosis by n-3 PUFAs is an improvement in blood rheology that might lower the risk on thrombotic vascular disease. Whole-blood viscosity is decreased by n-3 PUFAs in almost every study examining this subject (12-14). The increased incorporation of n-3 PUFAs in membrane phospholipids is thought to increase erythrocyte deformability (12, 14), possibly because of an increased membrane flexibility (51). An increase in erythrocyte deformability was also observed by investigators using filtration techniques (12, 14, 46, 52). With the new St George's Filtrometer, changes in erythrocyte deformability could not be seen with any dose of n-3 EEFA used. This is in agreement with de Leeuw et al (53) who used this technique in diabetic patients consuming 2.7 g n-3 EEFA in their triglyceride form daily for 10 wk. These conflicting results are probably due to differences in filtration technique.

Blood filtration reflects not only erythrocyte flexibility but also retention of blood components other than erythrocytes in the filter, eg, white cells (54). The St George's filtrometer is capable of differentiating between these two factors affecting blood filterability by measuring the initial filtration rate as erythrocyte variable when the filter is not yet occluded by blood components. By employing this method, it appeared that the erythrocyte deformability was not influenced by n-3 EEFAs.

Attenuation of chemotactic responsiveness of PMNLs was demonstrated after n-3 PUFA supplementation (16). This might be related to a reduced formation of leukotriene B₄, which is a powerful chemotactic compound (16). Furthermore, a decrease in chemiluminescence and superoxide production of phagocytosing PMNLs was reported as a result of fish-oil

supplementation, indicating a suppression of PMNL inflammatory potential (17). Knapp and FitzGerald (55) found no influence on the ability of PMNLs to kill *S aureus* in their study with seven healthy volunteers consuming 10 g EPA/d for 4 wk. We also did not observe the leukocyte killing capacity changed by EEFAs. It should be realized, however, that in our killing experiments with PMNLs, considerable interindividual and month-to-month variations were noticed as were also observed by other investigators (56, 57). Although no dose-related effect could be demonstrated, minor influences of fish oil on leukocyte killing capacity cannot be excluded.

In conclusion, the overall effect of n-3 PUFAs in their ethyl ester form in this study with healthy volunteers appeared to be beneficial in a dose-related manner, especially on triglyceride concentration and lipoprotein pattern. Doses > 3 g n-3 EEFAs/d did not result in higher efficacy concerning incorporation in plasma phospholipids and changes in lipids and lipoprotein concentrations. Further effects, such as a decrease in blood pressure, might be obtained with higher doses (58). However, it has become clear that the safety or higher doses is questionable, causing unfavorable changes in lipid profile in various groups of patients (7, 42) and higher glucose concentrations in subjects with impaired glucose tolerance (59). We, therefore, should focus on the possible beneficial effects of low doses of n-3 PUFAs, which can be taken conveniently for longer periods. From our study it appears that, at least when considering changes in lipid profile and the ability to incorporate n-3 PUFAs into plasma phospholipids, 3 g n-3 fatty acids is an appropriate dose in humans. E

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EXHIBIT U

Omega-3 Fatty Acids in Diabetes Mellitus

Gift from the Sea?

LLOYD AXELROD

The potential role of omega-3 fatty acids in the prevention of atherosclerotic disease in the nondiabetic population currently engenders interest, enthusiasm, and controversy. Some apparently beneficial effects of omega-3 fatty acids on platelet function, eicosanoid formation, plasma triglyceride levels, and blood pressure have been described in patients with diabetes mellitus. However, enthusiasm for the use of omega-3 fatty acids in diabetes has been dampened by reports of potentially deleterious effects of these agents, including increased plasma glucose, glycosylated hemoglobin, plasma total cholesterol and LDL cholesterol, and serum apolipoprotein B levels. These adverse effects have been achieved with large, perhaps excessive, doses of omega-3 fatty acids, in the range of 4–10 g/day. The magnitude of these adverse effects has been small (typically 10–36%). It cannot be assumed that the effects of omega-3 fatty acids are the same in patients with diabetes mellitus as in nondiabetic subjects or patients with primary hyperlipidemia. First, the biosynthesis and composition of fatty acids is abnormal in diabetic animals and possibly in diabetic patients. Second, many potential mechanisms implicated in the pathogenesis of atherosclerosis are present in diabetic but not necessarily in nondiabetic subjects. Third, the mechanisms of many of the risk factors in diabetic patients differ from the mechanisms of these abnormalities in nondiabetic subjects, reflecting the effects of insulin deficiency, hyperglycemia, and their sequelae. Finally, because diabetes is a heterogeneous group of diseases, the effects of omega-3 fatty acids must be addressed separately for patients with insulin-dependent diabetes mellitus, non-insulin-dependent diabetes mellitus, and possibly other forms of diabetes. Thus, it is not possible to assess the

potential risks and benefits of dietary fish and fish oils in diabetic patients. Studies are needed to determine the regulation of fatty acid synthesis and the fatty acid composition of phospholipids in diabetic patients under defined conditions of metabolic control and diet and to determine the effects of dietary fish and fish oils in appropriate quantities on the fatty acid composition of phospholipids, the mechanisms involved in the pathogenesis of vascular disease in diabetes, and the incidence of microvascular and atherosclerotic complications. *Diabetes* 38:539–43, 1989

The potential role of omega-3 fatty acids in the prevention of atherosclerotic disease in the nondiabetic population elicits great interest and enthusiasm among the scientific community and the public at large and considerable controversy with regard to dietary and pharmacological recommendations. The initial enthusiasm for this gift from the sea was soon followed by warnings about potentially deleterious effects unique to diabetic patients, e.g., worsening of hyperglycemia. What is the potential role of these marine lipids in the prevention of atherosclerotic disease and microvascular disease in patients with diabetes mellitus? The purpose of this article is to specify the kinds of information needed to answer this question and to assess the information available about the risks and benefits of omega-3 fatty acids in diabetes mellitus.

DIETARY POLYUNSATURATED FATTY ACIDS AND VASCULAR DISEASE

The idea that the consumption of dietary fish or fish oils rich in omega-3 fatty acids may exert a beneficial effect on human health first attracted widespread attention because of studies of the dietary composition of the Greenland Eskimos, a population with a low prevalence of atherosclerosis and an age-adjusted mortality rate from acute myocardial infarction that is ~10% that of the inhabitants of Denmark or North America, despite the fact that Greenland Eskimos consume

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as much fat and cholesterol as the Danes and the North Americans (1-4). The major difference was in the composition rather than the quantity of the dietary fats (2,3). The Danes consumed more than twice as much saturated fat and more omega-6 fatty acids than the Eskimos. The Eskimos consumed ~14 g/day of omega-3 fatty acids, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), derived predominantly from the seals and whales that are major components of the Eskimo diet. These provocative observations were made while the involvement of platelets and eicosanoids in the pathogenesis of atherosclerosis was being recognized.

It was quickly appreciated that dietary omega-3 fatty acids might alter eicosanoid synthesis and decrease platelet function and thereby decrease the risk of atherosclerosis. The appeal of the hypothesis grew with the recognition that omega-3 fatty acids not only modify platelet function and eicosanoid synthesis but also affect many other mechanisms involved in the pathogenesis of atherosclerosis (4,5). In other words, the prophylactic value of omega-3 fatty acids is potentially high because they act at multiple steps implicated in the pathogenesis of atherosclerosis (4,5). Omega-3 fatty acids inhibit arachidonic acid synthesis and incorporation into phospholipids, decrease platelet production of thromboxane A_2 (TXA₂), a potent vasoconstrictor and inducer of platelet aggregation, and increase production by platelets of TXA₃, which lacks biological activity. EPA is used for synthesis of prostaglandin I₃ (PGI₃), the activity of which is added to that of PGI₂, a potent vasodilator and inhibitor of platelet aggregation. Other proposed effects of omega-3 fatty acids include decreased platelet aggregation (and possibly other platelet functions), decreased plasma lipoprotein levels, increased deformability of the erythrocyte, decreased blood viscosity, decreased blood pressure, decreased blood pressure response to vasopressors, increased thrombolytic activity, alterations in leukotriene production, and decreased inflammatory cell activity. The effects of omega-3 fatty acids in the nondiabetic population have recently been reviewed (4-6).

Thus, a single dietary manipulation may modify multiple mechanisms involved in the pathogenesis of atherosclerotic disease. Because some of these mechanisms contribute to the development of diabetic microvascular disease, it is a reasonable corollary that omega-3 fatty acids may also delay or prevent the development of diabetic microvascular disease.

HAZARDS OF EXTRAPOLATION FROM NONDIABETIC TO DIABETIC PATIENTS

It cannot be assumed that the effects of omega-3 fatty acids are the same in patients with diabetes mellitus as in nondiabetic subjects or patients with a primary hyperlipidemia. First, the biosynthesis and composition of fatty acids is abnormal in diabetic animals and possibly in diabetic patients. Second, many potential mechanisms of atherosclerosis, e.g., hyperglycemia, increased platelet aggregation and platelet TXA₂ synthesis, and decreased erythrocyte deformability (with a consequent increase in blood viscosity), are present in diabetic but not necessarily nondiabetic subjects. Third, the mechanisms of many of the risk factors in diabetic patients, e.g., hyperlipidemia, increased platelet aggregation,

and decreased erythrocyte deformability, differ from the mechanisms of these abnormalities in nondiabetic subjects, reflecting the effects of insulin deficiency, hyperglycemia, and their sequelae. Finally, because diabetes mellitus is a heterogeneous group of diseases, the effects of omega-3 fatty acids must be addressed separately for patients with insulin-dependent diabetes mellitus (IDDM), non-insulin-dependent diabetes mellitus (NIDDM), and perhaps other forms of diabetes.

ABNORMAL FATTY ACID COMPOSITION OF PLASMA AND TISSUE LIPIDS IN DIABETES

The biosynthesis and composition of fatty acids in phospholipids is abnormal in diabetes (Fig. 1). Fatty acid composition is altered in the phospholipids of various tissues in experimental animals with diabetes mellitus, including plasma, liver, heart, kidneys, and erythrocytes (7-12). These changes probably reflect decreased activities of the microsomal Δ^9 -desaturase, Δ^6 -desaturase (7,8,12), and Δ^5 -desaturase (10,12) enzymes. The Δ^9 -desaturase enzyme converts saturated fatty acids into monounsaturated fatty acids. The Δ^6 -desaturase converts linoleic acid to γ -linolenic acid; this is the rate-limiting step in the conversion of linoleic acid to arachidonic acid. The Δ^5 -desaturase converts dihomo- γ -linolenic acid to arachidonic acid and eicosatetraenoic acid to EPA, which can be converted to DHA. Thus, these enzymes are necessary for the biosynthesis of arachidonic acid, EPA, DHA, and other unsaturated fatty acids.

In relative terms, the arachidonic acid content is often decreased and the omega-3 fatty acid content (including EPA and DHA) is often increased in the tissue phospholipids of diabetic rats (10). In absolute terms (based on measurements of the fatty acids of the total lipids in the carcass of the whole animal), all polyunsaturated fatty acids (omega-6 and omega-3) are decreased in diabetes (10).

Insulin therapy reverses and overcorrects the diminished Δ^9 - and Δ^6 -desaturase activities and restores the fatty acid composition to normal, except for the decrease in arachidonic acid (8,12). Furthermore, the changes in fatty acid composition in diabetes are influenced by diet, because restriction of food intake decreases the magnitude of these changes (8,10). Thus, the alterations in fatty acid composition in tissues from diabetic animals reflect the consequences of insulin deficiency and diet. The arachidonic acid deficiency in some tissues, e.g., platelets, could also reflect enhanced phospholipase activity and enhanced release and utilization of arachidonate.

These observations have been made in the rat. Studies on the fatty acid composition of platelets derived from diabetic patients have produced seemingly inconsistent findings. For example, arachidonic acid levels have been either decreased (13), increased (14-16), or unchanged (17-19). The extant studies have not been controlled for the degree of insulin deficiency or for the composition of the diet.

These findings have several implications. First, because fatty acid synthesis and composition are altered in diabetes, the effects of dietary modifications may be different in diabetic than in nondiabetic individuals. For example, if in relative terms arachidonic acid content is decreased and omega-3 fatty acid content is increased in diabetic patients (as is the case in animals), then additional supplementation

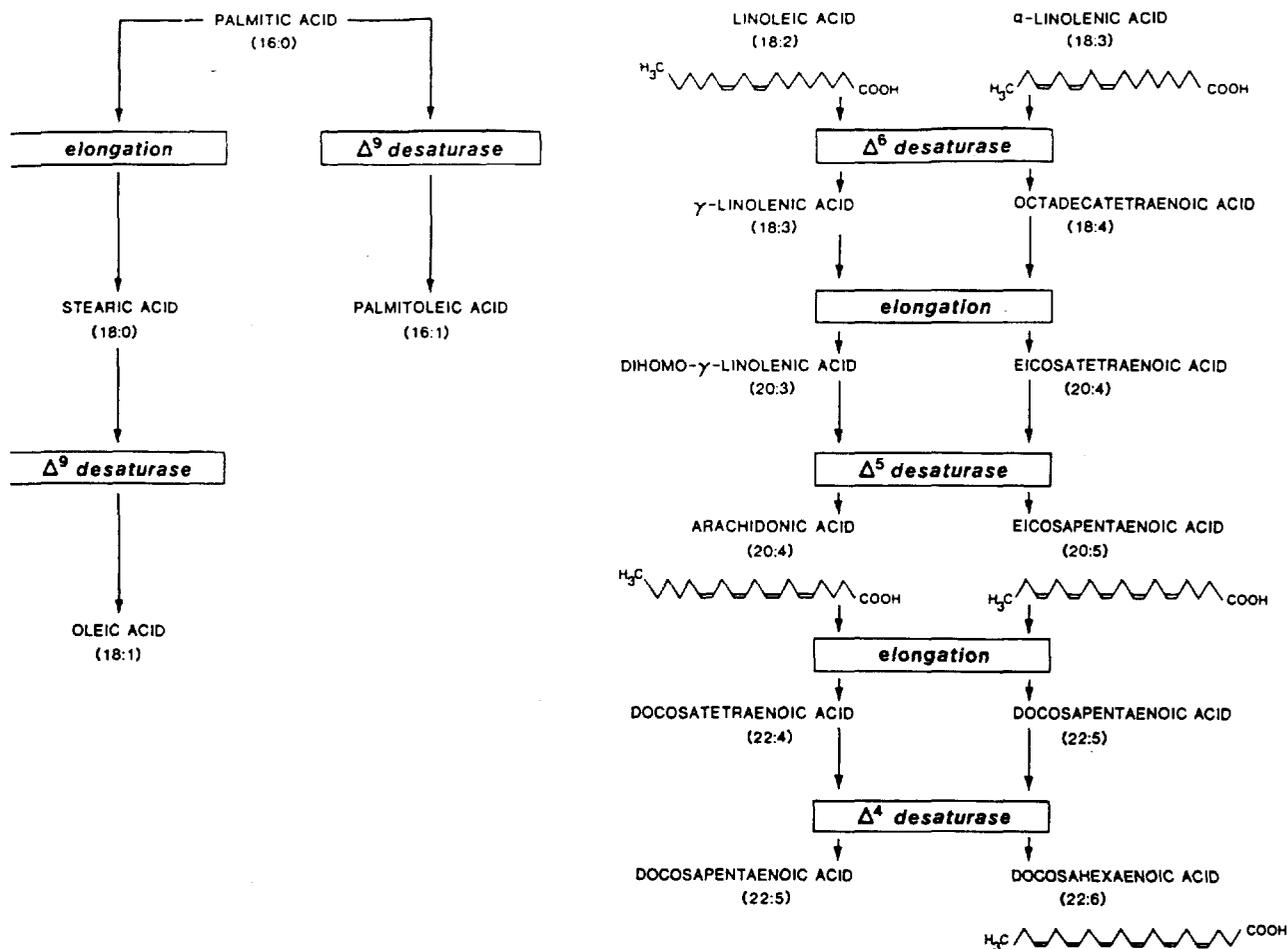


Fig. 1. Pathways of metabolism of fatty acids by chain elongation and desaturation. Fatty acids are divided into 4 families: n-9, n-7, n-6, and n-3 (ω -9, ω -7, ω -6, and ω -3). Number in each family name indicates position of 1st double bond, counting from methyl end of molecule. Chemical formula indicates number of carbon atoms and then number of double bonds. For example, arachidonic acid contains 20 carbon atoms and 4 double bonds: 1st is located after C-6, counting from methyl end. Chemical formula may also include location of 1st double bond counting from methyl end. Thus, arachidonic acid is also rendered frequently as 20:4 n-6, where number after n indicates position of 1st double bond, counting from methyl end. Spatial configurations of selected fatty acids are also depicted. Fatty acids are converted from one to another by sequence of elongation and desaturation steps. Desaturase enzymes are named by position at which double bond is introduced, counting from methyl end of molecule. Abnormalities in these pathways in diabetic animals are reported to occur at Δ^9 -desaturase, Δ^6 -desaturase, and Δ^4 -desaturase steps (see text).

th omega-3 fatty acids may be of little benefit. Alternatively, in absolute terms all polyunsaturated fatty acids (omega-6 and omega-3) are decreased in diabetic patients (as is the case with animals), then the net balance of omega-6 and omega-3 fatty acids and their biologically active products may be unusually susceptible to dietary manipulation in diabetic patients. Second, studies are needed in patients with diabetes to determine 1) the fatty acid composition of phospholipids under defined conditions of metabolic control and 2) the effects of fish and fish oils on phospholipid fatty acid composition under these conditions.

EFFECTS OF OMEGA-3 FATTY ACIDS IN DIABETES MELLITUS RELATIVE TO DOSE

Several studies of the effects of omega-3 fatty acids in diabetes mellitus have already been performed. Some apparently beneficial effects of omega-3 fatty acids on platelet

function, eicosanoid formation, plasma triglycerides, and blood pressure have been described (20-23). For example, 4.6 g/day of omega-3 fatty acids produced a significant reduction in platelet TXB₂ production in vitro and in the lag phase of platelet aggregation in patients with IDDM (20). Approximately 1.6 g/day of omega-3 fatty acids administered in the form of cod liver oil decreased TXB₂ generation by clotting blood in patients with IDDM but not in normal subjects (21). A dose of 9 g/day of omega-3 fatty acids decreased platelet aggregation and circulating triglyceride levels in patients with NIDDM (22). A dose of 2.7 g of omega-3 fatty acids appeared to decrease both the systolic and diastolic blood pressure in patients with NIDDM (23).

The enthusiasm displayed initially among investigators and in the news media for omega-3 fatty acids in diabetes was rapidly superseded by warnings about the potentially

deleterious effects of these agents, including increases in plasma glucose (24–28), glycosylated hemoglobin (25,26), plasma total cholesterol and low-density lipoprotein cholesterol (LDL-chol) (20,27), and serum apolipoprotein B (apoB) (23,26) levels. In general, the magnitude of these changes has been small (typically 10–36%). The changes in glucose metabolism are associated with increased hepatic glucose output, impaired insulin secretion, and unchanged glucose disposal rates (25). The total cholesterol and LDL-chol levels did not change in some studies (23–26). The increases in apoB levels are not specific for diabetic patients, because they also occur in nondiabetic subjects (29). The modest increases in plasma glucose and glycosylated hemoglobin levels probably could be prevented by conventional treatment for diabetes, i.e., other modalities of diet, oral hypoglycemic agents, or insulin. Thus, it may be possible to obtain the beneficial effects of omega-3 fatty acids without a deterioration of metabolic control. This possibility has not been investigated.

The putative adverse effects of omega-3 fatty acids have almost invariably been achieved with large doses (4–10 g/day). (An exception to this statement is a study that employed 2.7 g/day of omega-3 fatty acids per day but lacked a control group or a washout period [23].) These doses correspond to 0.33–0.83 kg (0.73–1.83 lb)/day of fish that is rich in omega-3 fatty acids, assuming a content of 1.2 g omega-3 fatty acids/100 g of fish (4,30). These doses are large in terms of the amount of fish that a person can reasonably be expected to eat and in terms of the benefits that have been ascribed to fish consumption vis-à-vis death from coronary heart disease. Thus, a recent study of a group of Dutch men described an inverse relationship between fish consumption and death from coronary heart disease during a 20-yr follow-up period (31). Mortality from coronary heart disease was >50% lower among those who consumed fish than among those who did not. The benefits of fish consumption were detected among subjects who consumed ≥ 30 g of fish per day, estimated to contain 0.2 g of EPA. Interpretation of this study depends on a dietary history obtained at the start of the 20-yr follow-up period and on the assumption that fish consumption remained relatively constant during this interval. Many of the effects of omega-3 fatty acids, such as their effects on plasma triglycerides and glucose metabolism, are related to dose in diabetic patients (26). An intake of 4 g/day in patients with NIDDM appears to have no effect on fasting plasma glucose and glycosylated hemoglobin levels (20,26).

These considerations suggest that the doses of omega-3 fatty acids used in patients with diabetes mellitus have been excessive and that beneficial effects may be achieved and potentially harmful effects may be averted at lower doses. Alternatively, the beneficial effects of dietary fish may not be due entirely to the presence of omega-3 fatty acids. This possibility seems unlikely, because virtually all of the known effects of fish on risk factors for vascular disease are reproduced by omega-3 fatty acids, but it has not been excluded (4). Excessive doses of omega-3 fatty acids are no more rational than excessive doses of aspirin in efforts to prevent vascular disease. The dose of omega-3 fatty acids that will give the optimal ratio of beneficial effects to harmful ones is unknown. Whether small quantities taken over a prolonged

period will produce the same effects as large quantities consumed over a brief period is also unknown. The relationship between dose and effect, a matter of fundamental importance in nutrition and pharmacology, must be defined for dietary fish and fish oils in patients with diabetes mellitus. The few studies of the effects of omega-3 fatty acids that have been performed in patients with diabetes mellitus do not define the role of these lipids in this disorder. Rather, they help to define the direction of future studies.

Studies are urgently needed to assess the effects of lower and more realistic doses of omega-3 fatty acids (provided as dietary fish or fish oils) on risk factors for vascular disease in diabetes to determine whether such doses will favorably alter some risk factors, e.g., platelet function and eicosanoid production, without having a deleterious effect on other risk factors, e.g., plasma glucose levels and lipoprotein metabolism. These investigations should also be controlled for the fat and calorie content of the fish or fish oils under study. The quantity of fish or fish oils administered should be related to the patient's size (e.g., body mass index), and studies should be performed for prolonged periods (months or years) to determine whether any observed effects are transient or long lasting.

From this discussion, it appears that it will be difficult to predict the effect of omega-3 fatty acids on the incidence of macrovascular and microvascular complications in diabetes, because the pathogenesis of these complications involves multiple variables, some that may change in an apparently favorable direction and others in an apparently deleterious way in response to these marine lipids. Whereas studies of the effects of omega-3 fatty acids on the mechanisms implicated in the pathogenesis of diabetic vascular complications are essential to an understanding of the biological effects of these substances, such studies probably will not reveal the net effect of omega-3 fatty acids on the incidence of vascular complications. There is no substitute for direct ascertainment of the effects of omega-3 fatty acids on the incidence, prevalence, and severity of vascular disease in patients with diabetes mellitus.

TABLE 1
Areas for future study of omega-3 fatty acids in diabetes mellitus

- The regulation of fatty acid synthesis and the fatty acid composition of phospholipids in plasma, platelets, and other tissues under defined conditions of metabolic control and diet:
- The effects of dietary fish and fish oils in appropriate quantities on:
 1. phospholipid fatty acid composition of plasma, platelets, and other tissues
 2. potential pathogenetic mechanisms
 - platelet function
 - eicosanoid (prostaglandin, thromboxane, and leukotriene) production
 - lipoprotein and apolipoprotein levels
 - erythrocyte deformability
 - blood viscosity
 - blood pressure
 - thrombolytic activity
 - inflammatory cell activity
 3. incidence of vascular complications
 - microvascular: retinopathy, nephropathy, and certain neuropathic complications
 - atherosclerotic

CONCLUSIONS

It is not yet possible to assess the potential risks and benefits of dietary fish and fish oils in patients with diabetes mellitus. Studies are needed to determine the regulation of fatty acid synthesis and the fatty acid composition of phospholipids in diabetic patients under defined conditions of metabolic control and diet. Studies are also needed to determine the effects of dietary fish and fish oils in appropriate quantities on the fatty acid composition of phospholipids, the mechanisms involved in the pathogenesis of vascular disease, and the incidence of microvascular and atherosclerotic complications in patients with diabetes mellitus (Table 1).

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EXHIBIT V

Can Garlic Reduce Levels of Serum Lipids? A Controlled Clinical Study

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PURPOSE: To assess the effects of standardized garlic powder tablets on serum lipids and lipoproteins, glucose, and blood pressure.

SUBJECTS AND METHODS: Forty-two healthy adults (19 men, 23 women), mean age of 52 ± 12 years, with a serum total cholesterol (TC) level of greater than or equal to 220 mg/dL received, in a randomized, double-blind fashion, either 300 mg three times a day of standardized garlic powder in tablet form or placebo. Diets and physical activity were unchanged. This study was conducted in an outpatient, clinical research unit.

RESULTS: The baseline serum TC level of 262 ± 34 mg/dL was reduced to 247 ± 40 mg/dL ($p < 0.01$) after 12 weeks of standard garlic treatment. Corresponding values for placebo were 276 ± 34 mg/dL before and 274 ± 29 mg/dL after placebo treatment. Low-density lipoprotein cholesterol (LDL-C) was reduced by 11% by garlic treatment and 3% by placebo ($p < 0.05$). There were no significant changes in high-density lipoprotein cholesterol, triglycerides, serum glucose, blood pressure, and other monitored parameters.

CONCLUSIONS: Treatment with standardized garlic 900 mg/d produced a significantly greater reduction in serum TC and LDL-C than placebo. The garlic formulation was well tolerated without any odor problems.

Garlic (*Allium sativum*) [1-3] has been used in herbal medicine for centuries for various ailments. In recent years, garlic has been the focus of serious medical and clinical attention because of reports of beneficial effects on several cardiovascular risk factors [4,5]. Garlic extracts have been reported to reduce levels of serum lipids [3-18], blood pressure [4,5,7-13,16,18], and plasma viscosity [19], inhibit platelet aggregation [9,16,18,20,21], increase fibrinolytic activity [9,21], and produce vasodilation [21-23]. Thus, garlic is assumed to have antiatherosclerotic properties, although its full potential in the prevention or treatment of circulatory or vascular diseases is yet to be defined.

Garlic's principal active agent appears to be alliin, a sulfur-containing compound that with its breakdown products, gives garlic its characteristic odor [1]. Alliin is formed enzymatically from an odorless precursor, alliin, when garlic cloves are mechanically disrupted. Since the alliin content of natural garlic may vary 10-fold and the quantity of alliin released can be influenced by specific extraction methods [24,25], standardizing garlic products by using their potential for releasing alliin has been suggested to ensure the accuracy of dosage and effectiveness in long-term therapy [26].

A dried garlic powder tablet standardized to provide 1.3% alliin, which corresponds to an alliin release of 0.6%, has been available in Germany as a dietary supplement over the counter (OTC) to improve cardiovascular risk factors [27]. More recently, these garlic tablets have been available OTC in the United States, but no health claims are made.

We conducted a 12-week, double-blind study comparing the effects of standardized garlic tablets (Kwai, Lichtwer Pharma GmbH, Berlin, Germany), 900 mg/d, with that of placebo on serum lipids, serum glucose, blood pressure, and other parameters in 42 subjects with hypercholesterolemia.

SUBJECTS AND METHODS

Subjects with known serum total cholesterol (TC) levels greater than or equal to 220 mg/dL were screened by a history and physical examination, Chem-23 (serum glucose, blood urea nitrogen, creatinine, sodium, potassium, chloride, carbon dioxide, calcium, phosphorus, TC, triglycerides [TG], total bilirubin, direct bilirubin, indirect bilirubin, alkaline phosphatase, serum glutamic oxaloacetic transaminase [SGOT], serum glutamic pyruvic

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transaminase [SGPT], γ -glutamyl transferase, lactic dehydrogenase, total protein, albumin, globulin, albumin/globulin ratio, uric acid, and iron), complete blood count (CBC), urinalysis, and electrocardiogram (EKG) prior to the study enrollment. Patients were excluded if they were older than 70 years or had a history of drug or alcohol abuse, impaired hepatic function test results (SGOT/SGPT) greater than 20% above normal, unstable angina, myocardial infarction, or coronary bypass surgery within 6 months, diabetes mellitus, known secondary hypercholesterolemia due to nephrotic syndrome or hypothyroidism, serum creatinine level greater than 2.0 mg/dL, or use of lipid-lowering agents within 1 month prior to enrollment. All subjects provided written informed consent before enrollment and were advised not to change their dietary habits or physical activity during the course of the study.

Qualifying subjects were then instructed to return to the clinic in a fasting state in 14 ± 2 days for a lipid profile (TC, TG, high-density lipoprotein cholesterol [HDL-C], low-density lipoprotein cholesterol [LDL-C]) and serum glucose measurement. Those with serum TC values of greater than or equal to 220 mg/dL at two consecutive visits were randomized to either placebo or matching garlic tablets, 300 mg three times a day, for 12 weeks, after all baseline parameters were obtained. During the double-blind phase, clinic visits were 6 weeks apart. Lipid profile and serum glucose determinations were repeated at Weeks 6 and 12. At the end of the double-blind phase, each subject had an exit physical examination, safety laboratory tests (Chem-23, CBC, urinalysis), and EKG. Two sitting blood pressure measurements and a pulse rate were obtained after 10 minutes of rest in the clinic using an appropriate-size cuff, a conventional mercury sphygmomanometer, and standard recommended technique [28]. Body weight, side effects, and compliance were also monitored at each clinic visit, and patients were questioned as to any changes in diet or physical activity.

All laboratory analyses were done by a CDC (Centers for Disease Control) and NWLRC (Northwestern Lipid Research Clinic, Seattle) standardized laboratory, SmithKline Beecham Labs. The serum TC [29] and TG [30] were measured by enzymatic procedures. HDL-C was isolated initially by precipitating LDL-C and very-low-density-lipoprotein cholesterol (VLDL-C) with phosphotungstate/magnesium chloride. An aliquot of the supernatant was then assayed for cholesterol content [31]. LDL-C was calculated by the Friedewald equation, $LDL-C = TC - HDL-C + TG/5$. Patients with TG greater than 400 mg/dL were excluded.

Statistical Analysis

The average of two visits prior to randomization represented the baseline value for the serum lipid,

TABLE I

Demographic Characteristics

	Standardized Garlic Tablets (900 mg/d)	Placebo
No.	20 (14 W, 6 B)	22 (15 W, 7 B)
Mean age (y)	48 \pm 15*	55 \pm 9
Sex	11 M, 9 F	8 M, 14 F
Body weight (kg)	78 \pm 17	77 \pm 14
Blood pressure (mm Hg)	128/81 \pm 11/7	126/82 \pm 9/7
Height (cm)	168 \pm 9	165 \pm 13
Nonsmokers	11	20

W = white; B = black; M = male; F = female.
*Mean \pm SD of mean.

glucose, blood pressure, body weight, and other parameters. The differences from baseline value at Weeks 6 and 12 were then calculated and these differences were analyzed for treatment effects by an analysis of variance, followed by Mann-Whitney and Wilcoxon U-tests [32].

RESULTS

The demographic characteristics of the study population are shown in Table I. The two treatment groups were fairly comparable for the listed variables. Mean age was slightly lower in the garlic-treated group and there were fewer smokers in the placebo group.

The effects of garlic tablets and placebo on levels of serum lipids, blood pressure, and body weight at baseline and at the end of 6 and 12 weeks of treatment are shown in Table II. Changes from baseline for serum TC and LDL-C at the end of 12 weeks are shown in Figure 1. No significant treatment differences in the measured parameters were seen at Week 6 between the placebo and garlic groups. At Week 12, however, serum TC was lowered by 6% with garlic tablets and 1% with placebo ($p < 0.01$). This reduction in serum TC was caused mainly by a reduction in LDL-C, which was decreased by 11% in the garlic-treated group and 3% in the placebo group ($p < 0.05$). There was a small, but nonsignificant, increase in TG in both groups. HDL-C, serum glucose, blood pressure, and body weight did not change significantly.

Only 1 of 20 patients treated with garlic tablets complained of increased belching with garlic taste. Two patients receiving placebo had mild abdominal discomfort. One patient taking placebo reported prolonged oozing from a razor cut during shaving, and another patient had a minor rash. In general, garlic tablets were quite well tolerated without any significant odor problems.

COMMENTS

The hypolipemic efficacy of garlic tablets, standardized to release 0.6% allicin, has been well studied in

TABLE II
Efficacy Variables*

	Standardized Garlic (900 mg/d) (n = 20)			Placebo (n = 22)		
	Before	6 Wk	12 Wk	Before	6 Wk	12 Wk
TC (mg/dL)	262 ± 35	248 ± 31	247 ± 40 [†]	276 ± 34	262 ± 38	274 ± 29
LDL-C (mg/dL)	188 ± 37	172 ± 33	168 ± 43 [†]	191 ± 34	180 ± 39	185 ± 25
HDL-C (mg/dL)	47 ± 12	45 ± 13	46 ± 13	49 ± 14	48 ± 15	50 ± 17
TG (mg/dL)	151 ± 81	166 ± 137	165 ± 86	195 ± 112	176 ± 107	199 ± 101
Glucose (mg/dL)	100 ± 11	102 ± 13	98 ± 12	98 ± 9	97 ± 7	97 ± 7
Blood pressure (mm Hg)	129 ± 13	129 ± 15	130 ± 17	128 ± 10	127 ± 12	127 ± 12
Body weight (kg)	82 ± 6	83 ± 8	81 ± 10	83 ± 8	81 ± 7	82 ± 6
Heart rate (beats/min)	79 ± 17	79 ± 17	79 ± 17	77 ± 14	77 ± 14	77 ± 15
	71 ± 8	66 ± 6	69 ± 6	72 ± 7	68 ± 6	70 ± 8

*Mean ± SD.

[†]p < 0.01 Kwai versus placebo for differences from baseline treatment effects.

[‡]p < 0.05 Kwai versus placebo for differences from baseline treatment effects.

TABLE III
Reported Efficacy of Garlic Treatment

References	Study Design	Sample Size	Dose of Garlic* (mg/d)	Treatment Duration (wk)	% Reduction		Drop in BP (mm Hg)
					TC	TG	
[7]	DB, standard	40	600	12	-6	-8	6/15
[8]	DB, placebo	40	600	12	-10	-8	9/16
[9]	Open	20	600	4	-11	+3	8/5
[10]	DB, placebo	261	800	16	-11.6	-16.8	N/A
[12]	DB, R, P	47	600	12	-12	-21	12/11
[13]	DB, placebo	40	900	16	-21	-24	4/3
[14]	Open	40	600	12	-6.4	-16	N/A

BP = blood pressure; DB = double-blind; R = randomized; P = placebo; N/A = not available.

*Given as Kwai.

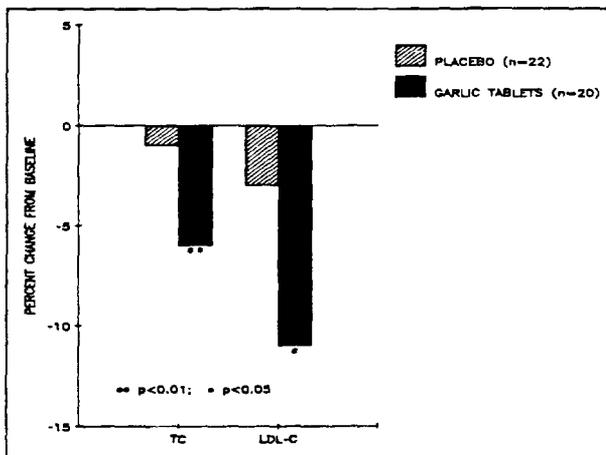


Figure 1. Percent reduction in serum TC and LDL-C after 12 weeks of treatment with standardized garlic powder tablets or placebo.

Germany, where it is available OTC as a dietary supplement to improve cardiovascular risk factors [27].

In this double-blind randomized study, 12 weeks of treatment with garlic 900 mg/d produced a modest but significantly greater reduction in serum TC and LDL-C than that with placebo. This reduction in serum cholesterol, although somewhat lower, is congruous with the data reported by others (Table III).

In a double-blind, multicenter study in 261 German patients with hyperlipidemia, Mader *et al* [10]

reported a 12% reduction in TC and 17% decrease in TG after 16 weeks of treatment with standardized garlic powder 800 mg/d. The subgroup with initial cholesterol levels in the range of 250 to 300 mg/dL showed the greatest response, with a 14% reduction in TC. The onset of the hypolipidemic effect was evident as early as 4 weeks and became progressive and greater with time. There was no special dietary monitoring. Vorberg and Schneider [13] reported even greater reductions, e.g., 21% in TC and 24% in TG following 4 months of treatment with standardized garlic powder 900 mg/d. A low TC response in our study may be partly related to the shorter duration of treatment as well as to the greater body mass indices of our population when compared with the German data [14]. Administration of 600 mg/d of standardized garlic for 12 weeks has resulted in reductions in serum cholesterol that have ranged from 6.9% to 12%, and in TG from 8% to 12% [7-9,12,14].

In our study, there was a slight increase in TG in both the placebo and garlic-treated groups. However, neither the changes from baseline nor the differences between treatment groups were significant. The reason for this apparent discrepancy in our TG data as compared with that of others is not clear. Voluntary modifications of diet may have partly accounted for the changes in TG reported by others [7-10,12-14].

The mechanisms or active ingredients by which garlic lowers serum cholesterol levels are not known. Inhibition of hydroxymethylglutaryl coenzyme A reductase [33] and cholesterol biosynthesis [34] has been suggested.

Most studies with standardized garlic powder also reported some reduction in blood pressure in hypertensive subjects (Table III). Most subjects in our study were normotensive and only three patients in each group were receiving antihypertensive therapy. These patients also had normal blood pressure during treatment. Data analysis with or without inclusion of these three subjects in each group showed no significant change from average baseline blood pressure. There were no significant changes in serum glucose levels or body weight. Various safety parameters and EKG also showed no significant changes.

Garlic powder, given in the form of tablets in our study, was well tolerated and only one subject reported increased belching and a garlic odor. Mader *et al* [10] reported a 21% incidence of garlic smell following 800 mg/d of these garlic tablets. However, in their study, 9% of patients receiving placebo also reported the same garlic odor. In fact, garlic odor has been one of the major concerns of treatment with garlic extracts. The general incidence in the German reports appears to be about 10% to 15%. The low incidence in our study may be partly due to the high "expectation" of garlic smell or taste from ingesting garlic pills, and hence not "viewed" by the subjects as an adverse effect.

Since 900 mg/d was very well tolerated in our study, higher dosages of standardized garlic powder are worth exploring. Also, controlled clinical trials of longer duration are needed to assess the long-term benefit of garlic on vascular and circulatory disease processes.

In conclusion, treatment with garlic tablets standardized to deliver 0.6% allicin, the assumed active ingredient of garlic, produced a significantly greater reduction in TC and LDL-C than that with placebo. Treatment with garlic 900 mg/d for 12 weeks was very well tolerated with no significant garlic odor problem. Further studies are certainly warranted.

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Exhibit W

A double-blind crossover study in moderately hypercholesterolemic men that compared the effect of aged garlic extract and placebo administration on blood lipids^{1,2}

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ABSTRACT A double-blind crossover study comparing the effect of aged garlic extract with a placebo on blood lipids was performed in a group of 41 moderately hypercholesterolemic men [cholesterol concentrations 5.7–7.5 mmol/L (220–290 mg/dL)]. After a 4-wk baseline period, during which the subjects were advised to adhere to a National Cholesterol Education Program Step I diet, they were started on 7.2 g aged garlic extract per day or an equivalent amount of placebo as a dietary supplement for a period of 6 mo, then switched to the other supplement for an additional 4 mo. Blood lipids, blood counts, thyroid and liver function measures, body weight, and blood pressure were followed over the entire study period. The major findings were a maximal reduction in total serum cholesterol of 6.1% or 7.0% in comparison with the average concentration during the placebo administration or baseline evaluation period, respectively. Low-density-lipoprotein cholesterol was also decreased by aged garlic extract, 4% when compared with average baseline values and 4.6% in comparison with placebo period concentrations. In addition, there was a 5.5% decrease in systolic blood pressure and a modest reduction of diastolic blood pressure in response to aged garlic extract. We conclude that dietary supplementation with aged garlic extract has beneficial effects on the lipid profile and blood pressure of moderately hypercholesterolemic subjects. *Am J Clin Nutr* 1996; 64:866–70.

INTRODUCTION

An increased serum cholesterol concentration is an important risk factor for the development of cardiovascular and possibly cerebrovascular disease. Similarly, enhanced platelet response to activating agents represents a distinct risk for thromboembolic events in the arterial circulation. Reduction of these and other risk factors through dietary modification, behavioral changes, and medicinal intervention has already substantially decreased the incidence and mortality from coronary and cerebrovascular disease. Supplementation of the diet with certain biofactors may further reduce such risk factors (1, 2). Garlic belongs to a group of dietary supplements that may lessen the incidence of cardio- and cerebrovascular disease by reducing cholesterol concentrations and decreasing platelet responsiveness to activating agents.

Several studies were published over the past two decades that ascribe a hypocholesterolemic action to garlic preparations

(3–14). Although there are substantial differences in the composition of garlic preparations, many of those currently on the market as well as fresh garlic have been reported to decrease total cholesterol significantly. A careful and critical review of the data from authentic scientific studies led one of us to estimate that dietary supplementation with ~10–15 g cooked garlic/d or equivalent amounts of garlic oil or aged garlic extract (AGE) can lower serum cholesterol by ~5–8% in the majority of hypercholesterolemic persons (1). Some investigators, however, have found no cholesterol-lowering effect of garlic (15). There is a lack of well-controlled intervention trials that can validate such an estimation, especially trials in which vital signs, body weight, blood chemistry results, and liver functions of the study subjects are carefully monitored. Changes in some of these variables can have substantial effects on the serum cholesterol concentration. For this reason we undertook a study in which all of these variables were monitored during an 11-mo study.

We performed a double-blind crossover study in a group of moderately hypercholesterolemic men who were given AGE over a period of 4–6 mo. We measured serum lipids, including total cholesterol, low-density-lipoprotein (LDL) and high-density-lipoprotein (HDL) cholesterol, and triacylglycerols, and monitored their blood pressure.

SUBJECTS AND METHODS

Study population

Fifty-six men aged 32–68 y were enrolled in the double-blind crossover study. A prerequisite for enrollment was normal results from a physical examination and a total cholesterol concentration between 5.7 and 7.5 mmol/L (220 and 290 mg/dL). All participants were advised to follow the National Cho-

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lesterol Education Program Step I diet (16) for the length of the study. During a 4-wk period baseline values of lipid profiles (total, HDL, and LDL cholesterol, and triacylglycerols) were analyzed at weekly intervals and the subjects were then randomly assigned into one of two study arms to receive either nine placebo or nine AGE capsules (Wakunaga of America Co, Mission Viejo, CA), which were taken in three divided doses with meals daily as a dietary supplement. Both types of capsules were identical in appearance and were custom-made for this study. Each garlic capsule contained 800 mg AGE powder whereas the placebo capsules contained 600 mg corn starch, 99.5 mg microcrystalline cellulose, 0.5 mg caramel, and 3.5 mg magnesium stearate. The first intervention period began in week 5 and lasted for 180 d, during which time lipids were analyzed six times. After that, individuals were changed to the supplement they did not receive during the first intervention period and continued the study for a total of 120 d, during which period blood lipids were measured five times. Measurements were performed by MetPath (Corning Clinical Laboratory, New Britain, CT). Cholesterol was measured enzymatically by using a cholesterol esterase test with a reproducibility of 2%. LDL-cholesterol values were calculated by using the Friedewald equation (17). Hemoglobin, hematocrit, and white blood cell values as well as serum creatinine and thyroid and liver functions were checked four times, once during the baseline study period, twice during the first intervention period, and once at the end of the second intervention period. Body weight was checked once during the baseline period, six times during the first intervention period, and five times during the second intervention period. Blood pressures were monitored by manual measurement with a sphygmomanometer.

This investigation was reviewed and approved by the Human Studies Review Board of the institution where it was conducted. All volunteers signed an informed consent form before enrollment in the study.

Statistical evaluation

All data are presented as means \pm SDs at each of the 15 sampling points. The latter are described throughout the paper as -4 to -1 for the four weekly baseline evaluations and as 1-11 for the 11 intervention-period measurements that were performed at 30-d intervals except for the first, which came 10 d after the intervention trial began. Each response variable was analyzed according to the methods described for crossover designs by Cochran and Cox (18). Analyses were conducted by using the general linear models procedure and plotting procedures from SAS software (19). Fisher's least-significant-difference test (18) was used to compare treatment regimens. *P* values < 0.05 were considered significant. We evaluated all data comparing the effect of dietary supplements within each study arm and also across the two study arms. Arm 1 study subjects began their intervention trial with AGE, whereas arm 2 study subjects started with placebos.

RESULTS

We screened 75 men, most of whom were former participants in the Pawtucket Heart Health Study and known to have moderately high serum cholesterol concentrations. Fifty-two met our entrance criteria and were enrolled. Of these individuals, 11 dropped out during the first or second intervention

period for a variety of reasons that included allergy to the coating material of the capsules, various gastrointestinal complaints, difficulties in taking the required number of capsules, and perception of unusual body odor. Forty-one men completed both arms of the study. There were no significant differences in any of the blood count or blood chemistry values in the study subjects for the placebo and AGE intervention periods nor between the latter and baseline. Similarly, thyroid screening test results were not affected by the study supplements. Note that animal experiments have shown that thyroid uptake of [31 I] becomes abnormal after injection of high doses of allyl sulfides (20). However, the AGE used in this study contains only trace amounts of these thioallyl compounds. Body weights of those who started with the garlic supplement were 85.8 ± 13.0 , 86.3 ± 10.1 , and 85.7 ± 10.6 kg, respectively, for the baseline, first, and second intervention periods. Study participants starting with the placebo supplement weighed 85.8 ± 16.3 , 86.8 ± 17.0 , and 86.0 ± 16.8 kg for the baseline, first, and second intervention periods.

Systolic blood pressures (Figure 1A and B) showed significant reductions with AGE supplementation not only compared with

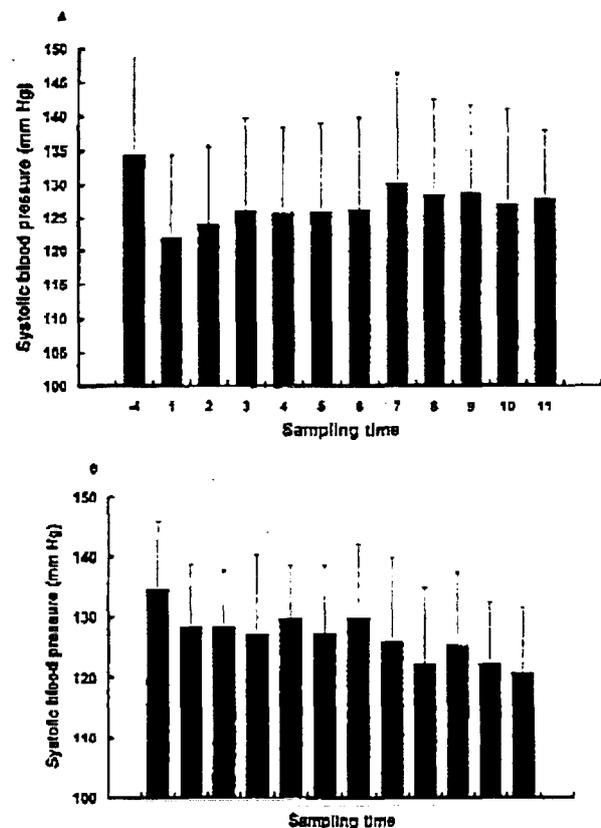


FIGURE 1. Systolic blood pressure readings in study subjects who began their intervention trial with aged garlic extract (AGE) (study arm 1: A) or with placebo (study arm 2: B) supplements. Within-study arm comparisons: AGE compared with baseline, *P* = 0.0001 (both study arms); placebo compared with AGE, *P* = 0.003 and 0.0001; and placebo compared with baseline, *P* = 0.0045 and 0.0003 for study arms 1 and 2, respectively. Across-study arm comparisons: AGE compared with placebo, *P* = 0.0001; AGE compared with baseline, *P* = 0.0001; and placebo compared with baseline, *P* = 0.0002.

baseline but also with placebo, and irrespective of whether such comparisons were made within or across each study arm. Significant reductions were also noted between baseline and placebo intervention periods. At maximal reduction during AGE administration, systolic blood pressures were 5.5% lower than the average pressure during placebo supplementation. Diastolic blood pressures (Figure 2) were also significantly reduced during the AGE compared with the placebo intervention period, but when differences within each study arm were evaluated separately, it became apparent that study subjects taking AGE supplements during the first intervention period showed no significant change in values from either baseline or the placebo period.

The lipid profiles of study participants are shown in Figures 3–6. Irrespective of whether the men started the first intervention period with AGE or placebo and whether comparisons were made within or across study arms, total serum cholesterol was significantly reduced with AGE administration (Figure 3, A and B). At the time of maximal reduction, total cholesterol concentrations of subjects averaged in both study arms were 6.1% below the mean concentrations of all study participants with placebo and 7.0% below their baseline values. LDL-cholesterol values for individuals taking AGE were also reduced compared with those taking placebo (Figure 4). Within-study arm analyses showed significant reductions after AGE in comparison with the placebo, but only in subjects starting their dietary intervention with placebo supplements did the difference between baseline and AGE become significant. The maximal reduction of LDL cholesterol was 4% when the average of both study arms was compared with baseline values and 4.6% compared with the average placebo concentrations. Lipid-lowering became apparent within one to two sampling periods, ie, 10–40 d, and reached a nadir \approx 3 mo after subjects switched to AGE. HDL-cholesterol and triacylglycerol concentrations were not significantly changed by either of the two dietary supplements (Figures 5 and 6, respectively). Comparisons of the ratios of HDL to total cholesterol across study arms showed a significant increase ($P = 0.0145$) after AGE compared with

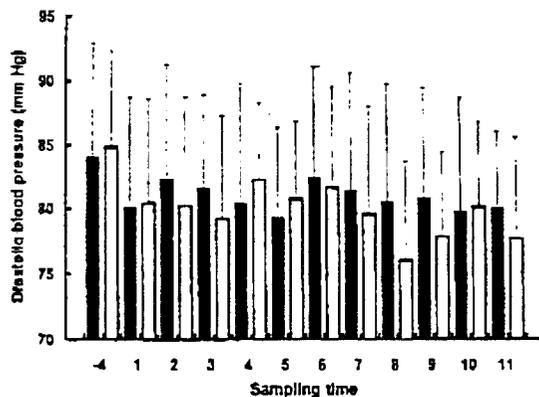


FIGURE 2. Diastolic blood pressure measurements in subjects of both study arms. Solid columns denote means + SDs of subjects starting on aged garlic extract (AGE) and open columns indicate means + SDs of subjects starting on placebo. Within-study arm comparisons: AGE compared with baseline, $P = 0.036$ and 0.0001 ; placebo compared with AGE, $P = 0.51$ and 0.0004 ; and placebo compared with baseline $P = 0.015$ and 0.0007 , for study arms 1 and 2, respectively. Across-study arm comparisons: AGE compared with placebo, $P = 0.026$; AGE compared with baseline, $P = 0.0001$; and placebo compared with baseline, $P = 0.0001$.

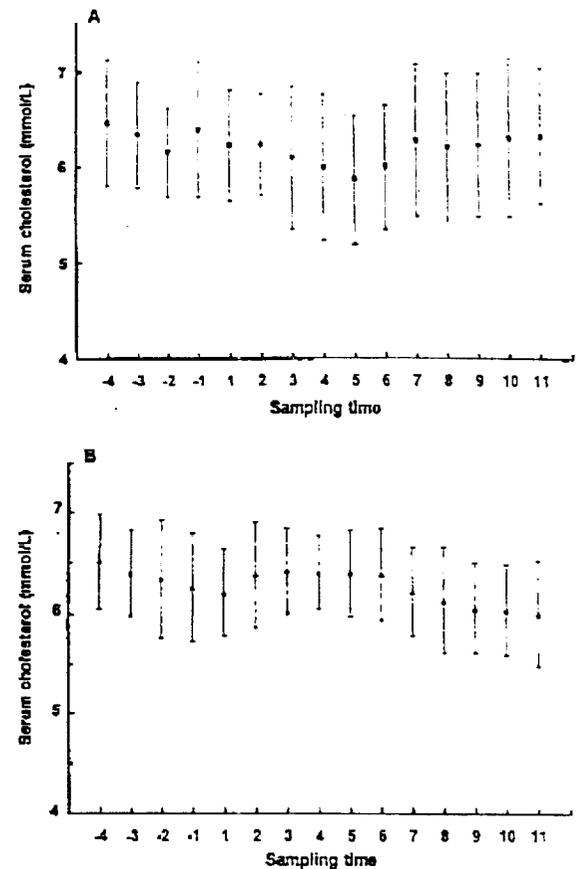


FIGURE 3. Serum total cholesterol concentrations of subjects starting their intervention trial with aged garlic extract (AGE) (study arm 1; A) or placebo (study arm 2; B). Within-study arm comparisons: AGE compared with baseline, $P = 0.0001$ for both study arms; placebo compared with AGE, $P = 0.0005$ and 0.0001 ; and placebo compared with baseline, $P = 0.30$ and 0.62 , for study arms 1 and 2, respectively. Across-study arm comparisons: AGE compared with placebo, $P = 0.0001$; AGE compared with baseline, $P = 0.0001$; and placebo compared with baseline, $P = 0.458$.

placebo supplementation (0.19 ± 0.04 compared with 0.18 ± 0.04).

Compliance, which was measured by pill counting, was somewhat better in participants who started with placebo than in those starting with AGE. Among the latter, 8 of 17 subjects showed pill counts that were within 5% of the expected value. More than one-half of the participants in this group had pill counts that were $\geq 20\%$ off the expected value. On the other hand, 8 of 24 subjects starting with placebo had pill counts that were $\geq 20\%$ off the expected value. A variety of reasons were given for poor compliance, the most common one was forgetting to take lunch-time doses of the supplement.

DISCUSSION

Many supplementation trials with garlic have been performed over the past 15 y. Recently, two meta-analyses evaluated the published trials on the basis of objective criteria for comparison (21, 22). The authors of both studies concluded that garlic supplements produced a reduction in cholesterol

AGED GARLIC EXTRACT AND HYPERCHOLESTEROLEMIA

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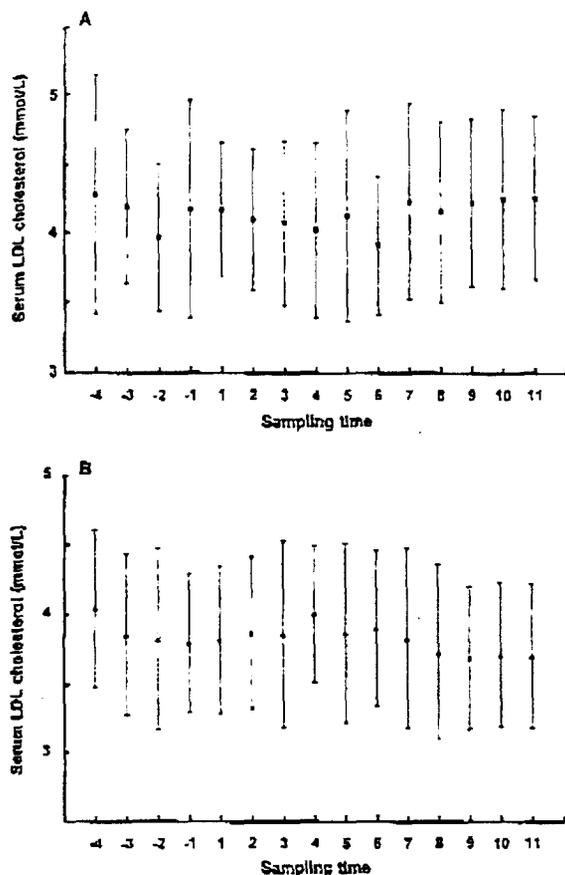


FIGURE 4. Serum LDL-cholesterol concentrations in study subjects starting the intervention trial with aged garlic extract (AGE) (study arm 1; A) or placebo (study arm 2; B). Within-study arm comparisons: AGE compared with baseline, $P = 0.179$ and 0.01 ; placebo compared with AGE, $P = 0.015$ and 0.0035 ; and placebo compared with baseline, $P = 0.36$ and 0.95 , for study arms 1 and 2, respectively. Across-study arm comparisons: AGE compared with placebo, $P = 0.004$; AGE compared with baseline, $P = 0.031$; and placebo compared with baseline, $P = 0.622$.

concentrations ranging from 9% to 12%. We believe that this range is too high, above the 5–8% reduction in total serum cholesterol estimated by Lin (1) in a critical analysis of the existing data. Triacylglycerol concentrations also decreased during garlic therapy by $\approx 13\%$ in eight trials, all of which used garlic powder preparations. HDL cholesterol was not significantly lowered however. In one study of 42 healthy adults who had cholesterol concentrations ≥ 5.7 mmol/L (≥ 220 mg/dL), LDL cholesterol was reduced 11% by garlic compared with 3% by placebo treatment, but there were no changes in HDL cholesterol or triacylglycerols (14). The reductions in total cholesterol obtained by nonpowder preparations of garlic were in general somewhat higher than those of powder preparations (22).

From the standpoint of acceptability of garlic as a dietary supplement, its composition and method of preparation are of great importance. Most of the garlic powder tablets contain substantial amounts of odorous compounds, eg, diallyl sulfides or polysulfides responsible for the characteristic aroma of crushed and stale garlic. On the other hand, steam-distilled garlic oils as well as oil-macerated garlic contain only polysulfides and other volatile thioallyls. During steam distillation

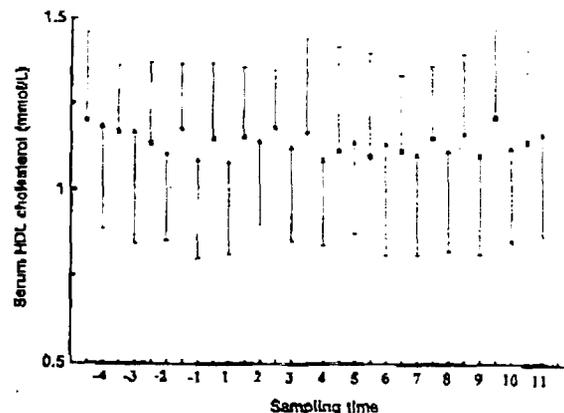


FIGURE 5. HDL-cholesterol concentrations of study subjects starting the intervention trial with aged garlic extract (AGE) (■) or placebo (▲). P values were not significant.

much of the alliin is converted to diallyl sulfides. Many of the thioallyl compounds are quite unstable. Allicin, the oxide of diallyl disulfide, is only a transient constituent of garlic preparations and under physiologic conditions does not pass through the intestine and enter the blood stream. Injected allicin is rapidly destroyed in the blood (1). Similarly, other oxygenated thioallyls, such as ajoene, are also unstable and have low bioavailability. The preparation used in this study has fewer unstable thioallyls but is relatively rich in *S*-allylcysteine with smaller amounts of *S*-allylmercaptocysteine. These compounds are the result of an aging process that leads to the reduction of alliin (*S*-allylcysteine sulfoxide). Aged garlic preparations are far less odorous than fresh garlic extracts or garlic powders. Nevertheless, in high concentrations some odor is associated with AGE capsules although it is somewhat different from the typical garlic odor. In our study most subjects ($\approx 70\%$) were able to clearly identify whether they were taking garlic supplements or placebo. This led to a relatively high rate of noncompliance.

Dietary supplementation with AGE did not change blood counts or chemistry test results, nor did it affect thyroid function. Garlic supplementation has been reported to produce a modest reduction in blood pressure (5, 6, 9). Our studies confirm this finding.

Consumption of AGE reduced total cholesterol and LDL cholesterol, which reached a maximum effect around the third month after AGE supplementation started. There was not a significant difference in HDL cholesterol or triacylglycerols between placebo and AGE periods nor between baseline and the intervention periods. The ratio of HDL to total cholesterol was significantly higher when subjects were consuming AGE compared with placebo. Although, in theory, this ratio should be a good predictor of risk for coronary artery disease, the inability to provide accurate HDL-cholesterol measurements has caused national expert panels to caution against use of this ratio (23). Even though our study showed a very significant AGE-induced reduction of total and LDL cholesterol, one wonders what effect might have been observed in individuals with better compliance. Whether a smaller dose of the aged garlic extract could have produced greater compliance and in turn a comparable reduction in cholesterol also remains unknown. However, it is apparent that removal of most of the

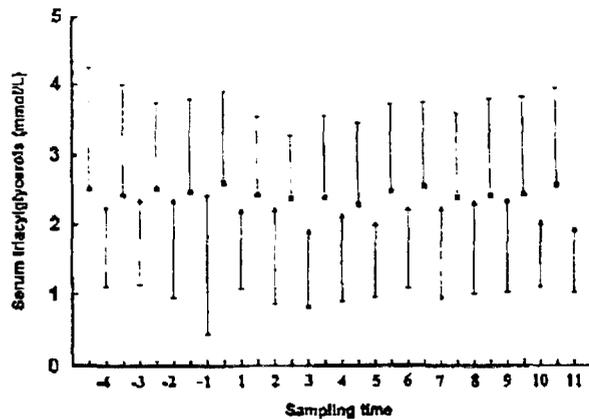


FIGURE 6. Serum triacylglycerol concentrations in study subjects starting the intervention trial with aged garlic extract (AGE) (■) or placebo (▲). *P* values were not significant.

odor-producing substances from the garlic preparation did not completely eliminate objections to the ingestion of garlic extracts in high doses by some of some participants. Although comparisons between this and other garlic studies are difficult to make in view of the differences in the garlic preparations administered, nevertheless, our results are comparable with the estimated hypocholesterolemic effects of garlic supplements (1). All of our study participants were men with moderately elevated cholesterol concentrations, in the range for which pharmaceutical intervention is not absolutely required.

In several animal studies in which AGE was administered in conjunction with cholesterol loading or a diet conducive to cholesterol elevation, it produced far greater reductions ($\approx 30\%$) in serum cholesterol (24). This difference in efficacy could be due to a difference in cholesterol homeostasis, slightly higher doses used in animal studies, and/or the diet. If the diet is the cause of the difference, then AGE could produce a larger cholesterol-lowering effect among individuals consuming excessive amounts of cholesterol or a diet that is conducive to hypercholesterolemia. The subjects in this study were advised to follow the National Cholesterol Education Program Step I diet, which is hypocholesterolemic.

It was reported that an odor-modified liquid garlic extract produced transient elevations in total cholesterol and triacylglycerol concentrations and the ratio of LDL to very-low-density lipoprotein during initial supplementation (7). This was later followed by a significant reduction below baseline values. We did not observe this phenomenon in our study but the average cholesterol concentration in our study population was considerably less than that reported in the above study. Neither did we observe the HDL cholesterol-elevating effect of the liquid garlic extract reported by the same authors.

Our results show that AGE is a safe supplement over extended periods of time and that it does not alter blood counts or change blood chemistry or thyroid function. Furthermore, our double-blind crossover study showed that garlic supplementation can produce significant reductions in total as well as LDL cholesterol but did not change HDL cholesterol and triacylglycerol concentrations. In addition, blood pressure is beneficially affected by garlic administration. ■

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Exhibit W-1

Effects of Dietary Garlic Supplementation in a Rat Model of Atherosclerosis

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Summary

In the present study possible antiatherogenic effects of dietary garlic were investigated in an experimental model which consists in the deendothelialisation by ballooning of the *a. carotis communis* of rats. 3 experimental groups were established: group I received a standard diet; the diet of group II was supplemented with 2% cholesterol and group III received 2% cholesterol and 5% dried garlic powder. Four weeks after ballooning plasma cholesterol, the average thickness of the neointima as well as the DNA content and the expression of collagens type I, III and IV in the ballooned arterial segment were determined. Furthermore, the specific activities of the enzymes glutathione peroxidase, glutathione disulfide reductase, glutathione-S-transferase and glucose 6 phosphate dehydrogenase were measured in homogenates of liver, heart and aorta. Hypercholesterolemia induced by cholesterol-feeding (group II 92 ± 18 mg/100 ml) was significantly reduced by garlic (group III 53 ± 19 mg/100 ml). Only little effects of garlic were seen in inhibiting neointima after ballooning. However, significant effects were found in protecting the enzymes of the glutathione dependent peroxide detoxification system, which is strongly impaired under hypercholesterolemia. Generally a normalisation, in some cases even an improvement beyond that, of the enzyme activities occurred in the garlic treated group.

This indicates that in the model of atherosclerosis used here garlic is effective in lowering plasma cholesterol and in improving peroxide detoxification; however, it has only little influence on the wound healing reaction and does not significantly inhibit the development of intima thickenings after ballooning.

Zusammenfassung

Wirkungen der Futteranreicherung mit Knoblauch in einem Arteriosklerosemodell in Ratten

1. Introduction

Experimental and clinical data suggest an antiatherosclerotic effect of dietary administered garlic which is predominantly postulated by its action on cardiovascular risk factors [1-6]. These effects include decrease of low density lipoprotein (LDL) and increase of high density lipoprotein (HDL) in plasma, decrease of thrombogenic activity, decrease of intracellular lipid accumulation of cultured vascular smooth muscle cells and inhibi-

In der vorliegenden Studie sollten antiatherogene Wirkungen von diätetisch verabreichtem Knoblauch untersucht werden. Durch Deendothelialisierung mittels eines Ballonisierungskatheters wurden Intimaläsionen in der *A. carotis communis* von Ratten induziert, die entsprechend der Fütterung in 3 Gruppen (jeweils $n = 10$) eingeteilt wurden: Gruppe I erhielt Standardfutter, Gruppe II mit 2% Cholesterin und Gruppe III mit 2% Cholesterin und 5% Knoblauchtrockenpulver angereichertes Futter. Vier Wochen nach der Ballonisierung wurden der Plasmacholesterinspiegel und die mittlere Dicke der Neointima, ihr DNA-Gehalt sowie die Expression der Kollagen-Typen I, III und IV im behandelten Arteriensegment bestimmt. Zusätzlich wurden in Homogenaten aus Leber, Herz und Aorta die spezifischen Aktivitäten der Enzyme Glutathion-Peroxidase, Glutathiondisulfid-Reductase, Glutathion-S-Transferase und Glukose-6-Phosphat-Dehydrogenase ermittelt. Die Ergebnisse zeigen, daß die durch Cholesterinfütterung induzierte Hypercholesterinämie (Gruppe II 92 ± 18 mg/100 ml) durch Knoblauch signifikant gesenkt wurde (Gruppe III 53 ± 19 mg/100 ml). Dagegen waren Hemmwirkungen auf die Bildung der Neointima nach Ballonisierung nur tendenziell erkennbar. Deutliche antioxidative Schutzwirkungen durch die Knoblauchfütterung zeigten sich bei den Enzymbestimmungen. Während in Gruppe II generell verminderte Aktivitäten der Glutathion-abhängigen Peroxid-Detoxifikation auftraten, wurde in Gruppe III eine weitgehende Normalisierung, z. T. sogar eine Verbesserung der Werte gefunden. Damit zeigte sich, daß im verwendeten Arteriosklerosemodell durch Knoblauch sowohl eine Senkung des Plasmacholesterins als auch eine verbesserte Peroxid-Entgiftung erzielt werden kann, wogegen die nach Ballonisierung auftretende Wundheilungsreaktion mit Neointimabildung nur wenig beeinflusst wird.

Key words: Atherosclerosis, experimental · Garlic, dietary, pharmacology · Lipid reducers

tion of cholesterol biosynthesis in liver cells in culture. Another aspect was provided by the finding that garlic exerts an antioxidative effect which could be important for the prevention of oxidative modification of LDL. In order to characterize further the antiatherogenic effect of garlic on the level of the arterial wall we determined its effects on plaque growth in carotid artery of rats deendothelized by ballooning. Additionally, we were interested in effects on enzymes related to the peroxide detoxification in liver, heart, and aorta. Whereas para-

tial effects of garlic in this model, the improvement of peroxide detoxification was highly significant.

2. Materials and methods

2.1. Animals and model of arteriosclerosis

Male Sprague-Dawley rats with a body weight of 250–300 g were purchased from Interfauna (Tuttlingen, FRG). The animals were housed (5 per cage) in a room maintained at 20–22 °C and kept at a 12 h light-dark cycle. Diets (see below) and water were given ad libitum. For the ballooning experiments, which were authorized by the Regierungspräsidium Tübingen, male rats were used according to published references and due to experiences in our laboratory [7, 8].

Arteriosclerotic lesions were induced within the common carotid artery by deendothelialization [7, 8]. In short, the animals were anesthetized by flumisonium/fentanyl and metomidate (Hypnorm[®] and Hypnodil[®], resp.; Janssen, Neuss, FRG, 0.5 ml/kg body weight i.m.). A midline incision was made in the neck and the left external carotid artery was exposed. A 2F Fogarty embolectomy catheter (American Edwards Lab, Santa Ana, CA, USA) was inserted from cranial and pushed through the common carotid artery to the level of the aortic arch. Then the catheter was inflated with 25 µl distilled water and 3 times gently withdrawn through the entire length of the artery. After removal of the catheter the external branch of the artery was permanently ligated. The contralateral artery remained untreated and served as intraindividual control.

2.2. Experimental groups

30 rats were randomly assigned to the following 3 experimental groups: group I received the rat standard diet ("semipurified 1533"); the diet of group II was supplemented with 2% cholesterol (Sigma, Munich, FRG), whereas group III received a diet containing 2% cholesterol and 5% dried garlic powder (charge n. 91110503, Lichtwer Pharma, Berlin, FRG, standardized to 1.3% alliin, corresponding to 0.6% alliin). All diets were prepared in pellets by Altromin (Lage, FRG). Each group consisted of 10 animals.

The feeding of groups II and III, respectively, began one week prior to the intervention. Feeding time after ballooning was 4 weeks in all 3 groups. Then the rats were sacrificed by an overdose of fentanyl/metomidate (2 mg/kg b.w. i.p.).

2.3. Determinations

After asphyxia blood was taken from the heart for preparation of plasma (addition of ethylene-diaminetetraacetate) which was used for determination of total cholesterol by the cholesterol oxidase-peroxidase method as given by the supplier (Boehringer Mannheim GmbH, Mannheim, FRG).

The carotid arteries were carefully prepared and excised. After stretching to their *in vivo* length, the arteries were cut in three 5 mm long rings. One segment was immersion fixed in 4% paraformaldehyde, embedded in paraffin and sections (4 µm thick) were cut using a microtome (Rotation Microtome 2040, Reichert-Jung, Heidelberg, FRG). After hematoxylin-eosin staining, the slices were used for histological examination on light microscopic level [8]. In two cross-sections obtained from each arteriosclerotic carotid artery average thickness of the neointima was measured using a projection screen (Zeiss, Oberkochen, FRG) adapted to the microscope. Intimal thickness was determined under "blind" conditions (i.e. without knowledge of the group) as a radial measure in each 45° sector of a corresponding coordinate system laying on the screen (total magnification 630×) and the means were derived.

The second arterial ring was quick frozen on a precooled metal block and used for determination of DNA by the fluorimetric assay with diaminobenzoic acid [9].

The third ring was again fixed in paraformaldehyde (4%), embedded, cut and used for semiquantitative determination of collagen types I, III and IV [10]. After treatment with protease (0.1%, Sigma, Munich, FRG) and incubation with pig serum (dilution 1:30), the cross-sections were treated with corresponding antibodies against the different collagen types (Dunn 1310, 1330, 1340, Aspach, FRG). Bridging antibodies were purchased from Sigma and the final detection was made by the alkaline phosphatase-anti alkaline phosphatase reaction (Dako D651, Hamburg, FRG). The intensity of color developed in the reac-

tioned on the origin of the samples.

After excision of the carotid arteries, the heart, aorta and liver were also removed from the animals. These organs were immersed in ice cold physiological salt solution and cleaned from blood, fat and connective tissue. Tissue homogenates, prepared in Potter-Fleichem glass homogenizers were obtained from samples of the left ventricular wall, from the liver and the aorta [11].

The homogenates were used for the determination of the specific activities of the enzymes glutathione peroxidase (GSHPOD), glutathione disulfide reductase (GSSG-Red) and glucose-6-phosphate dehydrogenase (G6PDH) [11, 12] as well as glutathione-S-transferase (GST) [13].

All chemicals, with the exceptions mentioned, were purchased from E. Merck (Darmstadt, FRG).

2.4. Statistics

All results were calculated as mean values ± SD. Differences between groups II and III were compared by Student's *t*-test, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Behaviour of the animals

Although the ballooning procedure with ligation of the external carotid artery represents a severe intervention, recovery of the animals is usually very rapid and normal behaviour (food intake, sleep-wake cycles etc.) is observed.

Only some animals of group II revealed a postoperative impairment for 5–7 days. However, after the experimental time of 4 weeks, no differences in growth and behaviour of the animals of the different groups were found.

3.2. Influence of garlic supplementation on plasma cholesterol levels

Plasma cholesterol of the rats of group I was found to be 65 ± 14 mg/100 ml (lowest 44.5, highest 89.0 mg/100 ml). Feeding the diet with 2% cholesterol (group II) increased the level up to 92 ± 18 mg/100 ml (63.2–116.5), whereas in group III 53 ± 19 mg/100 ml (22.0–82.5) was determined. This means that the dietary induced increase in plasma cholesterol is significantly reduced ($p < 0.05$) by the additional supplementation of garlic powder.

3.3. Influence of garlic supplementation on neointima formation

The effect of garlic on the growth of the neointima after endothelial denudation was characterized using three different parameters: the thickness of the intimal proliferation, its DNA content, as well as the occurrence and distribution of the collagen types I, III and IV. The results obtained are summarized in Tables 1 and 2.

Table 1a shows that the average thickness of the neointima reaches the highest value in group II. In group I and III the mean values are clearly lower, however, statistical significance of the differences was not obtained. It should be mentioned that the subendothelial space of the nontreated arteries is void of cells and that the intima consists only of the endothelial lining in direct neighborhood of the lamina elastica interna. In the ballooned arteries neointimal thickenings of about 10 to 20 intimal cell layers were found.

A similar result was obtained by measuring the DNA content of the vessel wall segments (Table 1b). Again the highest content was found in group II, but the decreasing effect of garlic in group III could not be established by statistical significance ($p < 0.1$).

The results of the immunohistochemical analysis of the different collagen types are summarized in Table 2. Des-

Table 1 Influence of dietary garlic supplementation on neointima formation in rat carotid artery induced by deendothelialisation - a) average thickness, b) DNA content

	Group I (normal diet)	Group II (- 2% cholesterol)	Group III (- 2% cholesterol + 5% garlic)
a) Average thickness of neointima (μm)	64 \pm 31	70 \pm 30	57 \pm 22
b) DNA ($\mu\text{g}/\text{mm}^2$) denuded artery	4.8 \pm 0.8	4.7 \pm 1.2	4.1 \pm 1.5
Control artery (without intervention)	3.9 \pm 0.7	4.0 \pm 1.7	3.7 \pm 0.8

The values represent mean values \pm S.D. (n = 10), the differences between group II and III reach p. values $<$ 0.10.

Table 2 Influence of dietary garlic supplementation on collagen typing during neointima formation in rat carotid artery induced by deendothelialisation

	Collagen Type I		Collagen Type III		Collagen Type IV	
	Intima	Media	Intima	Media	Intima	Media
Group I	++	-	-	-	+	-
Group II	++	++	--	-	+	-
Group III	+	+	--	+	-	-

The intensity of color development in the collagen staining reaction was subjectively evaluated by comparing slices from ballooned and control arteries, respectively. ++ = strong increase, + = moderate increase, - = no alteration

Table 3 Influence of dietary garlic supplementation on enzyme activities of glutathione dependent peroxide detoxification in homogenates of liver, heart, and aorta from rat

	Group	Liver	Heart	Aorta
GSHPOD	II	1.23 \pm 0.34	0.91 \pm 0.43	0.79 \pm 0.37
	III	1.59 \pm 0.57*	1.66 \pm 0.41*	0.65 \pm 0.27
GSSG Red	II	1.20 \pm 0.29	0.79 \pm 0.40	0.84 \pm 0.45
	III	1.97 \pm 0.45**	2.14 \pm 0.85**	1.71 \pm 0.50
GSH-S-Transf.	II	0.56 \pm 0.21	0.50 \pm 0.14	0.29 \pm 0.04
	III	0.98 \pm 0.38	0.91 \pm 0.50*	1.11 \pm 0.52**
G6P DH	II	0.42 \pm 0.15	0.75 \pm 0.22	0.43 \pm 0.10
	III	1.18 \pm 0.22**	1.02 \pm 0.32*	0.57 \pm 0.12*

All values represent mean values \pm S.D. (n = 10 in each case) which were related to those obtained from the normal diet (group I) and correspond (n = 100). Significance of the differences between groups II and III: * p $<$ 0.05, ** p $<$ 0.01 are indicated. The mean values of enzyme activities is found in group I are given in ml/mg protein as follows: liver, heart, aorta: GSHPOD (494, 273, 267), GSSG Red (42.5, 15.4, 17.8), GSH-S-Transf. (72.8, 22.5, 80.5), G6PDH (24.1, 11.2, 14.5).

pite the difficulty of subjective observation of the slices, a general increase of collagen content in neointima and in group II even in the media can be detected. This is mainly due to increased expression of type I and III under hypercholesterolemia. Administration of garlic (group III) caused a reduction of type I and IV in the neointima. Furthermore, the histological examinations showed that in the garlic treated group the stained collagens were not arranged in lamellae but were diffusely distributed in the extracellular space.

3.4. Influence of garlic supplementation on enzymes of glutathione dependent peroxide detoxification

The results of the determinations of the specific activities of glutathione peroxidase, glutathione disulfide reductase, glutathione-S-transferase, and glucose-6-phosphate dehydrogenase in liver, heart and aorta are summarized in Table 3. It can be seen that hypercholesterol-

emia generally effected these enzymes leading to decreased activities. Only in liver, GSHPOD and GSSG-Red were not influenced. However, since the activity of the NADPH₂ regenerating enzyme G6PDH is rate limiting in the complete reaction sequence, it is obvious that under hypercholesterolemia peroxide detoxification is impaired in all three organs. Also GST, which has a partial activity for peroxide detoxification is impaired under hypercholesterolemia.

Supplementation with garlic (group III) caused an enhancement of the activities of these enzymes (with the exception of GSHPOD in aorta). Yet, since the effects on the rate limiting enzyme G6PDH are most important, a generally improved capacity for detoxification of hydro/liperoxides can be evoked by garlic.

4. Discussion

In the present study three aspects of therapeutic effectivity of dietary administered garlic were investigated in the widely used model of atherosclerosis which consists in the ballooning-induced deendothelialization of carotid arteries of rats fed a cholesterol-enriched diet [7].

The first aspect is related to the cholesterol-lowering effect in plasma. As shown in other experimental models [14-16] or patients [17], a hypocholesterolemic action by garlic could be confirmed in our study, although in rats of group II only a moderate hypercholesterolemia could be induced by the diet. Explanations of the cholesterol-lowering effect could be provided e.g. by an increased excretion of cholesterol by feces [14, 15] or by an inhibition of its endogenous biosynthesis [5].

The second aspect is the effect of garlic on neointima formation after ballooning of the arteries. Both parameters investigated, average thickness as well as DNA content, show a tendency towards reduction of plaque growth by garlic administration, however, this difference could not be established statistically. One serious problem of the model used seems to be, that together with deendothelialization also more or less extended injuries to the media occur. In a series of experiments (n = 5) even a loss of DNA was detected in the ballooned arterial segments 14 days after intervention (unpublished observation). This means that the damage caused by the ballooning procedure to the arterial wall cannot be standardized and that the repair mechanisms lead to quantitatively different results. Whereas these repair mechanisms are only partly inhibited by garlic, clear plaque reducing effects were shown in rabbits fed with cholesterol-rich diet [15, 18]. Since under these conditions oxidation and modification of LDL seem to be more important for plaque formation than after deendothelialisation, one can conclude that the present model is not optimal for detecting inhibiting effects of garlic on neointima formations.

Nevertheless, the immunohistochemical determinations of collagen expression within the ballooned vessel wall show that garlic has a reducing effect on collagen content. During neointima formation a clear increase in collagen type I and, under hypercholesterolemia, type III was found. By garlic, collagen type I and type IV were reduced and arranged diffusely in the extracellular matrix.

A similar effect of garlic preventing collagen fibrosis in an experimental model of heart hypertrophy and insufficiency was described [19].

The last topic of interest - based on the observation of the antioxidative effect of garlic [6] - was the effect on the enzymes of peroxide detoxification. With respect to these we investigated not only glutathione-S-transferase, glutathione peroxidase and glutathione reductase but also glucose-6-phosphate dehydrogenase which in all

three organs has the lowest specific activity and thus represents the rate limiting step by regenerating NADPH₂. The results show that hypercholesterolemia has generally an impairing effect on these enzymes in rat heart, liver and aorta which is in most cases strongly overcome by garlic. Similarly, a restoration by garlic of glutathione peroxidase, glutathione-S-transferase and superoxide dismutase was found in ethanol poisoned rats [20].

The mechanisms by which garlic can influence these enzyme activities remain to be elucidated, furthermore it should be clarified, whether the effects described persist for a long time or are only transient.

Nevertheless, besides the inhibiting effect of garlic supplementation on enzymes of cholesterol biosynthesis [5] a protective effect via improved peroxide detoxification can be suggested, probably not only relevant in atherogenesis but also in other diseases with increased free radical/peroxide production.

Therefore further investigations are necessary with models of atherosclerosis in which LDL modification is of higher importance than in the wound healing process after deendothelialization by ballooning.

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Exhibit W-2

Garlic as a lipid lowering agent— a meta-analysis

ABSTRACT—Garlic supplements may have an important role to play in the treatment of hypercholesterolaemia. To determine the effect of garlic on serum lipids and lipoproteins relative to placebo and other lipid lowering agents, a systematic review, including meta-analysis, was undertaken of published and unpublished randomised controlled trials of garlic preparations of at least four weeks' duration. Studies were identified by a search of MEDLINE and the ALTERNATIVE MEDICINE electronic databases, from references listed in primary and review articles, and through direct contact with garlic manufacturers. Sixteen trials, with data from 952 subjects, were included in the analyses. Many of the trials had methodological shortcomings. The pooled mean difference in the absolute change (from baseline to final measurement in mmol/l) of total serum cholesterol, triglycerides, and high-density lipoprotein (HDL)-cholesterol was compared between subjects treated with garlic therapy against those treated with placebo or other agents. The mean difference in reduction of total cholesterol between garlic-treated subjects and those receiving placebo (or avoiding garlic in their diet) was -0.77 mmol/l (95% CI: -0.65 , -0.89 mmol/l). These changes represent a 12% reduction with garlic therapy beyond the final levels achieved with placebo alone. The reduction was evident after one month of therapy and persisted for at least six months. In the dried garlic powders, in which the allicin content is standardised, there was no significant difference in the size of the reduction across the dose range of 600–900 mg daily. Dried garlic powder preparations also significantly lowered serum triglyceride by 0.31 mmol/l compared to placebo (95% CI: -0.14 , -0.49). HDL-cholesterol was non-significantly lowered by 0.04 mmol/l (95% CI: -0.11 , 0.03 mmol/l). Side-effects from garlic therapy, other than odour, were rare. In conclusion, use of garlic therapy, either as dried garlic preparations (in doses as low as 600 mg per day) or as fresh, high allicin yielding garlic (10–20 g per day) appears significantly to reduce total serum cholesterol over a 1–3 months period. However, more rigorously designed and analysed trials are needed.

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Introduction

Garlic (*Allium sativum*) was used as a remedy for a wide variety of ailments from as early as 1500 BC [1]. Recently most attention has been paid to the possible cardioprotective actions of garlic [2]: these include lipid lowering action [1], antioxidant activity [3], antiplatelet action [4], favourable haemostatic effect [5], and haemodynamic properties [6].

Allicin, the principal active compound in a garlic bulb, is thought to be responsible for most of the pharmacological activity. Crushing the garlic clove activates the enzyme alliinase and converts alliin to allicin. In addition to allicin, other biologically active compounds can be extracted from garlic, including aluraprene and various oils, mucilage and albumin [2].

The first clinical trials of garlic appeared in the literature in the late 1970s, but many suffered from significant methodological shortcomings. These included inappropriate methods of randomisation, lack of controls, poorly characterised patient groups, short duration, insufficient statistical power leading to the likelihood of a type II error, and failure to undertake an intention-to-treat analysis. Because of these problems the authors of an overview of garlic in 1989 conclude that there was inadequate scientific justification to recommend garlic supplementation to reduce cardiovascular risk [2]. Thirteen clinical studies were identified in this earlier report, but only nine of them were randomised controlled trials. No quantitative techniques were used to estimate the size of an overall treatment effect.

Since then, a further nine randomised controlled trials have been published and commercial garlic preparations are now more widely available. On some of the dried powder preparations contain a standardised amount of allicin [7].

Garlic supplements may have an important role to play in the treatment of hypercholesterolaemia. At least 25% of men and women aged 25–59 years have total cholesterol concentrations exceeding 6.4 mmol/l [8], which is associated with a markedly increased relative risk of premature ischaemic heart disease [9]. Since garlic products are quite acceptable to the public it is important to establish their efficacy. It was therefore decided to undertake a meta-analysis to combine the evidence that now exists. We had three *a priori* hypotheses:

- garlic acts as a lipid-lowering agent in human subjects, reducing total cholesterol and serum triglyceride whilst elevating high density lipoprotein (HDL);

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- there is a dose-response relationship between the amount of garlic consumed and the degree to which blood levels are reduced; and
- the magnitude of the lipid lowering effect observed with dried garlic preparations is greater than with non-powder preparations.

Method

Identification of pertinent studies

A computerised literature search was conducted with DataStar on the MEDLINE database, using the terms (i) 'GARLIC' and (ii) 'LIPIDS' or 'BLOOD PRESSURE' in combination, to identify all published trials involving garlic between 1966 and July 1992. A search of the electronic ALTERNATIVE MEDICINE database, maintained by the British Library of Medicine, was also undertaken using the term 'GARLIC'. In addition, published reviews, reference lists from clinical trials, and conference abstracts were examined. To identify unpublished studies, letters were sent to manufacturers of garlic preparations and authors of published reports using the compound.

For inclusion in the meta-analysis, studies had to meet two methodological criteria. First, there had to be at least two treatment groups and, secondly, allocation to the groups must have been by formal randomisation. Studies which used historical controls were excluded. In addition, since the review was confined to the medium and long-term effects of garlic on serum lipids, trials of less than four weeks or which did not include measurement of serum lipids were also excluded.

Data extraction

Data were independently extracted from the published reports by both authors and disagreements resolved by discussion. For each trial, the following were documented:

- a country of origin;
- b study population;
- c number of subjects;
- d baseline cholesterol level;
- e type of garlic preparation (including whether or not the allicin component was standardised) and dose regimen used;
- f nature of the control group;
- g method of allocation;
- h extent of blinding;
- i parallel group versus cross-over design;
- j duration of treatment;
- k method of analysis;
- l laboratory method used to measure lipid levels;
- m withdrawals; and
- n adverse effects.

Reports which appeared only in non-English language journals were examined with the assistance of a translator.

In trials where either the methods were unclear or the results not expressed in a form which allowed extraction of the necessary key data, the individual investigators were asked by letter for the required information. Nine of the 13 investigators responded to our request, but only three could provide the data requested.

Quality assessment

The methodological quality of the studies included in the review was assessed using a simplified scheme described by Chalmers [10]. This involves assessing three dimensions of trial methodology that are important potential sources of bias:

- the quality of the random allocation (ie control of selection bias at entry);
- whether the primary analysis included every person entered into the randomised cohorts (ie control of selection bias after entry); and
- whether the assessors of outcome(s) were unaware of the group to which individuals in the trial had been assigned (ie control of bias in assessing outcome(s)).

A three-point rating scale was used for each of the three dimensions (ranging from a score of 3 if the effort to control potential bias had been maximal, through to 1 if there had been little or no such effort). In two studies where the methods used to control these sources of bias were unclear the investigator was asked for clarification [11,12].

Data analysis

The effect of garlic on serum cholesterol, serum triglyceride, and serum HDL-cholesterol was assessed independently. It was measured as the difference (in mmol/l) between the mean change in serum lipid values (baseline-final value) in the two groups. The variance of this difference should be calculated using the paired baseline and final lipid measurements for each individual, but only two trials [13,14] presented sufficient information to do this.

For the remaining trials a conservative approach was adopted: the variance of the difference between means was calculated assuming that the baseline and final lipid measurements were unpaired.

A standard error (se) of the size of the effect ($x_1 - x_0$) was calculated for each trial independently using the following formula.

$$se(x_1 - x_0) = \frac{sd_1}{n_1} + \frac{sd_0}{n_0}$$

where x_1 and x_0 are the mean changes in the treatment and control groups, respectively, sd_1 and sd_0 the standard deviations, and n_1 and n_0 the number of subjects in the same groups.

In the results section, a negative treatment effect

Table 1. Summary of randomised controlled trials not included in meta-analysis

Author Year [Ref.]	Country	Participants	No. of subjects	Type of garlic	Duration weeks	Results	Reason for exclusion
Lutomski 1984 [17]	Poland	CHD	82	Oil	12	CHOL: ↔ TRIG: ↔	Insufficient data
Ernst 1985 [11]	Poland	HL	10	Dried (S)	4	CHOL: ↓↓ TRIG: ↓↓ HDL: ↓	Method of allocation unclear Baseline results not available
Luley 1986 [12]	Germany	HL	31	Powder	6	CHOL: ↔ TRIG: ↓ HDL: ↑	Insufficient data
Rotzsch 1993 [18]	Germany	HL	24	Dried (S)	6	TRIG: ↓↓*	Total serum cholesterol not measured

CHD = subjects with pre-existing coronary heart disease; CHOL = cholesterol; HDL = HDL cholesterol; HL = hyperlipidaemic subjects; (S) = standardised allicin content; TRIG = triglyceride; ↓ = nonsignificant decrease; ↓↓ = statistically significant decrease; ↑ = increase; ↔ = unchanged

* Changes seen in response to a fat-rich diet.

indicates a lowering of serum lipids with active intervention. Where results were reported in mg/dl, these were converted to mmol/l. For the two cross-over trials, only data from the first phase of the study were included in the analysis in order to avoid problems of carry-over effects or treatment-period interactions. In all analyses we endeavoured to use data based on all cases allocated, rather than limit to those which complied with the randomised treatment regime. This was possible in 11 of the trials.

A technique described by Bracken [15] was used to pool the effect sizes from individual studies. This is based on a fixed-effect model which assumes that the pooled effect size reflects the typical effect only of trials entered in the analysis. The pooled effect size is a weighted average of individual effects, with weights inversely proportional to the variance of each individual effect. Ninety-five per cent confidence intervals were calculated for the pooled effect size. Tests of heterogeneity were performed using the Mantel-Haenszel method [16].

Results

Description of trials identified

A total of 25 randomised controlled trials were identified which included an examination of the effect of garlic on serum lipid levels (including one trial 'in press' and one published only as a letter to the editor [11]). In five trials the follow-up was less than a month, and in three the data were insufficient to quantify the treatment effect in a form suitable for comparison with the other studies [11,12,17]. These trials were excluded when attempts to obtain this

information from the investigators and the manufacturers proved unsuccessful (Table 1). The other excluded trial measured the effect of garlic therapy on HDL₂-cholesterol and triglycerides but not on total cholesterol [18]. The remaining 16 trials [13,14,19-32] (which included data from 952 subjects) were all eligible for inclusion. Their methodological characteristics are summarised in Table 2.

Fourteen trials used a parallel group design, and the remaining two were cross-over studies [20,25]. Two trials were conducted in an open-label fashion [13,21]. Further two were single-blind [19,23] and all the remainder were double-blind. Thirteen were placebo controlled. One trial compared raw garlic added to normal diet against a control group who were instructed to avoid garlic in their normal diet [21]. Agents used as the control group in the other two trials were bezafibrate [30], and a combination of a diuretic and reserpine, respectively [23]. All but one [13] of the trials were conducted in both men and women using a broad range of clinical entry criteria. None of the studies involving non-powder garlic preparations was conducted specifically in hyperlipidaemic patients. In contrast, five of the trials involving garlic powder preparations included patients on the basis of the lipid levels [25,27,28,30,32]. Only three trials required participants to have sustained elevation of serum lipids after a run-in phase [20,27,32]. In two trials this lasted only 14 days [27,32]; in the trial comparing garlic against bezafibrate [30], the run-in phase was 8 weeks, during which time patients were required to take placebo tablets (single-blind).

The total daily dose of garlic ranged from 600-900 mg of dried garlic powder (in the powder preparations), equivalent to 1.8-2.7 g of fresh garlic per day

Table 2. Summary of trials included in meta-analysis

Author Year [Ref.]	Country	Participants	Type of garlic	Dose per day	Control	Design	Blinding	No. of subjects	Duration	Baseline cholesterol (mmol/l)	Analysis
<i>Non-powder garlic preparations</i>											
Bhushai 1979 [13]	India	H	Fresh	10 g	N	PG	O	25	2M	6.19	NS
Bordia 1981 [19]	India	CHD	Oil	0.25 mg/kg	P	PG	S	68	10M	5.45	ORT
Barrie 1987 [20]	USA	H	Oil	18 mg	P	CO	D	20	4W	NS	NS
Lau 1987 [14]	USA	H	Extract	NS	P	PG	D	32	6M	7.92	ORT
Godkari 1991 [21]	India	H	Fresh	10 g	N	PG	O	60	2M	5.53	NS
<i>Garlic powder preparations</i>											
Sitprija 1987 [22]	Thailand	DIA	Spray	700 mg	P	PG	D	33	1M	4.72	ORT
Kandziora 1988 [23]	Germany	HTN	Dried (S)	600 mg	RD	PG	S	40	12W	7.21	ITT
Kandziora 1988 [24]	Germany	HTN	Dried (S)	600 mg	P	PG	D	40	12W	7.59	ITT
Pleengadthya 1988 [25]	Thailand	H	Spray	700 mg	P	CO	D	30	2M	7.32	NS
Auer 1990 [26]	Germany	HTN	Dried (S)	600 mg	P	PG	D	47	12W	NS	NS
Mader 1990 [27]	Germany	HL	Dried (S)	800 mg	P	PG	D	261	4M	6.84	ORT
Vorberg 1990 [28]	Germany	HL	Dried (S)	900 mg	P	PG	D	40	16W	NS	NS
Kiesewetter 1991 [29]	Germany	SA	Dried (S)	800 mg	P	PG	D	60	4W	NS	NS
Holzgartner 1992 [30]	Germany	HL	Dried (S)	900 mg	B	PG	D	94	12W	7.39	ITT
Santos 1993 [31]	UK	NS	Dried (S)	900 mg	P	PG	D	60	6M	6.98	ORT
Lau 1993 [32]	USA	HL	Dried (S)	900 mg	P	PG	D	42	12M	7.00	NS

B = bezafibrate, CHD = subjects with pre-existing coronary heart disease, CO = crossover study, D = double blind, DIA = diabetic subjects, H = healthy subjects, HL = hyperlipidaemic subjects, HTN = hypertensive subjects, ITT = intention-to-treat analysis, M = months, N = no garlic control, NS = not stated, O = open label, ORT = on randomised treatment, P = placebo, PG = parallel group study, RD = reserpine/diuretic combination, S = single blind, SA = subjects with increased spontaneous aggregation, W = weeks

In the non-powder preparations the daily dose ranged from 10 g of raw garlic to 18 mg of garlic oil. The median duration of therapy was 12 weeks (range four weeks to 10 months).

The quality assessment of the trials was generally poor (Fig 1), with the notable exception of the trial comparing garlic powder and bezafibrate which scored the maximum number of points for each criterion [30]. Only one other trial report provided any information on the techniques used to achieve effective randomisation [27], and only three formally stated that an intention-to-treat analysis had been used [23,24,30]. In six reports the analysis was confined to those patients receiving randomised therapy at the conclusion of the treatment period. In the remainder it was not possible to confirm from the report which type of analysis had been used. It is difficult to disguise garlic capsules or tablets because of their odour. Although several studies attempted to do so, it is unclear from the data whether this was effective. None of the trials referred to any attempts to assess compliance with therapy. Although the lack of blinding to the medication on trial may produce problems in comparing rates of adverse effects, it is less likely to affect interpretation of the serum lipid levels based on labora-

tory measurements. In each case, a standardised procedure was used which had been appropriately referenced.

Effects on total cholesterol

The mean difference in reduction of total cholesterol between garlic-treated subjects and those receiving placebo (or avoiding garlic in their diet) was -0.77 mmol/l (95% CI: -0.65 , -0.89 mmol/l). This effect was significantly greater among subjects receiving non-powder garlic preparations (-0.99 mmol/l; 95% CI: -0.83 , -1.16 mmol/l) than those receiving garlic powder preparations (-0.51 mmol/l; 95% CI: -0.33 , -0.69 mmol/l) (Fig 2); however, the non-powder preparations showed significant heterogeneity (chi-square: 21.87; 4 df $p = 0.00021$). All further analyses were therefore confined to the powder preparations only.

The effect of increasing dosages of garlic amongst these powder preparations did not produce any clinically or statistically significant difference in the effect across the dose range of 600–900 mg of dried garlic powder per day.

The effect of garlic therapy on serum cholesterol progressively increased between months one and three

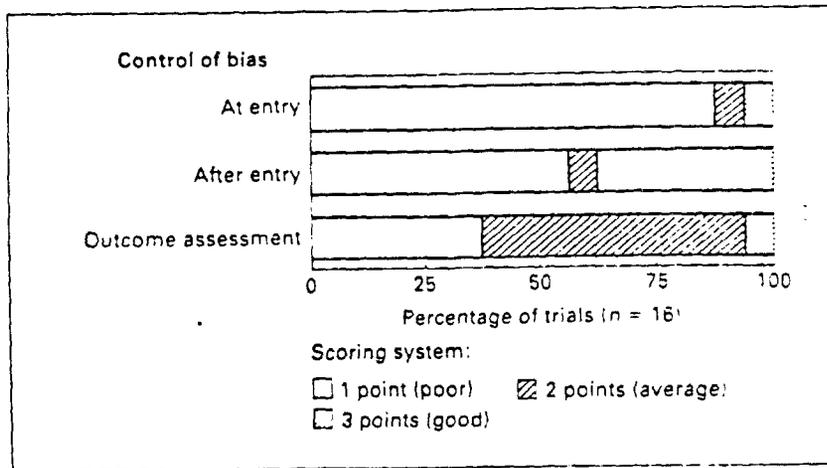


Fig 1. Overall methodological quality of trials included in the meta-analysis (n = 16), based on an assessment of control of selection bias at entry and after entry, and control of assessment bias. Percentage of trials scoring 3 points (full credit), 2 points (some credit), and 1 point (no credit). Results for individual studies are shown in Table 1.

(Fig 3). The reduction in serum cholesterol at six months was less than that seen at three months, but these data came from a single trial [31] and the confidence intervals were wide.

Effect on triglycerides and high density lipoprotein cholesterol

Eight trials (all garlic powder preparations) contributed data to the comparison of the effects of garlic against placebo on serum triglyceride levels [24-28, 31,32]. Garlic therapy reduces the serum triglyceride level by 0.31 mmol/l compared with placebo (95% CI: -0.14, -0.49), a reduction of 13%. Treatment with garlic powder results [23,25,31,32] in a small, insignificant reduction in HDL-cholesterol levels (-0.04 mmol/l, 95% CI: -0.11, 0.03 mmol/l). Only one trial has been reported so far which compares the effect of garlic therapy against a known lipid lowering agent, bezafibrate. Although both agents were effective, they did not differ significantly.

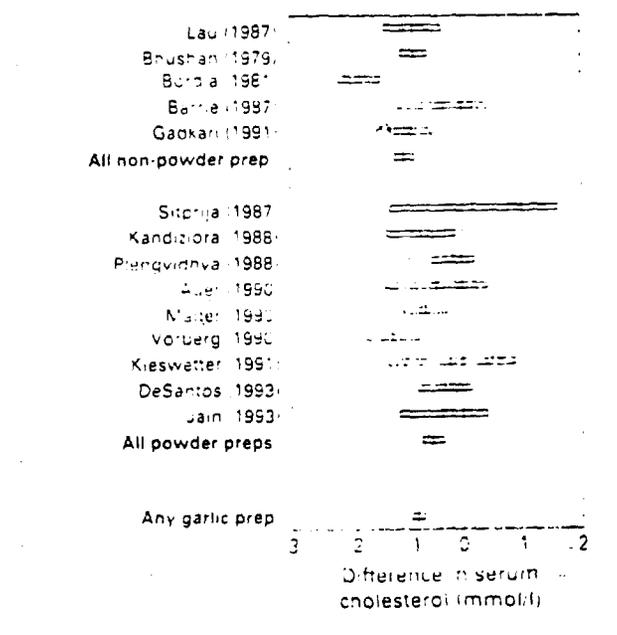
Adverse effects

Seven of the trials mentioned that adverse effects were not significantly increased as a result of garlic therapy, but only three documented these in detail. Among the studies using the dried garlic powder preparation, which is reported to be odour-controlled, only four kept records documenting the incidence of this effect [26,27,31,32]. In the largest of these trials [27], after 16 weeks therapy, 16% of subjects taking the active preparation complained of a garlic smell compared with 5% on placebo. When the results from the other trials were also included, the overall likelihood of experiencing odour when using a garlic preparation was nearly four times that with placebo (3.76, 95% CI: 1.82, 7.80). None of the studies reported any significant increase in other side-effects arising from garlic therapy.

Discussion

Garlic, in powder or non-powder form, can significantly lower serum lipid levels over a 1-3 months period. Serum cholesterol falls 8% with dried powder preparations and 15% with non-powder preparations, although the latter reduction must be interpreted with

Fig 2. Effect size (in mmol/l) of garlic therapy versus placebo (or avoidance of garlic) on total serum cholesterol for individual trials and overall for non-powder preparations, garlic powder preparations, and any preparation. Bars indicate 95% confidence interval. Tests for heterogeneity among sizes of treatment effects: (i) in non-powder preparations, chi-square 21.87; 4 df, p = 0.00021; (ii) garlic powder preparations, chi-square 15.36; 8 df, NS.



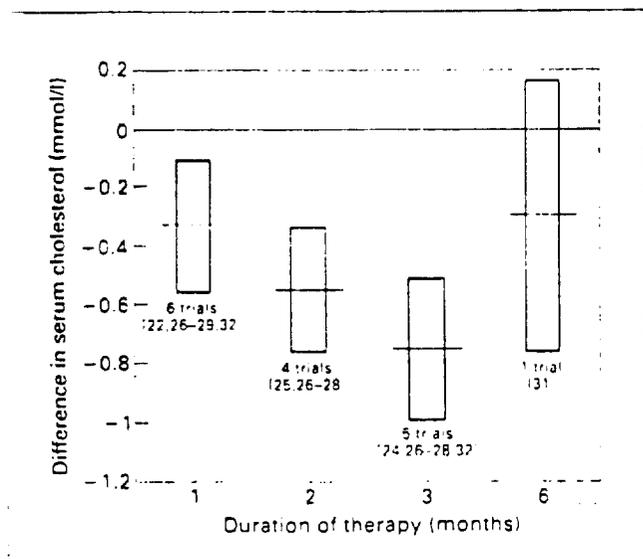


Fig 3. Summary effect size (in mmol/l) of garlic powders versus placebo on total serum cholesterol with 1, 2, 3 and 6 months' duration of therapy. Bars indicate 95% confidence interval. References to trials included are in square brackets.

caution, given the heterogeneity associated with the currently available trials. Serum triglyceride levels also drop significantly, whilst HDL-cholesterol is essentially unchanged. There is insufficient information to comment on the use of non-powder preparations in reducing other lipid parameters.

Amongst the garlic powder preparations these effects appear to be similar across the daily dose range of 600–900 mg. It is not possible to give an exact equivalent dose of fresh garlic, since there is considerable variation in clove size and allicin content depending on where the product is grown. However, 600 mg approximately equates to one medium size clove of fresh Chinese garlic per day (P. J. Josling, personal communication). Adverse effects, including odour, appear to be relatively uncommon with the standardised powder preparations, particularly in lower doses. However, the threefold increased likelihood of a garlic odour with the 'odour-controlled' dried garlic tablets may be socially unacceptable to some people.

Before generalising from the results of this meta-analysis to clinical practice, several important limitations need to be highlighted. The meta-analysis was based on published reports rather than on individual patient data (with the exception of two trials). A recent report suggests that such an approach may be misleading, because of problems associated with publication (i.e. patient exclusion, and length of patient follow-up) [33]. Although we tried to meet these concerns in part by approaching investigators to obtain additional unpublished data to clarify areas of uncertainty, the generally enthusiastic response of these investigators

was not matched with making the necessary data available. It is possible that there are also some unpublished trials that may show less favourable results which have not been identified despite systematic efforts.

The studies involving garlic which were identified have been largely restricted to Thailand, Germany and the USA. The total patient experience in randomised trials (1,365 individuals) is still quite small. Although uncontrolled studies suggest that dried garlic powder preparations are generally well tolerated with a low incidence of adverse effects, this needs to be confirmed in larger randomised trials. In any future trials the failure generally to incorporate an adequate run-in phase, which includes the use of dietary therapy, needs to be addressed, as also does the failure to assess the subjects' compliance with the treatment protocol.

Unfortunately, five of the trials were not analysed on an intention-to-treat basis because of lack of available data. The treatment effect may have been overestimated in these studies. On the other hand, the calculation of the standard deviation for the change in serum lipid values (baseline to final value) in both the active treatment and the control groups assumed a non-paired analysis in most cases, which would tend to overestimate the true standard deviation and therefore widen the confidence interval around the treatment effect.

The direction of the findings is supported by the other randomised controlled trials of shorter duration and those with insufficient data to be included in the formal pooling of the overall treatment effect (see Table 1). Failure to include these studies, particularly those with insufficient data, is therefore unlikely to have materially affected these results.

The similar effect on lipid levels of dried garlic powder and of bezafibrate is promising. Data from systematic reviews of the magnitude of lipid lowering with other non-pharmacological measures, such as oat products [34] and dietary advice [34], suggest much more modest reductions in total serum cholesterol. A step 1 lipid lowering diet reduced cholesterol by only 0–4% over a period of six months to six years [35]. Only when diets more intensive than step 2 were used was serum cholesterol reduced by 13–15% in various population sub-groups. Further trials are now required comparing dried powder garlic preparations with existing hypolipidaemic drugs after a period of appropriate dietary intervention.

Garlic is not a licensed medication and there is not enough evidence to recommend garlic therapy as an effective lipid lowering agent for routine clinical use. However, there is also no evidence to suggest it is harmful. The currently available data support the likelihood of garlic therapy being beneficial, at least over a few months. Resolving this situation will require further trials that avoid the methodological problems of earlier studies and, in particular, last long enough and have adequate statistical power to detect whether any clear-cut benefits arise from the use of garlic.

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EXHIBIT X

Parenteral Zinc Supplementation in Adult Humans during the Acute Phase Response Increases the Febrile Response

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ABSTRACT The acute phase response (APR) that follows injury or infection is characterized by a decrease in serum zinc concentrations, which we hypothesized benefits the host. Additionally, we proposed that preventing this decline by supplementing zinc would result in an exaggerated APR as indicated by elevated temperatures, increased serum cytokine concentrations, interleukin 6 and the acute phase protein (ceruloplasmin). A prospective, randomized, double-blinded, clinical trial was conducted. Patients on home parenteral nutrition with a diagnosis of catheter sepsis and patients with a diagnosis of pancreatitis, also on total parenteral nutrition (TPN), were recruited for the study. Following enrollment, block randomization was used to assign patients to receive 0 mg ($n = 23$) or 30 mg ($n = 21$) of zinc per day for the first 3 d of TPN. Blood samples for measurement of serum zinc, copper, ceruloplasmin and interleukin-6 were obtained upon enrollment and on d 1 through 3 of TPN. The highest temperatures reported on these days in the medical record were also recorded. Repeated measures ANOVA was used to determine differences in the primary outcome variables over time. No significant differences between groups were observed in serum interleukin-6 or ceruloplasmin concentrations. A significantly higher ($P = 0.035$) temperature was observed in the zinc-supplemented group compared with the control group on d 3 of parenteral nutrition. We conclude that parenteral zinc supplementation in patients experiencing a mild APR resulted in an exaggerated APR as evidenced by a significantly higher febrile response. *J. Nutr.* 127: 70-74, 1997.

KEY WORDS: acute phase response • zinc • humans • parenteral nutrition • sepsis

The acute phase response (APR)⁵ is a predictable, stereotyped metabolic response to infection and injury. It is stimulus nonspecific and is characterized by leukocytosis, fever, increased synthesis of acute phase plasma proteins, increased plasma copper concentrations, increased resting energy expenditure and depressed plasma iron and zinc concentrations (Beisel 1977). This response is mediated by a number of cytokines, including tumor necrosis factor, interleukin 1 (IL-1) and interleukin 6 (IL-6). Many of these cytokine-mediated alterations, including fever, hypoferrremia, leukocytosis and elaboration of acute phase proteins, beneficial to the host.

Zinc is involved in the elaboration of several cytokines.

The synthesis of interferon (Reardon and Lucus 1987) and leukocyte migration inhibitory factor are zinc dependent (Bendtsen 1980, Bendtsen and Mayland 1982). Zinc influences in vitro human leukocyte production of the cytokines tumor necrosis factor, IL-1 and IL-6 (Scuderi 1990). The regulation of leukocyte cytokine production by zinc suggests that the trace metal may play an important role in controlling the APR. During the APR, serum concentrations of zinc decline 10 to 69% because of hepatic sequestration by metallothionein (Schroeder and Cousins 1990). The decline in plasma zinc may serve as an important regulator of leukocyte cytokine production and ultimately influence or control the APR.

Despite the lack of knowledge concerning zinc sequestration during the APR, the importance of zinc for wound healing and in immunity has prompted some clinicians to routinely supplement parenteral solutions with additional zinc for catabolic, critically ill patients (Freund 1986, Weinsier and Morgan 1993). Current guidelines for parenteral trace elements recommend supplementing the standard daily dose of zinc (2 mg elemental zinc) with 2-4 mg ZnSO₄/d for catabolic patients (Shils et al. 1979). If the sequestration of zinc represents a host mechanism to control leukocyte cytokine production, prevention of the decline in serum zinc by use of supplemental parenteral zinc could be detrimental to the host.

¹ Presented at The American Society of Parenteral and Enteral Nutrition Clinical Congress January 16, 1996, Washington, DC. Braunschweig, C. L., Sowers, M. F., Kovacevich, D. S. & August, D. A. (1996) The metabolic role of zinc in the acute phase response. *J. Paren. Enteral Nutr.* 20: 30S (abs.).

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³ The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

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⁵ Abbreviations used: APR, acute phase response; IL-1, interleukin 1; IL-6, interleukin 6; TPN, total parenteral nutrition.

TABLE 2

Baseline characteristics of study groups following randomization for supplemental parenteral zinc¹

	Unsupplemented	Zinc supplemented	P value
Age, y	44 ± 3 (range 20-80)	42 ± 3 (range 27-74)	0.76
Sex			0.36
Male, n	8	9	
Female, n	15	12	
Disease status			0.35
Pancreatitis	21	17	
Catheter Sepsis	2	4	
Weight, kg	61.90 ± 3.77	70.00 ± 4.10	0.18
Interleukin-b, ng/L	84.4 ± 59.0	23.2 ± 4.6	0.11
Temperature, °C	37.23 ± 0.12	37.38 ± 0.18	0.59
Ceruloplasmin, U/L	130.0 ± 10	150.0 ± 10.0	0.29
Serum zinc, μmol/L	11.32 ± 0.76	14.38 ± 1.68	0.30
Serum copper, μmol/L	18.38 ± 1.41	19.62 ± 0.94	0.29

¹ Values are means ± SEM, n = 23 (un-supplemented), n = 21 (zinc supplemented). Originally 22 participants were randomized to each group; analysis it was discovered that one individual that had been randomized to receive zinc did not have zinc added to the TPN solution.

four time intervals were as follows: baseline, P = 0.48; d 1 TPN, P = 0.06; d 2 TPN, P = 0.25; d 3 TPN, P = 0.01. Temperatures of participants in the zinc-supplemented group became significantly more elevated over the 3 d of zinc supplementation during the APR than did temperatures of the unsupplemented group. No significant differences between study groups were observed for serum IL-6, ceruloplasmin or copper concentration (data not shown).

A subgroup analysis was undertaken that removed from analysis those participants with serum zinc concentrations greater than normal (serum zinc ≥ 19.98 μmol/L). This was done to examine the possibility that the amount of zinc administered resulted in serum zinc concentrations that exceeded normal concentrations and in turn induced an exaggerated APR. Despite the loss of power that occurred with the removal of seven participants (33% of the original supplemented group), the differences in temperature across the time intervals remained significant (P = 0.036, Table 4). The individual P values for differences between the two groups at the four time intervals were as follows: P = 0.90 at baseline, P = 0.08 on d 1 of TPN, P = 0.11 on d 2 of TPN, P = 0.029 on d 3 of TPN.

As stated, IL-6 concentrations were not significantly different between the study groups. To determine whether an associ-

ation between IL-6 concentrations and change in temperature existed, a correlation analysis was conducted. The correlation between fever and plasma IL-6 activity for all participants was r = 0.55 (P = 0.0002) on d 3 of TPN. When separated by treatment groups, the correlations between temperature and IL-6 concentrations were r = 0.46 (P = 0.04) for supplemented patients and r = 0.57 (P = 0.007) for those that did not receive zinc on d 3 of the study.

DISCUSSION

Participants supplemented with 30 mg zinc/d for the 3 d of TPN experienced significantly higher fevers than participants receiving no zinc. To our knowledge, this is the first report in a human population demonstrating that supplemental parenteral zinc, administered during a mild APR, influenced a febrile response. Temperatures in the supplemented group were higher on d 1 of zinc administration (P = 0.003) and retained this greater elevation throughout the study (P = 0.035). Temperatures of patients receiving zinc supplementation remained significantly greater than those of the control even when patients with initially elevated serum zinc concentrations were excluded from the analysis.

Our findings of a more pronounced febrile response

TABLE 3

Serum and urine zinc concentrations in controls and zinc supplemented groups receiving total parenteral nutrition (TPN) during the acute phase response¹

Measurement	Baseline	Day 1 TPN	Day 2 TPN	Day 3 TPN
Serum zinc, μmol/L				
Control	11.32 ± 0.76 (21)	11.47 ± 0.77 (20)	11.02 ± 0.77 (22)	11.48 ± 0.76 (21)
Zinc supplemented	14.38 ± 1.68 (19)	18.82 ± 1.84 ^a (20)	20.96 ± 1.84 ^a (20)	21.73 ± 2.00 (19)
Urinary zinc, μmol/L				
Control		9.79 ± 1.99 (22)	8.56 ± 1.53 (22)	6.12 ± 0.76 (22)
Zinc supplemented		19.12 ± 4.28 (20)	17.14 ± 2.60 ^b (20)	18.67 ± 2.00 (19)

¹ Values are means ± SEM, with n in parentheses. Letter superscripts indicate significant differences: ^a Difference between study groups (P = 0.0009, Student's t test); ^b Difference between study groups (P = 0.007, Student's t test); ^c Between-group effect over the 3 day study interval (P = 0.0004, repeated measure ANOVA).

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intravenous trace element supplementation (Snits et al. 1979), which recommends 5 mg zinc/d as a standard dose in TPN patients, with an additional 2-4 mg/d for catabolic patients. This continues to be the standard amount of zinc used in parenteral nutrition solutions today.

There are several reports of alterations in thermoregulation in zinc-deficient animals. Topping et al. (1981) found zinc-deficient rats had lower rectal temperatures than controls. When those animals were exposed to the cold, they were more prone to developing hypothermia than were zinc-adequate animals. More recently, Cossack (1991) reported alterations in the febrile response in zinc-deficient rats following the injection of *E. coli*. The zinc-deficient animals had a significantly lower febrile response 5-8 h after the endotoxin injection than did the pair-fed controls. Of note is the significant ($P < 0.001$) weight loss that occurred in the zinc-deprived rats during the 6-wk study period. At the end of the 6-wk study period average body weights were 142.2 ± 24.2 and 182.8 ± 7.7 g for zinc-deficient rats and pair-fed controls, respectively. Typically, animals fed zinc-deficient diets have lower weight gain than pair-fed controls (O'Dell and Reeves 1989). Many species respond to zinc deficiency with anorexia and cyclic feeding (Clegg et al. 1989). It is thought that the reduced food intake induces muscle catabolism and release of stored muscle zinc into the plasma. This zinc is then available for use by the liver and other tissues for zinc-dependent processes. Whether zinc deficiency per se or general protein energy malnutrition was responsible for the responses observed in Cossack's study cannot be determined.

Alterations in thermoregulation due to zinc deficiency have not been reported in humans. However, it has been known for many years that uncomplicated starvation in humans reduces core temperatures (Golden 1988).

In addition to temperature, serum IL-6 activity was determined as a measure of APR severity. Because of the great variability, no difference in IL-6 concentration was demonstrated between the control and zinc-supplemented groups. Numerous studies have observed associations between severity of illness or inflammatory process with IL-6 concentrations (Damas et al. 1992, Lesser et al. 1991, Nijsten et al. 1987, Viedma et al. 1992). Only Nijsten et al. (1987) reported a significant ($r = 0.61, P < 0.001$) correlation between IL-6 concentrations and body temperatures. In our study, correlations between temperature and IL-6 concentrations were significant in both the supplemented and control groups. To our knowledge, ours is the first study with patients experiencing a mild APR to demonstrate significant correlations between serum IL-6 concentrations and temperatures. Collectively, the significantly higher temperatures in the zinc-supplemented group and the correlation with IL-6 concentrations further corroborate the hypothesis that zinc supplementation during the APR results in a more exaggerated response.

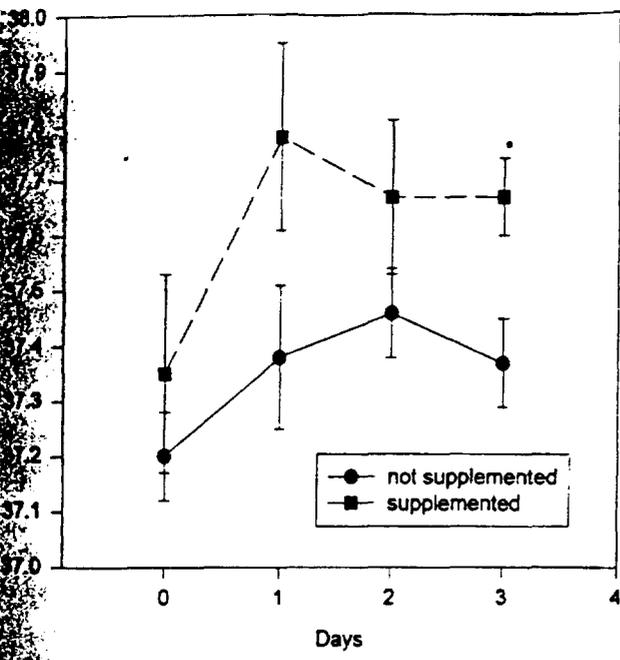


FIGURE 1 Temperature changes in zinc-supplemented vs unsupplemented humans from baseline to d 3 of total parenteral nutrition. Values are means \pm SEM, $n = 23$ (unsupplemented controls) or 21 (zinc supplemented). The value with the asterisk is significantly different from the value for the nonsupplemented group on d 3 of total parenteral nutrition ($P = 0.01$, Student's *t* test) with a between-group effect over the 4-d study interval ($P = 0.035$, repeated measures ANOVA).

Humans supplemented with zinc during an APR are consistent with the very limited animal literature. Alterations in the febrile response in rabbits following zinc administration during the APR were reported in 1978 by Mapes et al. (1978). The animals were supplemented with intravenous zinc chloride (1.6 mg/kg body wt) 1 h before intraperitoneal IL-1 infusions. Control rabbits that did not receive zinc before the IL-1 challenge had a monophasic fever that peaked 20 min after IL-1 administration and returned to basal levels within 70-80 min. When zinc was administered 1 h before the IL-1 challenge, fevers were approximately 40% higher than those obtained in control rabbits. The fevers in the zinc-treated animals lasted for 4-6 h before returning to basal levels. When a minimal amount of zinc chloride (0.04 mg/kg body wt) was given simultaneously with the IL-1, fevers of prolonged duration occurred, but the level of the fever did not differ from that in control animals. Results of this investigation preceded the panel of experts guidelines for

TABLE 4

Temperatures from baseline to d 3 total parenteral nutrition (TPN) in a subgroup of zinc-supplemented and unsupplemented patients during the acute phase response^{1,2}

Group	Baseline	Day 1 TPN	Day 2 TPN	Day 3 TPN
Controls	37.23 \pm .12	37.40 \pm 0.14	37.49 \pm 0.12	37.40 \pm 0.11
Zinc supplemented	37.25 \pm 0.17	37.85 \pm 0.20	37.80 \pm 0.15	37.82 \pm 0.14ab

¹ Includes only those individuals in the zinc-supplemented group that did not have greater than normal serum zinc concentrations.
² Values are means \pm SEM, $n = 23$ (unsupplemented controls) or 15 (supplemented). Letter superscripts indicate significant differences: * Difference between study groups on d 3 of TPN ($P = 0.029$, Student's *t* test); b Between-group effect over the 4-d study interval ($P = 0.036$, repeated measures ANOVA).

Zinc supplementation induces alterations in copper status potentially by a competitive interaction between zinc and copper for intestinal absorption. Zinc intake stimulates enterocyte metallothionein synthesis (Richards and Cousins 1975). Metallothionein sequesters copper much more avidly than zinc, making the copper unavailable for transfer and reducing copper absorption. Oral zinc supplements as low as 18.5 mg/d have been reported to induce copper imbalances (Festa et al. 1985). During the APR, endotoxin, cytokines and glucocorticoid hormones have all been demonstrated to increase metallothionein mRNA in the thymus, liver and bone marrow (Hambidge et al. 1986). This induction is associated with a redistribution of zinc (Cousins 1985, Cousins and Leinart 1988). The avid binding of copper by metallothionein, the enhanced production of this protein during zinc administration, and stress may lead to alterations in serum copper concentrations.

The gastrointestinal homeostasis of zinc absorption via induction of metallothionein was avoided in our study through the use of parenterally delivered zinc. Several weeks of oral zinc supplementation are typically required to induce reductions in copper and ceruloplasmin concentrations (Prasad et al. 1978). As would be expected, we did not observe any acute differences in serum copper or ceruloplasmin concentrations with our short-term parenteral zinc supplementation.

The use of zinc supplementation is expanding. In general, zinc is considered to be nontoxic and humans seem to tolerate fairly high intakes. The role of zinc in wound healing and in the immune system and the occurrence of zinc deficiency in patients with malabsorptive disorders, AIDS and diabetes are well established (King and Keen 1994). Oral zinc supplements are routinely recommended by physicians for patients with any of these conditions, presumably to enhance immune function.

The results from our study indicate that zinc supplements given parenterally to patients during a mild APR will result in an exaggerated APR. The effect of oral zinc supplementation on temperature during the APR remains unknown. Hempe et al. (1991) documented in rats that zinc absorption is not impaired during the APR; thus, oral zinc supplements may have the same impact on the APR as we observed. In some situations, such as in treatment of AIDS patients, the exaggerated response may prove to be beneficial. In other situations, in which reduced inflammatory response is desired, such as in treatment of patients experiencing a flare-up of Crohn's disease, ulcerative colitis or arthritis, an exaggerated APR may be detrimental. Future studies of these disease are warranted to delineate benefits achieved as well as to avoid any untoward effects that may occur from overzealous supplementation with this nutrient.

The effect of zinc supplementation during a severe APR has not been determined. Patients who may receive supplemental zinc include postoperative, trauma, burn and general intensive care unit patients. Many of the new enteral products that are marketed specifically for these patients contain more than 24 mg zinc, 4180 kJ. It is not uncommon for these patients to require 12,540 kJ/d and thus receive more than 72 mg zinc/d. Further clarification of the role of zinc sequestration in severe APR is needed prior to empiric supplementation in this population.

We have demonstrated in a prospective, randomized, double-blinded clinical trial that supplementation of 30 mg of parenteral zinc for 3 d in patients experiencing a mild APR resulted in a significantly higher febrile response. This level of zinc supplementation did not result in differences in serum copper and ceruloplasmin concentrations between study groups. We also found temperatures to be significantly correlated with serum IL-6 concentrations.

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Exhibit Y

Michael L. Macknin, MD, et.al.
Cleveland Clinic Foundation
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these studies showed that zinc had a beneficial effect (4-6) and four did not (7-10). In the studies that examined virus shedding (5, 7), zinc treatment had no effect on this shedding.

We designed a study similar to that of Godfrey and colleagues (6) and used the symptom score developed by these researchers. We emphasized starting treatment within 24 hours after the onset of symptoms, because Godfrey and colleagues found that early treatment was most effective. We used zinc gluconate lozenges, which appeared to be well tolerated and had the best bioavailability profile in previous studies. Other studies (4-7, 9) used lozenges containing 23 mg of zinc. To improve palatability, lozenges in our study contained 13.3 mg of zinc. This provided a local concentration of zinc ions of about 4.4 mmol/L, an amount greater than that necessary to suppress rhinovirus (0.1 mmol/L) (11, 12). The placebo lozenge contained 5% calcium lactate so that it had a medicinal taste similar to that of the zinc gluconate lozenge.

Ours was a pragmatic study designed to determine the efficacy of zinc gluconate lozenges in reducing clinical symptom scores under conditions that reflected usual medical care for the common cold (13, 14). We did not seek to define the mechanism of any zinc effect. Although virus cultures or serologic tests might have been desirable, we decided not to do these tests because they are almost never done in the course of standard care.

Methods

Study Design

We determined that a 50% reduction in the duration of symptoms (in days) would represent a significant clinical effect. A previous study of zinc gluconate given during the first day of cold symptoms suggested that the duration of illness was reduced from approximately 8 days to 4 days after treatment began (6). Our previous research on patients with colds who were seen at the Cleveland Clinic suggested that the mean duration (\pm SD) of cold symptoms was approximately 7 ± 6 days (15, 16). We chose a sample size of 100 patients so that we could detect a difference in the mean number of days of symptoms from 8 days in the placebo group to 4 days in the zinc group with a standard deviation of 6 days, a two-sided *P* value of 0.05, and an approximate power of 90%.

Patients were recruited from among the Cleveland Clinic staff through announcements in internal Clinic publications and by word of mouth. One hundred volunteers were enrolled between 3 October and 4 November 1994. All patients who completed

the study as specified by the protocol were enrolled in a raffle for one of two prizes: dinner for two or a trip for two to the Bahamas. The Institutional Review Board at the Cleveland Clinic Foundation approved the study, and participants gave informed consent at the time of enrollment. Participants were informed of the placebo-controlled, double-blind nature of the study.

Patients who volunteered for the study were enrolled only if they had had cold symptoms for 24 hours or less. Patients must have had at least two of the following 10 symptoms: cough, headache, hoarseness, muscle ache, nasal drainage, nasal congestion, scratchy throat, sore throat, sneezing, or an oral temperature greater than 37.7°C. Patients were excluded if they were pregnant, had a known immune deficiency, or had had symptoms of the common cold for more than 24 hours.

The zinc gluconate-glycine and placebo lozenges were supplied by the Quigley Corporation of Doylestown, Pennsylvania. The zinc lozenges consisted of a boiled hard-candy base prepared with approximately equal proportions of sucrose and corn syrup, zinc gluconate trihydrate (AKZO Chemie, Amersfoort, the Netherlands), a molar proportion of glycine (aminoacetic acid), and lemon and lime flavoring oils. The mixture was formed into lozenges that weighed 4.4 g and contained 13.3 mg of zinc. Placebo lozenges, also weighing 4.4 g, were prepared from the same flavored hard-candy base and contained 5.0% calcium lactate pentahydrate. Placebo and active lozenges were identical in weight, appearance, flavoring content, and texture. The zinc lozenges, however, were more astringent than the placebo lozenges.

A statistical consultant prepared a computer-generated randomization code and the packages of medication. The packages were identical in appearance except for the randomization numbers. The study medication was distributed by the study nurse, who was masked to treatment assignments. Patients were given 120 lozenges and were asked to dissolve 1 lozenge in their mouths every 2 hours while awake for as long as they had cold symptoms. The study nurse administered the first lozenge to assess initial tolerability. Participants were asked to take no other cold preparations during the study period. Acetaminophen samples and oral digital thermometers were given to the patients at the time of enrollment. All patients were called on the second day of medication use to make sure that they were not developing a more serious illness and to assess the adequacy of the masking through responses to a questionnaire. By assessing the adequacy of the placebo on the second day of treatment rather than only at the end of treatment, we hoped to decrease the likelihood that a rapid cure would help patients

in the zinc group correctly determine that they were receiving the active medication. This questionnaire was also administered at the end of treatment with the addition of questions about the occurrence of specific, previously described side effects of zinc therapy.

Patients returned to the Clinic for the final visit within 1 day of noting that their cold symptoms had resolved. At this visit, they returned unused lozenges so that adherence to the protocol could be checked through lozenge counts, and the study nurse confirmed that cold symptoms had resolved.

Patients were asked to complete a daily log documenting the severity of symptoms and the medications taken throughout the duration of their cold for as long as 18 days. Every day, patients graded each symptom as 0 for none, 1 for mild, 2 for moderate, or 3 for severe. Total symptom scores were calculated by summing the scores of the 10 symptoms for each day. Cold resolution was defined as resolution of all symptoms (a total symptom score of 0) or resolution of all but one mild symptom (a total symptom score of 1).

Statistical Analysis

The time to cold resolution was calculated as the number of days from study entry. Resolution rates were estimated using the Kaplan-Meier method, and resolution profiles were compared between groups using the log-rank test (17, 18). We estimated median resolution times using the method suggested by Lee (19). The effect of treatment on individual symptoms was examined by comparing the number of days with each symptom using the Wilcoxon rank-sum test. For analysis of treatment effect, we combined hoarseness, sore throat, and scratchy throat into a category called "throat symptoms" and nasal drainage and congestion into a category called "nasal symptoms." Plots of individual patient symptoms give the percentage of the baseline total severity score (sum of symptom scores for all patients) by assignment group and study day. When appropriate, we used the Fisher exact test and the chi-square test to analyze associations between the side effects and assigned groups. Patient adherence was examined by comparing the total lozenge counts between the two groups using the Wilcoxon rank-sum test.

These analyses were done using an intention-to-treat framework, regardless of patient adherence (20-22). Before the randomization code was broken, patients who received antibiotic therapy or whose condition was diagnosed by a physician as an illness other than the common cold were considered nonadherent. Patients who wrote their diaries from memory were also considered nonadherent. Patients were considered adherent if they took an average of

Table 1. Demographic Characteristics of 99 Volunteers Receiving Zinc or Placebo Lozenges for Treatment of the Common Cold

Variable	Placebo Group (n = 50)	Zinc Group (n = 49)
Age, y		
Mean \pm SD	37.9 \pm 9.2	37.5 \pm 7.5
Median (minimum-maximum)	37.6 (21.1-69.3)	36.1 (22.3-54.6)
Sex, n (%)		
Men	11 (22)	8 (16)
Women	39 (78)	41 (84)
Race, n (%)		
White	36 (72)	39 (80)
Black	7 (14)	4 (8)
Other	2 (4)	3 (6)
Unknown	5 (10)	3 (6)
Smokers, n (%)	6 (12)	5 (10)
Patients with allergies, n (%)	27 (54)	26 (53)

four or more lozenges per day for the first 4 days of the study (16 lozenges) and if they took no antibiotic agents.

Results

One hundred patients were enrolled in the study; 50 were assigned to the zinc group, and 50 were assigned to the placebo group. All patients were Cleveland Clinic employees older than 18 years of age. One patient in the zinc group withdrew from the study on the first day because she could not tolerate the lozenges; she did not complete the symptom diary. All other patients, as directly observed by the study nurse, indicated that they had good tolerance of the first lozenge. Demographic characteristics of the groups are given in Table 1.

The mean (\pm SD) and median symptom scores at baseline (the first measurement) were 8.6 ± 3.3 and 8 for the entire sample, 9.3 ± 3.6 and 8 for the placebo group, and 7.9 ± 2.8 and 8 for the zinc group. In practice, an increase in score from 8 to 9 entails scoring one symptom one grade higher or developing another mild symptom. Six hours after the study began, the mean symptom scores for the placebo group (9.3 ± 4.2 ; median, 9) and the zinc group (8.7 ± 4.0 ; median, 8) were closer.

The incidence of individual symptoms at baseline was similar in the two groups for all but two symptoms: sneezing (31 of 50 placebo recipients [62%] and 38 of 49 zinc recipients [77.5%]; $P = 0.09$) and sore throat (39 of 50 placebo recipients [78%] and 25 of 49 zinc recipients [51%]; $P = 0.005$). No patients had fever at baseline.

Eight patients (six in the placebo group and two in the zinc group) had colds that did not resolve while they remained in the study. Two of these patients (both were placebo recipients) completed the 18 days of the study, and the remaining six (four

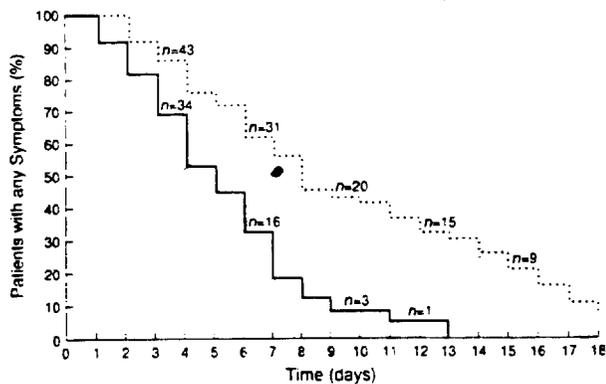


Figure 1. Kaplan-Meier curve for the duration of colds in 99 volunteers. Solid line = zinc group, dotted line = placebo group.

were placebo recipients and two were zinc recipients) dropped out after 7 to 16 days. In addition, one patient recorded his symptoms for 18 days but indicated that his cold resolved on day 19.

We used the Kaplan-Meier method to estimate the percentage of patients whose colds completely resolved (Figure 1) and almost completely resolved on each day of the study. The median time to resolution of all symptoms was 7.6 days in the placebo group and 4.4 days in the zinc group; the median time to resolution of all but one mild symptom (data not shown) was 7.5 days in the placebo group and 3.7 days in the zinc group. The results of the log-rank test and the plot of these distributions indicate that symptoms resolved significantly faster in the zinc group than in the placebo group ($P < 0.001$). This effect was also seen when the end of the cold was defined as almost complete resolution ($P < 0.001$). The study nurse directly observed whether the patients who returned their study forms and unused medication within 1 day of reported resolution of symptoms were free of symptoms.

Seventeen of the 100 patients (10 in the zinc group and 7 in the placebo group) were considered nonadherent. Of these 17, 6 (2 zinc recipients, 1 of whom also took antibiotic agents, and 4 placebo recipients) did not take enough medication for reasons that were not stated; 5 (all zinc recipients) stopped taking the lozenges because of adverse effects (bad taste in 3 patients, sore mouth in 1 patient, and a "lump in back of throat" in 1 patient); 4 (2 zinc recipients, 1 of whom also could not tolerate the taste of the medicine, and 2 placebo recipients) took antibiotic agents; 2 (both zinc recipients) reconstructed their diaries from memory; and 2 (1 zinc recipient and 1 placebo recipient) stopped keeping a record for reasons that were not stated. When data were analyzed after these 17 nonadherent patients were excluded, the study conclusions remained the same. No significant relation was seen between adherence status and group as-

signment ($P > 0.2$). Even when the 17 nonadherent patients were excluded, symptoms in the zinc group still resolved significantly faster according to both definitions of symptom resolution ($P < 0.001$). The median duration of symptoms for the adherent patients in the placebo and zinc groups was 7.2 and 3.9 days, respectively, for complete resolution and 5.7 and 3.4 days, respectively, for near-complete resolution.

Figures 2 and 3 show the percentage of the original symptom score (each day by group assignment) for nasal symptoms and throat symptoms. The zinc group had significantly fewer days with any symptom, nasal symptoms, throat symptoms, coughing, headache, hoarseness, nasal congestion, nasal drainage, and sore throat. The groups did not differ significantly in the resolution of muscle ache, scratchy throat, sneezing, or fever (Table 2).

We calculated the total number of lozenges from counts of returned lozenges and from patient diaries. When we found discrepancies, we used actual lozenge counts. During the entire study, the placebo group took a mean of 49 ± 30 lozenges (median, 42 lozenges) and the zinc group took a mean of 36 ± 22 lozenges (median, 28 lozenges) ($P = 0.03$). The placebo group took an average of 5 ± 2 lozenges per day (median, 5 lozenges per day), whereas the zinc group took an average of 6 ± 2 lozenges per day (median, 5 lozenges per day) ($P = 0.20$). Because their colds lasted longer, the placebo group used significantly more lozenges than the zinc group, but the number of lozenges per day of symptoms did not differ between the two groups.

Use of acetaminophen did not differ significantly between the two groups ($P = 0.10$): the placebo group took a median of 6 acetaminophen tablets, and the zinc group took a median of 4 tablets. Despite instructions to the contrary, 15 patients (10 placebo recipients and 5 zinc recipients) took other cold medications during the study ($P = 0.17$).

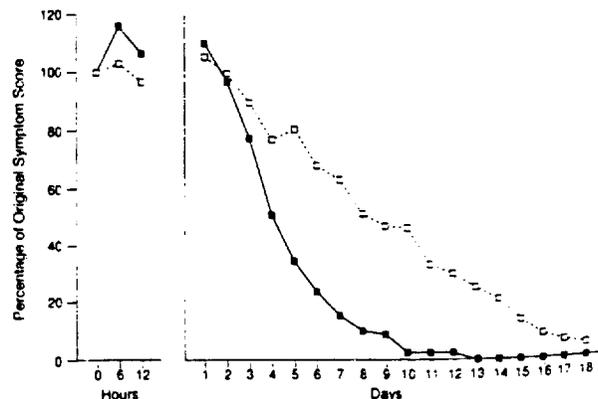


Figure 2. Percentage of the original nasal symptom score each day by treatment group. Nasal symptoms were nasal drainage and nasal congestion. Solid line = zinc group, dotted line = placebo group.

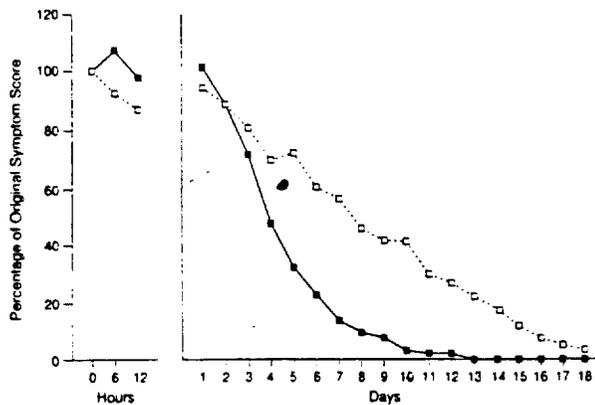


Figure 3. Percentage of original throat symptoms score each day by treatment group. Throat symptoms were hoarseness, sore throat, and scratchy throat. Solid line = zinc group, dotted line = placebo group.

Questions to evaluate the efficacy of masking to group assignment were asked after the first day of treatment and at the end of the study. Patients were asked to guess their assignment from among seven choices: certainly placebo, probably placebo, possibly placebo, do not know, certainly active, probably active, or possibly active. By assigning all guesses that mentioned "placebo" as placebo and all guesses that mentioned "active" as zinc, the following results were obtained. On the initial questionnaire, 50% of the placebo recipients (25 of 50) and 55.2% of the zinc recipients (27 of 49) correctly guessed their study assignment. At the end of the study, 54% of the placebo recipients (27 of 50) and 53.1% of the zinc recipients (20 of 49) correctly guessed their treatment assignment. Sixty-five of the 99 patients (65.7%) maintained their original guess at the end of the study. Because no clear pattern of movement of guesses was seen between the groups, masking appears to have been maintained during the study.

After the first day of treatment, 46% of the placebo recipients and 59% of the zinc recipients said that the study medication had helped alleviate their symptoms ($P = 0.19$). At the end of the study, 44% of the placebo recipients and 59% of the zinc recipients said that the study medication had helped improve the cold symptoms ($P = 0.13$). When the zinc and placebo groups were subdivided into the seven subgroups on the basis of how certain the patients were about their group assignment on the first day of treatment, the mean and median durations of symptoms in the zinc group were always shorter than those in the placebo group.

Thirty-eight of 50 placebo recipients (76%) and 28 of 49 zinc recipients (57%) described the taste of the lozenges as sweet. Patients were also asked to choose other tastes that applied to their medication, including sour, bitter, and salty. Eight placebo re-

cipients (16%) and 12 zinc recipients (25%) reported that the lozenges tasted sour; 6 placebo (12%) and 20 zinc (41%) recipients reported a bitter taste; and 4 placebo (8%) and 4 zinc (8%) recipients reported a salty taste. Many patients reported that the lozenges had an aftertaste. Thirty-four of 50 placebo recipients (68%) and 6 of 49 zinc recipients (12%) reported no aftertaste ($P < 0.001$). Twelve placebo recipients (24%) and 22 zinc recipients (45%) reported a mild aftertaste; 2 placebo (4%) and 17 zinc (35%) recipients reported a moderate aftertaste; and 1 placebo recipient (2%) and 3 zinc (6%) recipients reported a severe aftertaste. Two patients (1 in the placebo group and 1 in the zinc group) did not answer the question.

We ascertained side effects in two ways. During the study, we asked patients to list all of the side effects of their medication. This open-ended question was the only one asked during the study period. Seventeen of 49 zinc recipients reported that no side effects developed with their medication before the conclusion of the study. In these patients, the mean (4.7) and median (4.0) numbers of days until only one mild symptom remained was the same as the number in the 32 patients with identified side effects. The zinc recipients with and without identified side effects also had a similar mean (5.1 days and 5.5 days, respectively) and median (4.5 days and 6.0 days, respectively) time until symptoms completely resolved ($P > 0.2$).

The second method used to determine side effects entailed listing all of the common side effects of zinc and asking patients at the end of the study whether these or other side effects developed while they were taking the study medication (Table 3). As expected, patients described more side effects in

Table 2. Duration of Individual Symptoms of the Common Cold

Symptom	Duration in	Duration in	P value*
	Placebo Group (n = 50)	Zinc Group (n = 49)	
	<i>d</i> [†]		
Nasal symptoms‡	7.0 (4, 13)	4.0 (3, 6)	<0.001
Throat symptoms§	4.0 (3, 9)	3.0 (1, 5)	0.004
Cough	4.5 (1, 10)	2.0 (1, 6)	0.04
Headache	3.0 (1, 5)	2.0 (0, 3)	0.02
Hoarseness	3.0 (0, 8)	2.0 (0, 3)	0.02
Nasal congestion	6.0 (3, 12)	4.0 (2, 6)	0.002
Nasal drainage	7.0 (4, 11)	4.0 (2, 5)	<0.001
Sore throat	3.0 (1, 6)	1.0 (0, 3)	<0.001
Muscle ache	2.0 (1, 5)	1.0 (0, 3)	0.11
Scratchy throat	3.0 (1, 5)	3.0 (1, 4)	0.17
Sneezing	3.0 (1, 5)	2.0 (1, 4)	0.20
Fever	0 (0, 0)	0 (0, 0)	0.15†

* Wilcoxon rank-sum test.

† Values are expressed as the median (25th, 75th percentiles).

‡ Nasal symptoms were nasal drainage and nasal congestion.

§ Throat symptoms were hoarseness, sore throat, and scratchy throat.

¶ By the Fisher exact test.

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Table 3. Side Effects Reported by 99 Volunteers Receiving Zinc or Placebo Lozenges for Treatment of the Common Cold*

Variable	Placebo Group (n = 50)	Zinc Group (n = 49)
	n (%)	
Nausea ($P = 0.02$)†	2 (4.0)	10 (20.4)
Vomiting	1 (2.0)	0
Abdominal pain	1 (2.0)	3 (6.1)
Diarrhea	2 (4.0)	2 (4.1)
Constipation	0	1 (2.0)
Mouth irritation ($P > 0.2$)‡	10 (20.0)	12 (24.5)
Bad taste ($P < 0.001$)‡	15 (30.0)	39 (79.6)
Dizziness	0	0
Headache	0	0
Dry mouth	6 (12.0)	6 (12.2)
Other ($P > 0.2$)*	2 (4.0)	5 (10.2)
Number of side effects ($P < 0.001$)*		
0	19 (38.0)	5 (10.2)
1	26 (52.0)	19 (38.8)
2	2 (4.0)	17 (34.7)
3	3 (6.0)	8 (16.3)

* Statistical testing was done when adequate numbers of patients reported side effects

† By the Fisher exact test.

‡ By the chi-square test.

response to this question than in response to the open-ended question alone. Patients in the zinc group reported more side effects per person (25 zinc recipients and 5 placebo recipients had two or more side effects; $P < 0.001$), significantly more nausea (10 patients compared with 2 patients; $P = 0.02$), and more bad-taste reactions (39 patients compared with 15 patients; $P < 0.001$). The other symptoms described (vomiting, abdominal pain, diarrhea, constipation, mouth irritation, and dry mouth) did not differ significantly between the two groups.

Discussion

The common cold still has no definitive cure. At best, available over-the-counter medications minimally alleviate cold symptoms (23). Our study showed that the time to resolution of all symptoms was significantly shorter in the zinc group. The zinc group had significantly fewer days with coughing, headache, hoarseness, nasal congestion, nasal drainage, and sore throat but had more patients with side effects. The fact that zinc recipients and placebo recipients did not differ significantly in their subjective overall impression of whether the study medication had helped alleviate their cold symptoms is somewhat surprising. However, global assessment by patients may be based largely on subjective estimates of how long a cold "should" last rather than on objective knowledge of the duration; this created much variation in subjective estimates of whether the actual duration of the patients' cold symptoms were or were not "improved" with either treatment.

The results of our study are similar to those of

previous studies that showed a beneficial effect of using zinc for treating the common cold, particularly when zinc is started within the first 24 hours of onset of symptoms. Of the four studies that did not show a beneficial effect, three (7-9) were criticized for using a lozenge formulation that inactivated the zinc (24-26) and one (10) used a possibly ineffective dose of 4.5 mg of zinc per lozenge. Of the three studies that did show a beneficial effect, one (4) reported a strong treatment effect ($P < 0.001$) at 7 days (14% of zinc recipients compared with 54% of placebo recipients had symptoms at 7 days) but also noted a high rate of side effects in the zinc group. This finding caused some investigators to question the validity of the masking and therefore the validity of the study results (27). Al-Nakib and colleagues (5) used zinc gluconate lozenges in persons with experimentally induced colds and found no benefit in giving zinc prophylactically, but they did note a reduction in mean daily clinical scores compared with scores in placebo recipients on days 4 ($P < 0.01$) and 5 ($P < 0.05$) of treatment. The treatment was well tolerated, and the placebo lozenge was not distinguished from the zinc lozenge by taste or appearance.

Godfrey and colleagues (6) compared a nonchelating formulation, zinc gluconate-glycine, which releases 93% of contained zinc in saliva, with a placebo containing highly astringent tannic acid and saccharin. They reported a 26% reduction in the duration of colds when treatment was begun during the second day of symptoms and a 42% reduction (from 9.1 days to 5.3 days) when treatment was begun on the first day of symptoms. Our study was similar to that of Godfrey and colleagues; we used the same symptom score, emphasized starting treatment within 24 hours after onset of symptoms, and used a reduced dose of zinc to improve the palatability of the lozenge.

The mechanisms through which zinc affects the common cold remain to be determined, but several possibilities have been described. Zinc prevents the formation of viral capsid proteins, thereby inhibiting in vitro replication of several viruses, including rhinovirus (11, 12, 28-30). Zinc combines with the carboxyl termini (negatively charged canyons) of rhinovirus coat proteins, which may prevent the virus from combining with the tissue-surface protein (intracellular adhesion molecule type 1) and entering the cell. This process stops further reproduction (31, 32). Extracellular zinc may exert antiviral effects by stabilizing and protecting cell membranes by uncertain means (30, 33-36). In vitro studies have suggested that zinc may induce production of interferon (37). Zinc ions also have human prostaglandin metabolite-inhibiting properties at 0.01 to 0.1 mmol (38), which may also account for the

ability of zinc to help relieve symptoms of the common cold.

Our study has some limitations. First, we did not establish a microbiological diagnosis of the common cold. We relied solely on patients' subjective information and clinical evaluation by the study nurse. We elected not to do microbiological studies of rhinovirus because the expense of such studies is prohibitive, and our goal was only to determine whether zinc helped to relieve cold symptoms. The fact that the study was done early in the influenza season, when no cases of influenza had been reported at the Cleveland Clinic, supports the assumption that most of our patients did have a common cold. Doing the study at a different time of year could have involved different types of viruses, which might have altered the results. The absence of fever at baseline in all patients suggests that certain viruses, such as influenza, parainfluenza, and adenovirus, were unlikely causes of common cold in our patients. Second, the fact that more patients in the zinc group than in the placebo group had sore throats at baseline could suggest that different viruses were responsible for common cold in the two groups. This difference, however, diminished within the first 12 hours of the study. Third, we assessed compliance with the assigned treatment by reviewing patients' diaries and lozenge count. We did not check zinc or calcium blood levels, but it is difficult to predict whether these levels would have been meaningful, given that these elements have several other dietary sources.

The fourth limitation of our study is that our results cannot be applied to immunocompromised or pregnant patients, because neither group was included in our study. Fifth, we did not provide information on the cumulative effect of the repeated use of zinc or explore the possibility of development of resistance. We emphasize that we used only short-term zinc therapy for common colds. Habitual or long-term ingestion of large doses of zinc may be hazardous by causing imbalances in levels of copper (39) and possibly other nutrients. We also avoided zinc dosages greater than 150 mg/d, which have been associated with adverse effects (40). Sixth, although our results indicated clinical improvement when zinc was used to treat the common cold, we do not know the actual mechanism by which this occurred. Finally, if patients had complied with the protocol (one lozenge every 2 hours while awake), they would have taken seven or eight lozenges each day. Zinc lozenges were actually taken about four to eight times daily (median, five lozenges). This may raise concerns about compliance, but the number of lozenges taken appears to have been effective. A recent review (41) and a MEDLINE search showed no evidence that calcium lactate causes adverse ef-

fects in the doses used in our study. Thus, we do not believe that our results reflect an adverse effect of the placebo administration.

In our study, the only statistically significant adverse effects of zinc therapy were bad taste and nausea. Although the incidence of mouth irritation did not differ significantly between the two groups, mouth irritation may still be a clinically significant adverse effect because the placebo may also have been irritating. We assessed the possibility that patients withdrew from the study because of side effects before their colds had completely resolved. We hypothesized that patients who recognized side effects of medication before the end of the study may have decided to violate the protocol and discontinue their medications before they were completely well, either intentionally (because of the perceived unpleasantness of the side effects) or unintentionally (because the side effects masked their cold symptoms). No statistically significant association was seen between the presence or absence of medication side effects recognized before the conclusion of the study and the duration of the patients' illnesses. This is further evidence that patients adhered to the protocol and did not prematurely stop taking their assigned medication because of side effects. Individual patients must decide whether the possible beneficial effects of zinc on their cold symptoms outweigh the possible adverse effects.

Our data suggest that zinc gluconate in the form and dosage tested was helpful in reducing the duration of common cold symptoms. Although we used a lower dose of zinc, our results were nearly identical to those reported by Godfrey and colleagues in their subset of 44 patients who were randomly assigned to a treatment similar to ours after fewer than 24 hours of symptoms. In addition, multi-institution studies that obtain virologic data on the infecting organisms are needed to confirm our findings.

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Exhibit Z

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Zinc in the healing wound [Commentary]

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Outline

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Zinc was identified as an essential trace metal by Raulin (1) in 1869, yet it remains an enigma to medical science. It is the second most abundant trace metal in the human body and is present in all living cells and body secretions. Despite extensive research in recent years, scientists and clinicians are still unclear about the regulation of zinc uptake and excretion. Zinc concentrations in an adult range from 95 to 130 microgram/100 mL of serum, and as much as 20 percent of total body content is found in the skin, mainly in the form of zinc metalloenzymes. (2)

For more than 3000 years, zinc in the form of zinc oxide or calamine has been used in the treatment of skin wounds. Zinc in castor oil still has a special place in the treatment of nappy (diaper) rash. However, only during the past 40 years have serious clinical evaluations of topical or systemic zinc supplementation been conducted in patients with bed sores, venous ulcers, incisional wounds, and pilonidal cysts. (3,4) After more than 100 published reports, the precise role of supplementary zinc therapy remains unclear. Early studies claiming that oral zinc accelerated wound healing by as much as 43 percent (3) have not been confirmed, and zincated bandages were not shown to be beneficial in many subsequent trials. (5,6) (In individuals with dietary zinc deficiency or hereditary hypozincaemia, zinc therapy is indicated in wound healing.) Nevertheless a vast range of zincated bandages, dressings, emollients, and creams are available commercially, although we still do not know how much additional zinc a wound needs to enhance healing, or to what extent zinc is absorbed and used from the commercial products.

Scientific progress in understanding the importance of zinc in the health and wellbeing of skin has been hampered by the low concentrations present in some skin samples (less than 10 microgram/g wet tissue weight) and the lack of sufficiently sensitive analytical techniques. With the introduction of atomic absorption spectrophotometry, more accurate information about zinc metabolism can be obtained. We now know that zinc is a constituent of more than 70 metalloenzymes--notably, carbonic anhydrase, DNA and RNA polymerases, reverse transcriptase, proteases, and enzymes that have a central role in the reconstruction of the wound matrix. (7) At the August, 1995, meeting of the European Tissue Repair Society (ETRS), great emphasis was placed on the regulation of these zinc metalloenzymes (8) in the reconstruction and function of collagen in scar tissue. Copper ion, which also has a special role in collagenesis as a compound of lysyl oxidase, is necessary in the cross-linking of collagen fibres. Interaction between copper and zinc can occur, such that excess of one ion can impair essential processes modulated by the other. To obtain a favourable environment, clinicians need to create a balance between zinc and copper, and maybe other ions such as calcium, magnesium, and iron, to

optimise wound healing.

Meanwhile, scientific evaluation of zinc in human wound healing is severely restricted owing to the wide individual variations in zincaemia and tissue zinc content and lack of uniformity in wounds available for study. Limited investigations have been conducted in animal models and in cultured human skin cells. Zinc oxide is beneficial in healing incisional wounds in rats, (9) and enhances the degradation of wound debris in pig skin. (10) Experimental studies showed that topical zinc oxide reduced the initial haemorrhagic phase and promoted the regrowth of damaged skin and hair.

Although the use of animal models for skin healing in man can be criticised on account of interspecies differences in the overt appearance of the skin, its hair cover and glandular structure, fundamental patterns of cell proliferation and migration, and repair following injury are similar. Studies reported at the ETRS meeting show that zinc concentrations change within the naturally healing wound according to events in progress--haemostasis, inflammation, granulation tissue formation, re-epithelialisation, and normalisation. (11) Whereas demands for calcium are higher during haemostasis and later stages, (12) zinc concentrations increase during the formation of granulation tissue, scar tissue, and re-epithelialisation. There are no good data on the relative needs for copper, magnesium, and iron. Experimental studies show that zinc concentrations in the wound margin and wound care are 15-20 percent higher than in intact skin. This increase is furnished by plasma zinc and presumably by higher gastrointestinal zinc uptake. Consequently, if zinc is made available by topical therapy at an appropriate time, one would expect wound healing to be advanced. However, too much zinc at early stages after a wound is believed to upset calcium concentrations and retard healing. The form of presentation of zinc--aqueous paste, cream in amphiphilic vehicle, emollient, bandage--will have a profound influence on the amount and rate at which zinc is absorbed and hence its influence on the wound microenvironment.

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Exhibit AA

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Prevention and treatment of osteoporosis. [Science and Practice]

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In a series of elderly patients entering long-term care, osteoporosis, virtually universally present, was diagnosed in only 4% (1). Did the clinicians not recognise the disorder or did they assume that the skeletal changes were simply part of the ageing process? Whatever the answer, the problem was clearly not being addressed. Yet osteoporosis is both preventable and treatable.

Recognition of patients with osteoporosis ¹

Osteoporotic fractures mainly affect the spine (crush fractures), hip, and distal radius (Colles' fracture). However, since the bone loss is a generalised process, fractures can occur in any bone (2). The possibility of underlying osteoporosis should therefore be considered whenever any elderly person, or a patient with a predisposing condition, presents with a fracture.

Symptom-free individuals who are worried about their risk of osteoporosis—commonly women seen about the time of the menopause—until lately constituted more of a challenge to their doctor. However, we now know that a measurement of bone mass can predict the risk of future fractures (3). When a clinician is concerned about the risk of the disorder, measurement of bone mass with one of the non-invasive techniques (3) will permit quantitative assessment of the patient's risk, and targeted intervention if required. Some (4) have recommended screening the entire female population at or close to the time of menopause; this may be a sensible strategy but is currently prohibited by cost in most

countries.

Intervention in symptom-free individuals ¶

The first stage of intervention consists of behaviour modification, and the second of pharmacological methods. The many so-called risk factors that might increase the risk of osteoporotic fracture in an individual are discussed in the accompanying article (p 797). In clinical practice, the weight that can be given to any single group of risk factors is far from clear. Nevertheless, many of these factors are common to other diseases or markers of poor health, and their elimination often carries more benefit than mere reduction in fracture risk. Thus, patients should be encouraged to eliminate cigarette and alcohol use, improve their diet, and exercise more. Cigarettes and alcohol are associated with an increased risk of osteoporotic fracture, alcohol not least because of the high prevalence of use among the elderly and the resultant increase in the risk of falls that can accompany overindulgence.

Dietary modification ¶

Improvements in diet include ensuring an adequate calcium intake and reducing salt, animal protein, and caffeine, each of which may be a minor risk factor in the pathogenesis of osteoporosis (5). The importance of calcium intake is often debated. However, since an insufficient supply of the mineral from nutritional sources will result in skeletal calcium being used to maintain serum calcium concentration within its tightly controlled range, an adequate calcium intake is sensible. The difficulty is to define what we mean by adequate for any individual patient. Within the USA, an adequate intake is generally thought to be one that delivers 1000 mg of elemental calcium per day. For teenagers, pregnant and lactating women, and those at particular risk of osteoporosis, the recommendation increases to 1500 mg per day. Although some researchers dispute these figures, these amounts of calcium can be achieved by dietary modification and may have some effect in reducing the rate of bone loss, especially among older individuals and those with very low intakes (6). Consequently, I believe this policy is appropriate and worthwhile. Peak bone mass, which is largely under genetic control, may be increased by modest increments in calcium intake during the preteen years (7). Encouraging a healthy and active childhood is likely to ensure that the genetic potential is attained.

Calcium supplements ¶

For those who cannot or will not modify their diet to achieve the recommended intake, calcium supplements are often advised. In many circumstances this may be a reasonable approach. Recent data show some reduction in bone loss (8), but there is a danger that the patient will identify the supplement as a preventive agent that will protect her (completely) against osteoporotic fracture and that she need take no further action. This view is mistaken. Epidemiological studies of the effects of calcium on fractures have given conflicting results and no controlled clinical trial has been carried out with fracture, rather than bone loss, as the outcome.

When advised, calcium supplementation should follow simple guidelines. The aim is to achieve a total intake of 1000-1500 mg per day. Thus, supplementation need only add to dietary intake, which usually means that an individual will require a supplement of 400-600 mg per day. This is readily achieved with calcium carbonate, the salt that is usually available. In our clinic we recommend one chewable calcium tablet with each meal, since that delivers 200-300 mg with an acid load, which mimics dietary intestinal calcium. Another way is to give the supplement at night to reduce the increase in bone remodelling that occurs during sleep (9). No studies have rigorously tested whether there are significant differences in effect with these different regimens, or with different calcium salts.

Exercise ¶

Recommendations for exercise are likewise variable. In general, weight-bearing exercise is thought to be important, and many physicians simply recommend an increase in walking or aerobic activity. However, any effect of activity on skeletal status is likely to be small, and would only be maintained while the level of activity continues. A more important, but largely ignored, benefit of increasing the level of daily activity, especially among elderly people, is that the consequent improved strength, increased stability, and better balance will reduce the frequency of falls and the risk of associated injury.

Oestrogen ▯

Numerous studies have confirmed the cause and effect relation between loss of ovarian function and acceleration of bone loss. Several controlled clinical trials have shown that oestrogen intervention slows or eliminates bone loss, especially in the early years after the menopause (10). This effect is apparent at all skeletal sites, including the vertebral bodies and the femoral neck. Efficacy seems to depend on the amount of oestrogen delivered and not on the route of administration. From data obtained with transdermal oestrogen preparations, we know that circulating oestradiol concentrations in the early to mid follicular range are sufficient to exert a skeletal effect (11). Oral doses of 0.625 mg of conjugated equine oestrogens likewise prevent bone loss, as do similar doses of other oestrogens (12).

Epidemiological data suggest that oestrogen use is associated with a reduction of about 50% in the risk of hip fracture, and probably a similar if not greater reduction in other osteoporotic fractures (10). In the few studies that have examined the issue, the effects seem to be maximal in those in whom treatment is initiated early in the postmenopausal period. Since the duration of therapy required to give maximal reduction of risk is unknown, patients should be treated for as long as possible, at least for 5-10 years. However, oestrogen therapy is also effective in older individuals and those with established osteoporosis (13). In such patients oestrogens stabilise skeletal mass, with an increment of about 5% in vertebral bone mass and a reduction in the risk of recurrent fracture.

Oestrogens are potent hormones with numerous effects. In prescribing for patients with osteoporosis, these other benefits and potential risks must be discussed with the patient to ensure a decision that is as informed as possible, something that is especially important for compliance. The possible reduction in risk of cardiovascular disease (14) and effects on menopausal and urogenital symptoms should be reviewed. The increased risk of endometrial hyperplasia and thus the need for a progestagen (15), or for endometrial monitoring by ultrasound or sampling, should also be discussed. The most difficult issue is that of breast cancer (16). We still do not know for sure whether oestrogens increase the incidence of breast cancer. In general it seems that the risk is slightly increased, especially after long-term use, without an increase in mortality. Although the overall benefit/risk equation seems to favour the use of oestrogens in most individuals, many patients may be deterred by this possibility from embarking on hormone replacement therapy.

Calcitonin ▯

The wide spectrum of oestrogen effects, and the likelihood that a proportion of patients cannot or should not take these hormones, increase the need for alternatives that can be used specifically to protect those most at risk of osteoporosis. Such agents—calcitonin and the bisphosphonates—target only the skeleton. Consequently, determination of risk or presence of osteoporosis is required before they are used.

Calcitonin is a peptide hormone synthesised and secreted by the C-cells of the thyroid. The main and clinically the most important action of calcitonin is to inhibit bone resorption by inhibiting recruitment and activity of osteoclasts (17). Several calcitonins are available (salmon, human, eel, porcine) but the more potent salmon variety is most widely used. Until lately the main drawback with calcitonin was the requirement for parenteral administration, but an intranasal spray is now available. Although absorption can be erratic, preliminary data suggest that sufficient calcitonin is absorbed to reduce the rate of bone loss among postmenopausal women and the nasal preparation may be of value in treatment (18).

Confirmatory investigations are now underway, as are studies to determine whether the reduction in bone loss is accompanied by a reduction in risk of fracture. Resistance may develop to the effects of calcitonin, especially to the non-human types. Neutralising antibodies are sometimes present, but their role in resistance is unclear (19). Further data are required to determine the specific effects of calcitonin, especially in the oestrogen-dependent phase of bone loss.

Bisphosphonates ▯

Bisphosphonates are analogues of pyrophosphate that preferentially inhibit bone resorption (20). Several are at various stages of clinical development, and one, etidronate, is available in some countries for treatment of osteoporosis. The main advantage of these compounds is their availability when given by the oral route, although absorption is modest and variable, with intestinal side-effects noted with some compounds. Their potential disadvantages include their lengthy retention in the skeleton, and inhibition

of mineralisation, which for etidronate occurs at close to the dose effective in preventing bone loss. Bisphosphonates accumulate in the acid environment between the bone and osteoclasts, inhibiting these cells from resorbing mineral (20). Controlled clinical trials indicate that the net effects on bone mass are very similar to those of oestrogen or calcitonin (21). Although some data support reduction in the risk of fracture with etidronate administration, this conclusion is far from secure. Thus evaluation of other second and third generation bisphosphonates is important to determine whether these agents can conclusively prevent fractures. With the multiplicity of side-chains attached to the carbon atom, it is possible that there will be differences among the compounds in this class, not only in potency but also in effects. These agents have the potential to become effective low-cost oral drugs specific for bone and able to reduce bone remodelling and bone loss. Theoretically, one could completely eliminate the remodelling process, especially with the more potent compounds, and there might be long-term effects after cessation of treatment.

Other antiresorptive agents ▽

Several pharmacological agents have similar effects on the skeleton to those of oestrogen, calcitonin, or bisphosphonates and fall into the general classification of antiresorptive agents. Progestagens (22) act in a similar fashion to oestrogen at doses in excess of those used clinically to protect the endometrium. Since these doses are also associated with considerable biochemical side-effects, including reductions in high-density lipoprotein and increases in low-density lipoprotein, it is unlikely that they will be of value in osteoporosis prevention. Anabolic and androgenic agents likewise reduce bone remodelling (23) and loss of bone mass but also have unwanted effects on lipoproteins, and the androgenic side-effects reduce their usefulness in the postmenopausal population. For hypogonadal men who become osteoporotic testosterone is the treatment of choice. Tamoxifen seems capable of preserving bone mass (24), perhaps by acting as a weak oestrogen in bone, although this drug has lethal effects on osteoclasts in vitro, a feature not seen with oestrogens (25). The active metabolite of vitamin D, 1,25-dihydroxycholecalciferol (calcitriol), probably acts by improving calcium absorption and increasing the availability of dietary calcium, but direct bone effects have not been excluded (26). Calcitriol is sufficiently effective at increasing calcium absorption that there is a significant risk of hypercalciuria and hypercalcaemia at doses only slightly above those used in clinical trials (27). Consequently, its role if any in the treatment of this disease is likely to be small. There is now evidence that a significant proportion of the elderly population, especially institutionalised individuals, may have vitamin D insufficiency (1). Addition of modest amounts of vitamin D (800 units; 20 µg) to a calcium supplement can significantly reduce the risk of hip and non-vertebral fractures among institutionalised elderly people; this result indicates that all persons over the age of 70 should receive a vitamin D supplement, along with calcium. Thiazide diuretics also seem to reduce fracture risk (24), and in non-controlled studies are associated with preservation of bone mass; (30) a controlled trial is required before their use can be recommended.

Agents stimulating bone formation ▽

Fluoride is commonly used in Europe but has not been approved by the Food and Drug Administration in the USA. Two controlled studies in which high doses of sodium fluoride were evaluated could not identify a reduction in the risk of vertebral fractures and suggested that there might be an increase in the risk of peripheral fractures (31,32). Impressive and continuous increments in bone mass in the spine were observed for the 4 years of one of these studies. Less well controlled studies seem to show a reduction in recurrent fracture risk (33), but the concern about an increase in the risk of hip and other non-vertebral fractures with fluoride (34) limits its use. New bone formed under the influence of fluoride is abnormal and may weaken rather than strengthen the tissue (35), and calcium supplements must be given to correct the mineralisation of the new bone formed. Use of lower doses of fluoride, or alternative preparations such as the monofluorophosphate, may produce a more effective response (36).

Parathyroid hormone-Evidence of cancellous bone preservation in mild primary hyperparathyroidism (37) has added interest to the uncontrolled data that show significant increases in vertebral bone mass with the use of daily injections of the aminoterminal fragment of human parathyroid hormone (38). However, no fracture data are available, and reductions in cortical bone mass seen in one study indicate the need for more intensive evaluation of this agent.

Growth factors such as IGFs and TGF- Beta are abundant in marrow and synthesised by various cells including osteoblasts. These agents may have potential in the treatment of osteoporosis if they can be administered to target the skeleton and eliminate the potent extraskeletal effects. They are being evaluated with this in mind.

Clinical guidelines ㄱ

Prevention ㄱ

Prevention is likely to remain the most effective method of dealing with osteoporosis. Preventive strategies include ensuring adequate calcium and exercise, and the avoidance of habits detrimental to the skeleton (cigarettes and alcohol). For all (children and adults) an adequate calcium intake, preferably from dietary sources, is important. The actual calcium intake required by any individual cannot be determined by the clinician (there is no serum ferritin for calcium) and thus the general guidelines (above) are used. Recommendations vary by country and by culture.

The most effective point at which to intervene is around the time of the menopause. A pretreatment bone density measurement is a useful guide to the need for therapy. The lower the bone density at the menopause, the greater the risk of osteoporosis, and a reduction in density of 10% (1 SD) increases risk by about 100%. If bone density is less than 1 SD below the average value for young normal individuals oestrogen intervention should be strongly considered (3). Evidence of increased bone turnover may in the future be used along with bone density to predict risk more accurately.

Standard doses of oestrogen (conjugated equine oestrogens 0.625 mg, or its equivalent) preserve bone mass in most women (probably >90%). If bone mass is monitored, and is shown to continue to decline, an increase in dose may be required. The route of administration is not important-oestrogen may be given transdermally, subcutaneously, or percutaneously as well as orally, with equal skeletal response. If the patient has her uterus, a progestagen should be added either cyclically (eg, medroxyprogesterone acetate 5 mg per day for 12 days) or continuously (medroxyprogesterone acetate 2.5 mg/day), depending on the patient's preference. If the patient with a uterus does not wish to take a progestagen, follow-up with intravaginal ultrasound or endometrial biopsy is essential. Duration of treatment is not entirely clear. Data suggest at least 5-10 years, but a much longer time may be required. Bisphosphonates and intranasal calcitonin may in the future be alternatives to oestrogen for prevention. For patients who have had breast cancer, tamoxifen as adjuvant therapy should also provide some protection against osteoporosis.

Treatment ㄱ

For the patient who presents with a symptomless vertebral fracture, or a bone mass below the range of young normal, treatment is directed at maintaining skeletal mass. Oestrogens remain first-line therapy, with calcitonin or perhaps bisphosphonates as the alternatives (this depends on country and approval status). All patients should be given sufficient calcium to bring total intake to 1500 mg/day, together with a vitamin D supplement (800 units). The doses of oestrogen that are effective are similar to those used in prevention, although it is possible that higher doses are required for a greater proportion of individuals. Calcitonin is given in doses of 50-100 units subcutaneously daily or on alternate days. 100-400 units of the intranasal spray may be required. Dosing for bisphosphonates is not clearly established. Etidronate in the clinical trials was given cyclically (400 mg daily for 2 weeks every 3 months), but cyclical treatment may not be required for other bisphosphonates.

Testosterone in replacement doses is the treatment for hypogonadal men with osteoporosis. For other male patients calcitonin or perhaps a bisphosphonate are the available options.

For patients presenting with acute pain from a vertebral fracture, treatment is directed toward symptom relief and mobilisation. Analgesics should be prescribed, and the analgesic effects of calcitonin may reduce the requirements for narcotics in some individuals. Mobilisation may require the temporary use of a back support, and should be supervised by a trained physical therapist.

Patients who present with a hip fracture are usually over 75 years and there is no real evidence that

antiresorptive agents are beneficial for this group. Calcium and vitamin D remain the mainstay of therapy, together with measures to reduce the risk of falls and to protect the patient from the effects of falls when they occur.

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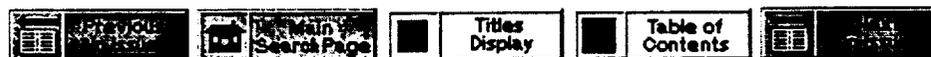
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Dietary calcium and hip fracture risk: the NHANES I Epidemiologic Follow-Up Study.

Looker AC, Harris TB, Madans JH, Sempos CT

Division of Health Examination Statistics, Centers for Disease Control, Hyattsville, MD 20782.

The effect of dietary calcium on hip fracture risk was examined prospectively using the NHANES I Epidemiologic Follow-Up Study cohort, which is derived from a nationally representative sample of the United States population. A cohort of 4342 white men and postmenopausal women ages 50-74 years at baseline (1971-1975) were observed through 1987 for up to 16 years of follow-up. Quantitative estimates of calcium intake were obtained at baseline from a 24-h recall, while weekly frequency of dairy food consumption was obtained from a qualitative food frequency. By 1987, 44 men and 122 women had experienced a hip fracture according to hospital records or death certificates. In the total sample of women the risk of hip fracture was only slightly lower for the highest quartile compared with the lowest. However, although not statistically significant, the age-adjusted risk of hip fracture was approximately 50% lower in the highest quartile of calcium intake compared with the lowest quartile in the subgroup of women who were at least 6 years postmenopausal and not taking postmenopausal hormone. The low relative risk observed among men, although interesting, must be interpreted cautiously due to small sample size. Adjusting for other risk factors did not appreciably change the results for either sex. The pattern of relative risks for calcium quartiles and by selected cutpoints was not consistent with a dose-response effect of calcium. Our results suggest that calcium may lower hip fracture risk in late menopausal women.

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Am J Epidemiol 1997 Jan 15;145(2):117-123

Dietary factors and the incidence of hip fracture in middle-aged Norwegians. A prospective study.

Meyer HE, Pedersen JI, Loken EB, Tverdal A

National Health Screening Service, Oslo, Norway.

Dietary data from a prospective study were used to relate factors influencing calcium balance (estimates of dietary calcium intake, protein intake from nondairy animal sources (meat, fish, and eggs), and coffee consumption) to the incidence of hip fracture. During the years 1977-1983, women and men born between 1925 and 1940 and living in one of three Norwegian counties were invited to a cardiovascular screening that included a dietary survey. The attendance rate at screening was 91.1%, and 90.7% of these persons (19,752 women and 20,035 men) filled in and returned a semiquantitative dietary questionnaire. This cohort was followed for an average of 11.4 years (range, 0.01-13.8 years) with respect to hip fracture, defined as cervical or trochanteric fracture. During follow-up, 213 hip fractures were identified, excluding fractures associated with high-energy trauma and metastatic bone disease. There was no clear association between calcium intake or nondairy animal protein intake and hip fracture in this cohort. However, an elevated risk of fracture was found in women with a high intake of protein from nondairy animal sources in the presence of low calcium intake (relative risk = 1.96 (95% confidence interval 1.09-3.56) for the highest quarter of nondairy protein intake and the lowest quarter of calcium intake vs. the three lower quarters of protein intake and the three higher quarters of calcium intake). Women who drank nine or more cups of coffee per day also had an increased risk of fracture, while there was no association between coffee consumption and hip fracture in men. Although these findings do not necessarily imply causal relations, they suggest the presence of risk factors for hip fracture that act through a negative calcium balance in this population.

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Biochemical and Molecular Roles of Nutrients

Energy Restriction and Zinc Deficiency Impair the Functions of Murine T Cells and Antigen-Presenting Cells during Gastrointestinal Nematode Infection^{1,2}

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ABSTRACT This study examined whether the impaired immune responses in zinc deficient- and/or energy-restricted mice exposed to a challenge infection of *Heligmosomoides polygyrus* might be associated with reduced numbers of spleen cells, altered proportions of spleen cell subpopulations and/or altered function of the T cells or antigen-presenting cells (APC). Female BALB/c mice were given free access to either a zinc-sufficient (60 mg zinc/kg diet, Zn⁺) or a zinc-deficient diet (0.75 mg zinc/kg diet, Zn⁻) or were pair-fed (PF) the zinc-sufficient diet. Significant differences in parasite burdens were observed. Worm numbers were lowest in Zn⁺ mice, intermediate in the PF mice and highest in the Zn⁻ mice, showing that both zinc deficiency and energy restriction reduced protective immunity against the gastrointestinal nematode *H. polygyrus*. Although the absolute numbers of spleen cells were reduced in both Zn⁻ and energy-restricted (PF) mice, neither deficiency altered the phenotypic distribution of the subpopulations of positive marker cells in the spleen. In vitro functional assays using a 1:1 ratio of APC:T cells showed that T-cell proliferation in response to parasite antigen (Ag) was impaired by a dietary effect of zinc deficiency on T cells and of energy restriction and zinc deficiency on APC function. Consequences of the nutritional deficiencies on cytokine production in response to parasite antigen were more complex: zinc deficiency reduced T-cell function [interleukin-4 and interleukin-5 (IL-4 and IL-5) production], and both nutritional deficits depressed APC functions [IL-4, IL-5, and interferon- γ (IFN- γ) production] and T-cell function (IFN- γ production). Thus, this study showed that zinc deficiency and energy restriction played identifiably distinct roles in regulating host immune responses against the gastrointestinal nematode *H. polygyrus*. *J. Nutr.* 128: 20–27, 1998.

KEY WORDS: • zinc deficiency • energy restriction • cytokines • mice • parasitic infection

Suboptimal intake of zinc has rapid and adverse effects on the defense systems of rodents, primates and humans (Keen and Gershwin 1990). In addition to the well-documented consequences of zinc deficiency on T cells (Keen and Gershwin 1990), zinc is important in the regulation of certain macrophage functions (James et al. 1987, Wirth et al. 1989), and in the production and development of B cells (King et al. 1995). Importantly, T-cell mediated responses are critical for host protection against parasitic infections (Mitchell 1980), and any impairment of T cells or the cells that present antigen

to them, caused by the nutritional deficiencies, is therefore expected to cause a decrease in host protection. Yet, surprisingly few studies have examined the effect of dietary zinc deficiency on host-parasite interactions (El-Hag et al. 1989, Fenwick et al. 1985, Wirth et al. 1989). Even fewer studies have attempted to isolate the effect of zinc deficiency per se from the effect of reduced food intake that accompanies zinc deficiency and that also impairs immune function (Luecke et al. 1978, Minkus et al. 1992).

To date, attempts to determine the direct cause of the impaired T-cell-dependent immune responses at the cellular level have been done in zinc-deficient animals only in the absence of infectious agents and have resulted in mixed conclusions. Most researchers agree that a reduction in lymphocyte numbers is a major factor and that this reduction is independent of the specific cell population (Cook-Mills and Fraker 1993, Keen and Gershwin 1990). However, there is less agreement regarding the effects of zinc deficiency on lymphocyte function. When tested on a per cell basis, some studies report no change in T-cell function (Cook-Mills and Fraker 1993) whereas others indicate an impaired ability to proliferate in response to mitogens (Zanconica et al. 1982). James et al. (1987), in particular, reported that zinc deficiency impaired T

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cell proliferative response to the mitogen phytohemagglutinin (PHA).³ Defective T-cell-dependent responses may also result from nutritional impairment of antigen presentation to T cells as shown by reduced T-cell mitogenesis when macrophages from zinc-deficient mice were mixed with T cells from control mice (James et al. 1987).

Our laboratory is involved in ongoing research concerning the effects of nutritional deficiencies on host defense mechanisms using the gastrointestinal trichostrongyloid nematode of mice, *Heligmosomoides polygyrus*. This parasite is used as a model for chronic gastrointestinal helminthiasis (Monroy and Enriquez 1992). CD4+ T cells, which are the principal effector T cells mediating host protective immune responses against *H. polygyrus* infection (Urban et al. 1991a), can be divided into two, or possibly three, functional groups defined by their patterns of cytokine secretion. Th1 cells secrete interleukin-2 (IL-2), interferon-gamma (IFN- γ), and lymphotoxin, whereas Th2 cells secrete interleukin-4 (IL-4), IL-5, IL-9 and IL-10. Both types express IL-3, granulocyte-macrophage colony stimulating factor (GM-CSF), and tumour necrosis factor (TNF). A putative third type of Th cells has been identified on the basis of production of TGF- β , which is thought to be involved in mucosal T helper function and down-regulation of Th1 responses (Chen et al. 1994). The cytokines produced by each of the two main CD4+ T subsets, Th1 and Th2, work by down-regulating the proliferation or functional activity of the opposing subset (Mosmann and Moore 1991). Thus, Th1 cells produce IFN- γ , which preferentially inhibits proliferation of Th2 cells, whereas Th2 cells, which are responsible for generating strong humoral immunity against helminth infections (Sher and Coffman 1992, Urban et al. 1992), synthesize both IL-4, which preferentially stimulates the generation of Th2 cells, and IL-10, which downregulates cytokines of Th1 cells by affecting APC (Sher and Coffman 1992). IgE and IgG1 are regulated by IL-4, eosinophils are regulated by IL-5, and all have been linked with host protective immunity against this parasite *H. polygyrus* (Urban et al. 1991a, 1991b and 1992). Current evidence suggests that the key protective component of this response is IL-4 (Urban et al. 1993). In adequately nourished mice, primary *H. polygyrus* infection stimulates a CD4+ T cell response that has the following Th2 characteristics: increased IL-4 gene expression and protein secretion, elevated levels of IL-3, IL-5 and IL-9 mRNA, eosinophilia and a vigorous B-cell response, resulting in substantial increases in serum IgE and IgG1 (Svetic et al. 1993, Urban et al. 1992, Wahid et al. 1993). Recently, we demonstrated that zinc deficiency and energy restriction enhanced the survival of *H. polygyrus* during both a primary (Shi et al. 1994 and 1995) and a challenge (Shi et al. 1995) infection. During primary infection (Shi et al. 1994), zinc deficiency, independent of energy restriction, resulted in a decreased delayed type hypersensitivity response in vivo and impaired production of IL-4 and IFN- γ by spleen cells in vitro. The observed decreases in serum levels of total IgE and IgG1, impaired eosinophilia and impaired production of IL-5 were attributed to a combined effect of zinc deficiency and energy restriction.

In this study, the potential cellular defects of zinc deficiency and energy restriction on different compartments of the im-

mune system were examined in mice with a challenge infection of *H. polygyrus*. The objective was to obtain evidence that would indicate whether the impaired immune responses that occur during the challenge infection in energy-restricted and/or zinc-deficient mice might be associated with reduced numbers of spleen cells, altered proportions of spleen cell subpopulations and/or altered function of splenic T cells or antigen-presenting cells (APC). To determine if energy restriction and/or zinc deficiency affected T cell function, APC from Zn+ mice were paired with T cells from different dietary groups; T cell proliferation in response to mitogen and parasite antigen was assayed, and cytokine production was quantified in vitro. The same protocol was used to examine the function of APC, except that control T cells from Zn+ mice were paired with APC from different dietary groups. Three Th2 cytokines (IL-4, IL-5 and IL-10) were assayed because of the key role that the Th2 response plays in host-parasite defense mechanisms to *H. polygyrus* (Urban et al. 1992). In addition, IFN- γ was measured because it is an important stimulatory cytokine for a Th1 response, and its overexpression could down-regulate the Th2 cytokines required for the resolution of this gastrointestinal nematode infection (Sher and Coffman 1992, Urban et al. 1992).

MATERIALS AND METHODS

Mice and diets. To achieve our objectives, we required a strain of mouse in which protective immunity exerts its effect within a few weeks of challenge infection rather than days or months, and in which a Th2 response phenotype was expected. The BALB/c mouse was chosen. This mouse strain is classified as an "intermediate responder" to the *H. polygyrus* parasite because worm expulsion normally occurs within 4 wk of challenge infection (Monroy and Enriquez 1992, Wahid et al. 1993). The immune response of BALB/c mice has been well studied and shown to be Th2 dominant (Urban et al. 1991a, 1991b and 1992), although infection also can induce production of the Th1 cytokine, IFN- γ (Shi et al. 1994, Urban et al. 1992).

Three-wk-old female BALB/c mice (Charles River, St. Constant, QC, Canada) were acclimated to a 14-h light:10-h dark cycle and temperature-controlled (22–25°C) animal room for 3 d while fed the zinc-sufficient diet. Mice were housed individually in Nalgene cages (Fisher Scientific, Montreal, QC) with stainless steel grids. To minimize environmental zinc contamination, all cages, grids, feeders and water bottles were acid-washed and rinsed with deionized water before use and were changed frequently. In addition, plastic filter tops were placed over the cages. All mice had free access to deionized water. On d 3, mice were weighed and assigned to three different dietary groups: zinc sufficient (Zn+, 60 mg Zn/kg diet), zinc deficient (Zn–0.75 mg Zn/kg diet) or pair-fed (PF, 60 mg Zn/kg diet), for which individual mice were matched to a Zn– mouse. Each mouse in the PF group was fed the Zn+ diet daily in amounts equal to that consumed in the previous 24 h by its Zn– paired mate. Mice were fed a powdered, biotin-fortified, egg-white-based diet, formulated to meet 1978 NRC requirements for the laboratory mouse (Table 1). Zinc was added as zinc sulfate to provide 60 mg zinc/kg diet for the Zn– diet, an amount twice the recommended level (NRC 1978). All other nutrients were included at levels sufficiently above recommendation to ensure that a 30% reduction in food intake would not generate any other specific nutrient deficiencies in the PF or Zn– mice. Diets were offered in mouse powder feeders (Laboratory Products, Montreal, Canada) specifically designed to minimize food spillage. Mice in Zn– and Zn– diet groups were allowed free access to diets. Daily food intake was determined by subtracting the amount of food remaining in the feeder from the amount given the previous day. Body weights were measured weekly, and total body weight gain was determined. All procedures were approved by the McGill Animal Care Committee according to the Canadian Council on Animal Care (1984).

Infection protocol. To stimulate a strong host protective immune response against *H. polygyrus* infection, an anthelmintic-abbreviated immunizing protocol was used. Four weeks after consuming the exper-

³ Abbreviations used: Ag, antigen; APC, antigen-presenting cell; BSA, bovine serum albumin; ConA, concanavalin A; FITC, fluorescein-isothiocyanate; GM-CSF, granulocyte-macrophage colony stimulating factor; HBSS, Hank's balanced salt solution; IFN- γ , interferon-gamma; IL, interleukin; L₃, third-stage larvae; L₄, fourth-stage larvae; mAb, monoclonal antibody; MLN, mesenteric lymph node; pc, post challenge infection; PE, phycoerythrin; PF, pair-fed (energy restricted); PHA, phytohemagglutinin; Th, T helper; TNF, tumor necrosis factor; Zn+, zinc sufficient; Zn–, zinc deficient.

TABLE 1
Composition of diets¹

Ingredient	Zinc sufficient (Zn-)	Zinc deficient (Zn-)
	g/kg	g/kg
Egg white ¹	180	180
Corn oil	100	100
Cornstarch	442.7	443
Sucrose	200	200
Alphacel ¹	30	30
Choline bitartrate	2	2
AIN 76 vitamin mix ²	10	10
AIN mineral mix ³ (minus zinc)	35	35
ZnSO ₄ · 7H ₂ O ⁴	0.264	0
Biotin	0.02	0.02
Menadione	0.00045	0.00045
BHT ⁵	0.01	0.01

¹ ICN Biochemicals, Division of ICN Biochemicals, Cleveland, OH.

² Vitamin mix (mg/kg diet), as formulated by ICN Biochemicals: thiamin hydrochloride, 6.0; riboflavin, 6.0; pyridoxine hydrochloride, 7.0; niacin, 30.0; D calcium pantothenate, 16.0; folic acid, 2.0; biotin, 0.2; vitamin B-12, 10.0; retinyl palmitate (250,000 u/g), 16.0; tocopherol power (250 u/g), 200.0; cholecalciferol (400,000 u/g), 2.52; menadione, 0.05; powdered sugar, 0.038.

³ Mineral mix (g/kg) as formulated by ICN Biochemicals: dicalcium phosphate, 17.5; sodium chloride, 2.59; potassium citrate, 7.7; potassium sulfate, 1.82; magnesium oxide, 0.84; manganous carbonate, 0.12252; ferric citrate, 0.21; copper carbonate, 0.01052; potassium iodate, 0.00036; sodium selenite, 0.00036; chromium potassium sulfate, 0.01924; powdered sugar, 4.13.

⁴ Zinc concentration by analysis (ICN Biochemicals) was 0.75 mg/kg diet in Zn- diet. Zn+ diet by analysis contained 60 mg/kg diet.

⁵ BHT, butylated hydroxytoluene.

imental diets, mice from each dietary group were infected with 100 infective third-stage *H. polygyrus* larvae (L₃). On d 9 and 14, mice were treated orally with pyrantel pamoate (Pfizer Canada, Kirkland, Canada), at a dose of 172 mg/kg body weight, to eliminate adult parasites from the intestine. One week later (d 21), mice were reinfected with 100 L₃ parasites.

Three weeks postchallenge infection, mice (8 per dietary group) were killed and the spleens were removed and weighed. The spleen cell suspensions were prepared, and the total number of cells per spleen was quantified. Spleen cell suspensions were used for flow cytometric analysis and for the functional studies of T cells and APC. At necropsy, the intestine was removed from each mouse and frozen at -20°C. At a later time, worms were removed and counted.

Liver zinc and copper analysis. Livers were freeze-dried to constant weight, fat extracted with petroleum ether and wet-ashed in nitric acid. Zinc and copper concentrations were analyzed by flame atomic absorption spectrophotometry (Perkin-Elmer 3100; Perkin-Elmer Canada, Montreal, Canada) and expressed as milligrams per kilogram fat-free dry weight.

Parasite and parasite antigen. *Heligmosomoides polygyrus* L₃ were obtained by culturing the feces of stock CD1 mice (Charles River) on moist filter paper for 7 d. The cultured larvae were suspended in deionized water (100 larvae/20 µL), and administered by gavage to mice using a pipette. The accuracy of the dose was estimated by direct counts of the number of larvae in each of five sham doses that were dispensed into plastic petri dishes before the experimental infection.

Parasite antigen (Ag) was prepared using fourth stage larvae (L₄) of *H. polygyrus* obtained from the intestines of mice infected 5 d previously. Parasites were homogenized in physiologic saline on ice for 10 min. The homogenate was centrifuged at 1500 × g at 4°C for 1 h, and the supernatant recovered and sterilized with the use of a 0.2-µm Acrodisc (Gelman Sciences, Ann Arbor, MI). Protein concentration was determined by using a commercially available assay (Bio-Rad, Mississauga, Canada).

Spleen cell preparation. Single cell suspensions were prepared aseptically by passing individual spleens in Hanks' balanced salt solution (HBSS, Gibco, Burlington, Canada) through a fine, stainless steel screen. Red blood cells were lysed with NH₄Cl (0.17 mol/L) for 5 min and the cells were washed twice with HBSS. Membrane debris was removed by filtering the cell suspension through sterile gauze. Cell viability was determined by trypan blue exclusion and was always >90%. The total nucleated splenocyte suspension was kept in complete RPMI 1640 medium: RPMI 1640 (Gibco) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Gibco), 2% HEPES buffer (Gibco), 100,000 IU/L penicillin and 100,000 µg/L streptomycin (Gibco).

Flow cytometric analysis. We also examined whether in addition to reduction in the cell numbers there were alterations in the composition of the lymphocyte populations as indicated by flow cytometric analysis of spleen cells from the different dietary groups. Spleen cell suspensions prepared as above were diluted to a concentration of 2 × 10⁶ cells/L. Aliquots of 0.5 mL were transferred to microtubes and blocked in sorting buffer [PBS, with bovine serum albumin (BSA), 10 g/L, and sodium azide, 5 g/L] with normal goat serum (1:40). After two washes at 300 × g with cold sorting buffer, cells were incubated for 30 min on ice in 50 µL of sorting buffer containing 1 µg monoclonal antibody (mAb)/10⁶ cells of either fluorescein-isothiocyanate (FITC)-conjugated anti-CD3 (clone 145-2C11), anti-B220 (clone RA3-6B2), anti-Mac-1 (clone M1/70), and anti-Ia^b (clone AF6-120.1) mAb, or FITC-conjugated anti-CD3 combined with phycoerythrin (PE)-conjugated anti-CD4 (clone RM4-5) or anti-CD8 (clone S3-6.7) mAb (Pharmingen, San Diego, CA). The cells were washed twice and resuspended in 150 µL of 5 g/L paraformaldehyde in PBS. One- and two-color flow cytometric analyses were performed immediately after labeling, using a Coulter EPICS Profile II (Coulter, Hialeah, FL).

T cell and APC enrichment. To obtain cell populations of both T cells and APC from each mouse, a nylon wool column separation technique was used. This method is known to yield a population of spleen cells containing 85–90% T cells, sufficiently enriched for most analytical purposes (Julius et al. 1973). Spleen cell suspensions, pooled from groups of two mice, were adjusted to a density of 1 × 10¹⁰ cells/L in complete RPMI 1640 medium, plated in 96-mm tissue culture dishes (Becton Dickinson Labware, Lincoln Park, NJ) and incubated for 2 h, at 37°C in 5% CO₂. After incubation, the nonadherent cells were removed by gently washing the surface of each dish with 37°C complete RPMI 1640. The adherent cells were collected using a cell scraper and formed part of the APC source. The nonadherent cells were enriched for T cells by passage through a 10-mL syringe column containing 0.7 g nylon wool (Polysciences, Warrington, PA). The column was incubated for 1 h at 37°C, and T cells were eluted with warmed medium. Antibody staining with anti-CD3 and flow cytometry revealed that cells were ~55% T cells.

The adherent cells from the nylon wool columns were collected by adding cold medium. These cells, together with the cells obtained from plastic adherence, were used as the source of APC. Both adherent and nonadherent cell populations were washed with the complete RPMI medium and their viability was determined by trypan blue exclusion. APC suspensions were incubated in tubes wrapped in aluminum foil in the presence of mitomycin C (Sigma, Mississauga, Canada) at a final concentration of 25 mg/L for 20 min at 37°C to inhibit DNA synthesis (Wunderlich et al. 1992). The effectiveness of this treatment was evidenced by the low proliferation of the cells (18.5–29.5 Bq to concanavalin A (Con A) stimulation, and 7.4–11.1 Bq to Ag stimulation). After incubation, the cells were washed three times with an excess of complete RPMI medium, counted and adjusted to a concentration of 5 × 10⁶ cells/L. Flow cytometry analysis showed that there were ~80% Ia^b cells in the APC population.

T-cell proliferation. For the in vitro functional studies of T cell and APC, a 1:1 ratio of APC to T cells was chosen on the basis of the strong proliferative responses seen when such a ratio was used to examine mitogenesis in nutritionally adequate mice (Croft et al. 1992 and 1994).

In our study, 100 µL of mixed cell suspensions, containing 2.5 × 10⁵ APC and 2.5 × 10⁵ T cells, were added to each well in a 96-well, flat-bottom microtiter plate (Falcon Labware, Oxnard, CA), in the presence of 100 µL of complete RPMI 1640 medium containing

TABLE 2

Effect of zinc deficiency and energy restriction (PF) on nutritional status of BALB/c mice and parasite numbers at 3 wk postchallenge infection with 100 *Heligmosomoides polygyrus* L₃¹

Dietary groups	Initial body weight	Body weight gain	Total food intake	Liver zinc ³	Liver copper ³	Worms/mouse
	g	g/9 wk	g/9 wk	µmol/kg		
Zn-	11.8 ± 0.5	8.2 ± 0.3 ^a	198 ± 4.0 ^a	1.66 ± 0.06 ^a	0.55 ± 0.03	3.5 ± 1.0 ^c
PF	11.5 ± 0.5	5.7 ± 0.3 ^b	163 ± 3.0 ^b	1.51 ± 0.05 ^a	0.42 ± 0.05	7.5 ± 1.0 ^b
Zn-	11.8 ± 0.4	3.6 ± 0.4 ^c	164 ± 3.0 ^b	1.34 ± 0.05 ^b	0.44 ± 0.03	16 ± 2.0 ^a

¹ Values are mean ± SEM. *n* = 8. Different letters in a column represent significant differences (*P* < 0.05). Zn+, zinc-sufficient mice; (60 mg Zn/kg diet); Zn-, zinc-deficient mice (0.75 mg Zn/kg diet).

Con A (Calbiochem, La Jolla, CA) (1 mg/L), or parasite Ag (6.5 mg/L), at concentrations shown to give optimal stimulation (data not shown), or medium only as a negative control. After the cells were incubated at 37°C in 5% CO₂ atmosphere for 48 h, 1 µCi (37 kBq) [³H] thymidine (methyl-³H, specific activity, 1 Ci/mmol; ICN Biomedicals Canada, Montreal, Canada) was added to each well, and cells were cultured for another 18 h. Cells were harvested onto glass fiber filters with a Titertek Cell Harvester (Skatron Instruments, Sterling, VA). Radioactive emissions were counted in a liquid scintillation counter (1219 Rackbeta; LKB Wallac, Turku, Finland). Results were expressed as kilobecquerels. All samples were assayed in triplicate.

Cytokine production. The mixed cell suspensions, containing equal numbers of APC and T cells, were adjusted to a final concentration of 5 × 10⁶ cells/L in the complete RPMI 1640 medium. Aliquots of 1.0–2.0 mL were incubated in 24-well flat-bottom tissue plates (Becton Dickinson Labware) with parasite Ag (at a final concentration of 6.5 mg protein/L) prepared as above, or with Con A (final concentration of 5 mg/L) (Calbiochem). The cell suspensions were incubated at 37°C in 5% CO₂ atmosphere with humidity. Forty-eight hours later, the cell suspension was centrifuged at 350 × *g*, 4°C, for 10 min. The cell-free supernatants were recovered and stored at -70°C until they were assayed for IL-4, IL-5, IL-10 and IFN-γ.

Cytokine ELISA. Two-site sandwich ELISA were used for all cytokine determinations. The following paired Abs were used: anti-mouse IL-4 mAb (BVD4-1D11, Pharmingen) and biotinylated anti-mouse IL-4 mAb (BVD6-24G2, Pharmingen) for IL-4 quantification, anti-mouse IL-5 mAb TRFK-5 (Pharmingen) and biotinylated TRFK-4 (Pharmingen) for IL-5; anti-mouse IL-10 mAb JESS-2A5 (Pharmingen) and biotinylated anti-mouse IL-10 mAb SXC-1 (Pharmingen) for IL-10; and anti-mouse IFN-γ mAb R4-6A2 (Pharmingen) and biotinylated anti-mouse IFN-γ mAb XMGI.2 (Pharmingen) for IFN-γ quantification. Streptavidin-horse radish peroxidase conjugate (Gibco) was used as the secondary layer, and the reaction was visualized with ABTS (Bio-Rad). The concentrations of cytokines were calculated from standard curves generated with the use of known concentrations of recombinant murine IL-4 (Genzyme, Markham, Canada), recombinant murine IL-5 (Pharmingen), recombinant murine IL-10 (Pharmingen) and recombinant murine IFN-γ (Genzyme). The sensitivities of the assays were as follows: IL-4, 0.05–0.1 µg/L; IL-5, 0.03–0.05 µg/L; IL-10, 0.1–0.2 µg/L; IFN-γ, 0.11–0.22 µg/L.

Statistical analysis. To test for dietary effects, one-way ANOVA on untransformed homogeneous data was performed. All tests of the data were done using SYSTAT version 5.03, Evanston, IL (Wilkinson 1991) with Tukey's post-hoc pairwise comparison (Lund and Lund 1955). All differences were considered significant at the 0.05 level.

RESULTS

Host nutritional status and *H. polygyrus* numbers. Dietary zinc deficiency significantly reduced the food intake and growth of the Zn- group (Table 2). Zinc deficiency was confirmed by a significantly lower liver zinc concentration in zinc-

deficient mice than in either PF or Zn+ mice (Table 2). Pair-feeding also reduced the body weight gain of mice. Therefore, this PF group was truly energy restricted, having a significantly greater energy deficit per gram of body weight compared with either the control or Zn- mice, which did not differ (data not shown). By inference, the Zn- mice were not additionally energy restricted when their food intake per gram of body weight was compared with the intake of the Zn+ mice, even though, according to the classic pair-feeding protocol, absolute food intake of the isoenergetic diets and therefore total energy intake was significantly lower. We concluded that the Zn- mice were singularly zinc deficient. Measurements of worm burdens showed that very few worms remained in zinc-sufficient mice killed 3 wk postchallenge infection (pci); twice as many worms were obtained from PF mice, and twice as many again were obtained from Zn- mice (*P* < 0.05) (Table 2). This showed that protective immunity was reduced by energy restriction and impaired even further by zinc deficiency.

Spleen cell population. Energy restriction and zinc deficiency reduced both the size of the spleen relative to body weight and the total number of spleen cells (Table 3). Zinc-deficient mice had significantly fewer spleen cells than both PF and Zn+ mice, and PF mice also had a lower number of the cells than Zn+ mice. Neither zinc deficiency nor energy restriction had a significant effect on the phenotypic distribution of cells bearing the following markers: CD3+, CD3+CD4-, CD3+CD8+, B220+ or Mac 1+ (Table 3). Therefore, a selective shift in immune cell populations was not induced by either zinc deficiency or energy restriction.

APC function. When Con A was used as the mitogen, similar proliferative responses were observed among the dietary groups, regardless of the source of APC in the culture (Fig. 1A). In contrast, when parasite-specific Ag was used as the stimulus, T-cell proliferation was significantly lower in the presence of APC from both Zn- or PF mice than in cultures with APC from Zn+ mice (Fig. 1B). Thus, these results show that energy restriction and zinc deficiency affected the ability of APC to stimulate proliferation of T cells in response to parasite-specific Ag, when a 1:1 ratio of APC to T cells was used.

The function of APC was also evaluated by comparing the ability of APC from different dietary groups to induce T-cell secretion of cytokines in response to parasite-specific Ag or Con A (Table 4). After stimulation with Ag, both zinc deficiency and energy restriction impaired the ability of APC to induce IL-4 production. A significantly lower concentration of IL-4 was detected in cultures with APC from Zn- mice compared with those from PF and Zn+ mice, but also a lower level of IL-4 was produced in cultures containing APC from

This observation is consistent with most nutritional studies showing a reduction in lymphocyte numbers in zinc-deficient animals (Cook-Mills and Fraker 1993). Although the precise nature of splenic involvement in immunity against the intestinal nematode is not yet clear, the spleen responds to *H. polygyrus* infection, as evidenced by the pronounced splenomegaly that accompanies infection (Parker and Inchley 1990, Shi et al. 1994). It has been suggested that this occurs when the mesenteric lymph nodes (MLN) are unable to process all of the parasite antigen (Parker and Inchley 1990). An effective immune response requires not only a sufficient number of normally functioning lymphocytes, but also a balanced composition of lymphocyte populations. Neither zinc deficiency nor energy restriction altered the proportion of total T cells, their CD4⁺ and CD8⁺ subsets, B cells or macrophages. This is consistent with data from infected rodents showing that, except in the most severe cases, zinc deficiency does not alter the composition of splenic lymphocytes in the uninfected model (King and Fraker 1991); it is also consistent with studies showing that energy restriction has no effect on the ratio of CD4⁺ to CD8⁺ spleen cells, despite the generalized increase in the percentage of cells carrying these markers (Woodward et al. 1995).

The similar composition of spleen cell populations among our dietary groups suggested that the impaired T-cell proliferation and cytokine production seen in our in vitro assays may have reflected a functional defect of T cells or APC. At 3 wk postchallenge, which was the time of peak response in zinc-sufficient mice, T cells and APC were removed from mice from the different dietary groups and mixed in vitro. Zinc deficiency exerted a strong effect on T cells and T-cell proliferation. Defects in DNA synthesis (Vallee and Falchuk 1993), changes in cell membranes and signal transduction pathways (Csermely and Somogyi 1988) or perturbations in parasite antigen and class II receptors or co-stimulatory CD28 and/or CTLA-4 molecules on T cells, and B7 molecules on APC (Lu et al. 1994) are potential underlying mechanisms that could result in the low T-cell proliferation associated with zinc deficiency.

Our results show for the first time in infected mice that energy restriction impaired the ability of APC to stimulate T cell proliferation in response to parasite Ag. Energy restriction has also been shown to cause a defect in the interaction of macrophage and T cells in uninfected mice (Christadoss et al. 1984). Although altered immunity has been reported in situations of reduced food intake (Luecke et al. 1978), the mechanisms by which energy restriction affects the immune response have not yet been determined. The results presented in our study suggest that functional impairment of APC may be one of the possible cellular mechanisms for the decreased T cell-dependent immune function commonly seen in the energy-restricted host (Woodward et al. 1995).

Both zinc deficiency and energy restriction impaired the Ag-specific response, whereas when the T cell mitogen, Con A, was used, the responses were indistinguishable among dietary groups regardless of the source of T cells or APC. Con A is a polyclonal activator for many populations of T cells, including both CD4⁺ and CD8⁺ T cells, whereas parasite Ag preferentially stimulates sensitized CD4⁺ T cells. Thus, CD4⁺ T cells are the most activated cell types during *H. polygyrus* infection and are likely to have elevated rates of proliferation and secretion, making them more sensitive to nutritional deficiencies. The Con A results initially were surprising, given that James et al. (1987) reported that zinc deficiency reduced the ability of macrophages to activate T-cell proliferation to PHA, whereas we tested the proliferative response to the mito-

gen Con A and parasite antigen using a different ratio and different cell populations. James et al. (1987) used pure macrophage populations, whereas we used total APC populations containing perhaps 10–15% macrophages. Therefore, our protocol was more likely to detect nutrient effects on B cells, the predominant cells in the APC population, rather than the relatively smaller population of macrophages in the study of James et al. (1987).

Cytokines are powerful regulators of immune responses (Mosmann and Moore 1991, Urban et al. 1991b and 1995), and our study shows that both zinc deficiency and energy restriction dramatically impaired cytokine secretion by perturbing APC and T-cell function. Con A stimulation after energy restriction reduced the ability of APC to stimulate T-cell production of IL-5 and IFN- γ , whereas energy restriction reduced the ability of T cells to produce IL-10. Cytokine production in response to parasite antigen was different. IL-4 secretion was reduced not only by the effects of zinc deficiency on T cells, but also by the combined effects of energy restriction and zinc deficiency on APC induction of T cells. Decreased IL-4 levels in the supernatants could reflect effects of zinc on one or more of the steps involved in transcription, translation (Vallee and Falchuk 1993) or in transport of the cytokine to the cell membrane, its passage through the membrane or its release from the cell surface. The effect of zinc on APC stimulation of T cells was consistent with that of James et al. (1987), in which macrophage function was impaired, after 12 wk of zinc deficiency, but differed from that of Cook-Mills et al. (1991) in which APC from Zn⁻ mice were able to process and present antigen efficiently. Reduced IL-4 production in the Zn⁻ host would be expected to prolong parasite survival and also to cause a decreased IgG1 and IgE response, because IL-4 stimulates B cell class switching to IgG1 and IgE secretion (Mosmann and Moore 1991). Our in vivo data had previously shown that all of these defects occurred in Zn⁻ and energy-restricted mice (Shi et al. 1994) after a primary infection. Now we can conclude that similar changes occur during a challenge infection.

Dietary defects in zinc and energy also produced changes in IL-5 production. Zinc deficiency down-regulated IL-5 production through an effect on T cells, whereas both zinc deficiency and energy restriction down-regulated IL-5 production through effects on APC function. Because IL-5 regulates development and function of eosinophils (Coffman et al. 1989), the reduced IL-5 production was presumably the cause of the low eosinophilia that we previously reported in both Zn⁻ and PF mice (Shi et al. 1994).

In conclusion, we have presented evidence that host protective immune responses against *H. polygyrus* challenge infection were significantly impaired by dietary zinc deficiency and energy restriction, using a 1:1 ratio of APC:T cells. In agreement with previous studies (Shi et al. 1994), we attributed these impaired immune responses in part to reduced numbers of lymphocytes caused by both zinc deficiency and energy restriction. In addition, we demonstrated that the reduced capacity of APC to induce T-cell responses in vitro was caused by both energy restriction and zinc deficiency, whereas functional defects in the T cells were caused by zinc deficiency. Although we studied only one ratio of T cell to APC, there is always the possibility that a different ratio could produce different results. We have no evidence that either deficiency altered the proportion of cell subpopulations in the spleen. Our results provide novel information concerning the underlying cellular mechanisms whereby zinc deficiency and energy restriction impair host protective immunity against the intestinal parasite, *H. polygyrus*, at the cellular level by profoundly perturbing T-cell and APC function.

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EXHIBIT CC

Symposium: Nutritional Advances in Human Bone Metabolism

Transport of Vitamin K to Bone in Humans¹

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ABSTRACT Molecules with vitamin K activity are important for optimal bone health. The major compound of this group in bone is vitamin K₁ (phyloquinone), which is derived exclusively from plant foods in the diet. Vitamin K₁ is absorbed along with dietary fat from the small intestine and transported by chylomicrons in blood. In serum obtained after an overnight fast from healthy men more than half of the vitamin K₁ was recovered from the density fraction that contains chylomicrons and chylomicron remnants (CR), and only a quarter was associated with the major lipoprotein in serum, low density lipoprotein. The concentration of vitamin K₁ in serum is closely related to the triglyceride concentration. Another determinant of vitamin K₁ concentration in serum is the presence of specific variants of apolipoprotein E (apoE). ApoE is a small protein through which the vitamin K-rich CR bind to lipoprotein receptors. The three most common variants of apoE promote CR clearance from circulation with very different efficiency, in the order E2>E3>E4. The variant that promotes CR clearance best is associated with low vitamin K₁ concentration in serum and increased response to vitamin K antagonists. Vitamin K₁ concentration in serum is linked to vitamin K status of bone. The bone protein osteocalcin tends to be less completely carboxylated in people with low vitamin K concentrations in serum. Many hemodialysis patients with a history of bone fractures have indications of poor vitamin K status. The same patients also appear to have a greatly increased prospective bone fracture risk. *J. Nutr.* 126: 1192S-1196S, 1996.

INDEXING KEY WORDS:

- phyloquinone • vitamin K • lipoprotein
- apolipoprotein E • bone fracture

Until recently, the only known function of vitamin K was its role in blood coagulation. Infants during a few weeks after birth are at risk from hemorrhage due

to lack of vitamin K; newborns in many countries now routinely receive intramuscular or oral doses of vitamin K as a prophylactic measure with great success. It is extremely rare, however, that older children or adults who are otherwise healthy develop a bleeding problem because of low vitamin K intakes. Intestinal bacteria provide some vitamin K in addition to dietary sources (Conly and Stein 1992), although the actual amounts are not known (Lipsky 1994). On the basis of this knowledge, an average intake of 1 µg vitamin K/kg body weight was assumed to be sufficient to maintain optimal health (Subcommittee on the Tenth Edition of the RDAs 1989).

In the last two decades several novel vitamin K-dependent proteins have been identified that are not related to blood coagulation. Attention has since focused increasingly on the importance of vitamin K for bone health, and it has been recognized that vitamin K supplies may be suboptimal in bone but sufficient to maintain normal blood coagulation. The following review will discuss the transfer of vitamin K from the sites of intestinal absorption to bone and how this is modulated by common genetic variation.

Bone tissues need vitamin K

Vitamin K participates in the residue-specific carboxylation of proteins that thereby acquire the ability

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to bind calcium (Furie and Furie 1990). Bone cells produce several of these calcium-binding proteins including osteocalcin, matrix-Gla protein (MGP)³ and protein S (Vermeer et al. 1995). The precise function of these proteins in bone is still unclear. Both osteocalcin and MGP are thought to facilitate bone mineralization. It has also been suggested that osteocalcin links osteoblast and osteoclast activity, based on the observation that osteocalcin aids in the recruitment of osteoclast precursors. Protein S has recently been identified as a ligand of tyrosine-kinase type receptors that modulate cell proliferation.

All of these activities require the carboxylated forms of the proteins. During synthesis the nascent proteins are processed by a vitamin K-dependent microsomal carboxylase. Protein carboxylation cannot proceed in the absence of vitamin K. Cortical bone contains as much vitamin K as liver (Hodges et al. 1993, Usui et al. 1990), which is several times the concentration in blood. However, the minimal amount of vitamin K that a bone cell needs to support carboxylase activity is not known.

Whenever a glutamate residue is carboxylated one molecule of vitamin K is oxidized to the 2,3-epoxide form at the same time. Vitamin K-2,3-epoxide may then be regenerated again by vitamin K-2,3-epoxide reductase that resides in the immediate vicinity of the carboxylase. About 40 mmoles of Gla are excreted daily (Ferland et al. 1993), and an equimolar amount vitamin K is oxidized. Daily vitamin K requirements, in contrast, are not more than 0.2 μ moles (Suttie 1987). It is reasonable to assume, therefore, that an average vitamin K molecule is recycled several hundred times. Nonetheless, without an outside supply, bone cells become vitamin K depleted and function less well. An important question is, therefore, how individual bone cells acquire vitamin K and which factors influence the amount that is transferred.

Lipoproteins are the main vehicle of vitamin K in blood

Fat in bone stores some vitamin K (Hodges et al. 1993) and may provide this to bone cells. Ultimately, however, all vitamin K is transported to bone by way of the blood circulation. Actually, several different forms of vitamin K are present in bones and other tissues. The dominant form is phylloquinone (vitamin K₁), which is exclusively derived from foods of plant origin (Shearer 1995). In addition, several species of menaquinones with vitamin K activity also are present in bone, and these may originate from enteric bacteria

or from fermented foods. All natural forms of vitamin K are extremely lipophilic and therefore not miscible with aqueous solution such as blood. Vitamin K is not bound to proteins like some of the other fat-soluble vitamins but in blood is exclusively associated with lipoproteins. Table 1 shows the distribution of phylloquinone in serum density fractions that were obtained from healthy volunteers after they had fasted overnight. A very similar distribution has been found in a larger group of hemodialysis patients, both in respect to vitamin K₁ and vitamin K₂ menaquinone-7 (Kohlsmeier et al., unpublished data). Vitamin K was predominantly associated with the triglyceride-rich lipoproteins (TRL); much smaller fractions were carried by low density lipoproteins (LDL) and high density lipoproteins (HDL).

The TRL fraction actually comprises two families of lipoproteins: very low density lipoproteins (VLDL) and VLDL-derived lipoproteins from the liver, and chylomicrons and chylomicron-derived lipoproteins that are of intestinal origin. These two metabolically diverse families of lipoproteins are not well resolved with current techniques. However, results from previous investigations can aid in the interpretation of the findings. Upon oral consumption, radiolabeled vitamin K is absorbed from the small intestine, becomes associated with chylomicrons in blood and disappears from circulation at the same rate as chylomicrons (Ichihashi et al. 1992, Shearer et al. 1974). This pattern is consistent with the assumption that chylomicrons and chylomicron remnants are the main carriers of vitamin K in blood. Furthermore, much less vitamin K is found in the LDL fraction than in the TRL fraction. Because VLDL are the direct precursors of LDL (Mahley et al. 1989), the low vitamin K content of LDL indicates that the hepatic VLDL carry similarly small amounts of vitamin K.

The metabolism of chylomicrons has been investigated extensively both in humans and in animals (for a review see Mahley et al. 1989). Chylomicrons form in the intestinal mucosa in response to the presence of intracellular triglycerides and are secreted into intestinal lymph ducts. As chylomicrons enter arteriovenous circulation, lipoprotein lipase starts cleaving the triglycerides they carry. At the same time there is a selective exchange of lipids and apolipoproteins with high density lipoproteins (HDL). Within a few minutes the large, triglyceride-rich chylomicrons are thus converted into much smaller, triglyceride-depleted lipoproteins, the chylomicron remnants (CRs), which differ considerably both in composition and in metabolic properties from their precursors. CRs contain apolipoprotein E that they acquire both from HDL in circulation and from hepatocytes when they enter the space of Disse in the liver (Shimano et al. 1994). Through the mediation of apolipoprotein E (apoE) CRs bind to lipoprotein receptors embedded in cell membranes and they are taken up into these cells by pinocytosis.

³ Abbreviations used: CR, chylomicron remnants; HDL, high density lipoproteins; LDL, low density lipoproteins; MGP, matrix-Gla protein; TRL, triglyceride-rich lipoproteins; VLDL, very low density lipoproteins.

TABLE 1
Phylloquinone in the main lipoprotein fractions of fasting healthy subjects^{1,2}

	Total serum	TRL	LDL	HDL
Phylloquinone, nmol/l	0.85 ± 0.52	0.45 ± 0.29	0.16 ± 0.14	0.16 ± 0.0
Phylloquinone, % of total	100	51.4 ± 17.0	25.2 ± 7.6	23.3 ± 10.5
Cholesterol, mmol/l	5.3 ± 1.4	0.9 ± 0.5	3.2 ± 1.3	1.1 ± 0.4
Triglycerides, mmol/l	2.1 ± 1.1	1.7 ± 1.1	n.d. ³	n.d.

¹ Values are means ± SD, n = 10.

² Lipoprotein fractions were prepared by sequential ultracentrifugation at densities $d = 1.006 \text{ kg/l}$ and $d = 1.067 \text{ kg/l}$ at 4°C and $95,000 \text{ g}$ for 18 h each time (Havel et al. 1955). Phylloquinone was measured by a multistage procedure with final analysis by high performance liquid chromatography (HPLC) with dual-electrode electrochemical detection (Shearer 1991). In outline the stages were (a) extraction of lipids from plasma or lipoprotein fractions with further purification by (b) sorbent extraction using silica cartridges and (c) normal phase HPLC. The final electrochemical detection stage (d) was coupled to a reversed phase HPLC column which resolved phylloquinone from MK-7 and the internal standard (MK-6) used to correct for losses.

³ n.d. = not determined.

TRL = triglyceride-rich lipoproteins; LDL = low density lipoproteins; HDL = high density lipoproteins.

Factors that influence vitamin K concentrations in blood

Being firmly associated with the lipoproteins of intestinal origin, vitamin K essentially shares the metabolic fate of chylomicrons and CRs. It may thus seem redundant to note that triglyceride concentrations closely correlated with vitamin K concentrations (Sadowski et al. 1989, Saupé et al. 1993) because chylomicrons are very triglyceride-rich lipoproteins. However, in blood obtained after an 8 to 12-h (overnight) fast most of the triglyceride is associated with VLDL or LDL and not with lipoproteins of intestinal origin. The association of triglyceride and vitamin K concentrations cannot be a direct one; but it is more likely to be due to the fact that VLDL and chylomicrons share catabolic pathways and therefore compete for the same enzymes and activators. Individuals with impaired VLDL catabolism and hypertriglyceridemia also have elevated concentrations of CRs (Slyper 1992); those with low serum triglyceride concentrations, on the other hand, tend to clear all triglyceride-rich lipoproteins more rapidly and have lower CRs concentrations.

Many acquired and constitutional factors are known to affect the clearance of chylomicrons and CRs including a common polymorphism of apoE (Mahley et al. 1989). ApoE is a 32-kD protein with genetic variation of the amino acids 112 and 158. Three variants with different isoelectric points are regularly encountered in populations worldwide (Hanlon and Rubinsztein 1995), and these are designated E2, E3 and E4 on the basis of their electrophoretic mobility. The combination of the parental genes allows for six possible phenotypic combinations (in order of frequency E3/3, E3/4, E2/3, E4/4, E2/4, E2/2). The rate at which CRs are cleared from circulation is faster in people with one or two gene copies coding for the variant E4 than in people who have only E3; CRs clearance is slowest in those with one or two gene copies of E2 (Cortner et al. 1987,

Weintraub et al. 1987). Thus, 8 h after a meal the serum concentrations of both chylomicrons and CRs tend to be lowest in those with the E4 variant and highest in those with the E2 variant.

A corresponding relationship between ApoE phenotype and phylloquinone concentration has been observed in patients on chronic maintenance hemodialysis treatment (Kohlmeier et al. 1995a, Saupé et al. 1993). Phylloquinone concentrations in patients with the genotypes E3/4 and E4/4 were less than half compared with patients with the phenotype E3/3, where the concentration in patients with the genotypes E2/3 and E2/3 tended to be higher. In cardiological patients with normal renal function the anticoagulant response to anti vitamin K therapy also was related to the apoE genotype (Kohlmeier et al. 1995b). Although on the same anticoagulant regimen patients with the genotypes E3/4 and E4/4 had a lower prothrombin content than the others, suggesting the decreased availability of vitamin K to hepatocytes for the synthesis of functional coagulation factors. The reason for lower serum concentrations and decreased availability of vitamin K in liver tissue appears to be the accelerated clearance of vitamin K-rich CRs in people with the E4 variant as detailed above. Once the vitamin K is taken up into the liver (or other tissues), no efficient mechanism exists that could transfer the intact molecule to other organs. In the liver vitamin K is catabolized into metabolically inactive forms that are excreted both in the bile and urine (Shearer et al. 1974) and are thus irretrievably lost.

Transfer of vitamin K from blood to bone tissue

Bone marrow is an important site of chylomicron and CR removal (Hussain et al. 1989). Animal experiments indicate that nearly all intestinal lipoproteins are removed from circulation by liver, spleen and bone marrow within <1 h. The reason for the preferential

delivery of intestinal lipoproteins to these three tissues is fairly obvious. Most tissues are separated from the bloodstream by layers of endothelial and other intervening cells. The receptor-bearing cells in liver, spleen and bone marrow, in contrast, have direct access to circulating blood and can thus bind and acquire lipoproteins preferentially. Among the bone marrow cells that have such direct access to intestinal lipoproteins are stromal and mesenchymal stem cells that are the obligatory precursors of all bone-forming cells (Baron 1993). Responding to appropriate stimuli these precursor cells migrate to sites of bone resorption where they line the activated bone surface and form osteoid tissue. These cells will carry whatever amounts of vitamin K they extracted from circulation while still residing in bone marrow. The migration of precursor bone cells from the marrow into bone may thus be the final step of vitamin K transport from the circulation to bone tissue.

This postulated mode of vitamin K transfer directly from circulating blood to osteoblast precursor cells provides an explanation why bone responds to vitamin K supplementation much more slowly than liver. The function of coagulation factors in vitamin K-deficient patients is usually restored by vitamin K supplementation within a matter of days (Suttie 1987). The effect of vitamin K supplementation on bone, on the other hand, appears to be barely half maximal after 1 mo (Knapen et al. 1993). If active bone-forming cells had direct access to the vitamin K-rich lipoproteins in blood like hepatocytes, a similarly rapid normalization within a few days should have been observed. A substantial delay would be expected, on the other hand, if the vitamin K supplement acts mainly on cells in the bone marrow that are subsequently recruited into bone tissue.

The precise mode of vitamin K transfer from circulation blood to bone-forming cells is of great importance for the understanding of vitamin K effects on bone and needs to be further explored. However, little doubt exists that the concentration of vitamin K in blood and its availability to bone are linked. The relationship that is consistently found between percent carboxylated osteocalcin and vitamin K concentration in serum attests to this link (Saupe et al. 1993). Osteocalcin is almost exclusively derived from new synthesis in bone. When osteocalcin is released to blood it has a half-life of only a few minutes. Furthermore, the calcium-binding form of osteocalcin is more completely carboxylated than the nonbinding osteocalcin. Therefore, the ratio of calcium phosphate (hydroxylapatite) binding to nonbinding osteocalcin in serum has been used to assess vitamin K status of bone (Vermeer et al. 1995).

More recently, the concentration of vitamin K₁ in serum was found to be a major determinant of bone fracture history in hemodialysis patients and the overriding risk factor for bone fractures in these patients (Kohlmeier et al. 1995c). Patients with a history of bone

fractures had significantly lower vitamin K₁ concentrations in serum (0.85 vs. 1.62 nmol/l, $P < 0.001$) and a lower percentage of hydroxylapatite-binding osteocalcin (48 vs. 55%, $P < 0.01$). During the 2 y after biochemical measurements 12% of the patients (8 of 68) suffered bone fractures, and all of these had vitamin K₁ concentrations < 1 nmol/l at the outset. Their low percentage of hydroxylapatite-binding serum osteocalcin (mean 42%) indicated that they had a much poorer vitamin K status in bone than patients who did not suffer a fracture.

Several studies have now demonstrated that vitamin K is important for human bone health and that increased dietary vitamin K intakes can improve mineral retention in some (Vermeer et al. 1995). Knowledge about the journey of this essential nutrient from the intestine to bone cells remains sketchy. Lipoproteins of the chylomicron family appear to be the main vehicle of vitamin K transport in blood. As a consequence, any factor that influences chylomicron metabolism is also likely to affect vitamin K transport and its availability to bone. The apoE polymorphism is probably only one, albeit important, example of such factors that influence vitamin K status of bone. Other polymorphisms of proteins involved in chylomicron metabolism, such as apoCII, apoCIII, apoAIV or lipoprotein lipase, may also prove to be important.

Although this review has focused on factors that influence vitamin K transport to bone, the importance of adequate dietary availability must not be ignored. ApoE genotype is a strong determinant only of interindividual differences in vitamin K₁ concentration (which is considerable) but not of intraindividual variation (Kohlmeier et al. 1995a). There is little doubt that in the absence of external vitamin K supplies everybody eventually becomes vitamin K deficient. Knowledge of specific genetic dispositions may enable us, however, to identify those that are most likely to be at risk, and ensure adequate vitamin K intakes to override a possible metabolic disadvantage. Studies are needed to further explore the impact of apoE and similar factors on nutrient sufficiency in healthy people of all ages and in those who may be most vulnerable to vitamin K deficiency (newborns and patients on antibiotics). Future investigations will also have to determine whether a higher dose of dietary vitamin K can offset the low serum vitamin K concentrations and increased bone fracture risk related to the apoE4 variant.

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EXHIBIT DD

Symposium: Nutritional Advances in Human Bone Metabolism

Effects of Vitamin K on Bone Mass and Bone Metabolism¹

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ABSTRACT Vitamin K is involved in blood coagulation and in bone metabolism via the carboxylation of glutamate residues in (hepatic) blood coagulation factors and (osteoblastic) bone proteins. The bioavailability of nutritional vitamin K depends on the type of food, the dietary fat content, the length of the aliphatic side chain in the K-vitamin and probably also the genetically determined polymorphism of apolipoprotein E. Although undercarboxylation of blood coagulation factors is very rare, undercarboxylated osteocalcin (bone Gla-protein) is frequently found in postmenopausal women. Supplementation of these women with extra vitamin K causes the markers for bone formation to increase. In parallel, a decrease of the markers for bone resorption is frequently seen. Insufficient data are available to conclude that the regular administration of vitamin K concentrates will reduce the loss of bone mass in white women at risk for developing postmenopausal osteoporosis. *J. Nutr.* 126: 1187S-1191S, 1996.

INDEXING KEY WORDS:

• vitamin K • bone mass • bone metabolism

Vitamin K is involved in the synthesis of a number of well-defined proteins, the vitamin K-dependent step being the addition of an extra carboxyl group at the γ -position of glutamate residues (Vermeer 1990). The product formed is an unusual amino acid called γ -carboxyglutamate (Gla).³ There are two physiological processes in which Gla-proteins are known to play a role: blood coagulation and bone metabolism (Hauschka et al. 1989, Shearer 1995). Those participating in blood coagulation are of hepatic origin, whereas the bone Gla-proteins are synthesized by the bone-forming cells (osteoblasts). Recent publications indicate that Gla-proteins may also be involved in the regulation of cell growth (Manfioletti et al. 1993, Varnum et al. 1995).

The human vitamin K supply may originate from two sources: the intestinal flora and the diet. Vitamin K₁ (phylloquinone) originates from green vegetables, whereas vitamin K₂ (menaquinones) is present in meat, fish and fermented foods (Hirauchi et al. 1989, Sakano et al. 1988). Also, the bacteria in the gut produce menaquinones, but the question whether these products substantially contribute to human vitamin K supply has remained a matter of dispute (Lipsky 1994). On the other hand, the vitamin K stores in human liver consist for >90% of menaquinones and for <10% of phylloquinone (Shearer 1992). Whether these menaquinones can be recruited for the formation of Gla-residues in proteins is unclear.

It is generally accepted that a dietary vitamin K intake of $\sim 1 \mu\text{g}/\text{kg}$ body weight \cdot d is required to ascertain the regular carboxylation of the various blood coagulation factors. Hence vitamin K deficiency is defined as a state in which undercarboxylated blood coagulation factors appear in the circulation (Vermeer and Hamulyák 1991). During recent years increasing evidence has shown that complete carboxylation of the bone Gla-proteins may require a higher vitamin K intake. Based on the Gla-content of the bone protein osteocalcin a substantial part of the population must be characterized as biochemically vitamin K deficient. In this paper we will review the available data.

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³ Abbreviations used: Gla, γ -carboxyglutamate; HA, hydroxyapatite; iOC, immunoreactive osteocalcin; ucOC, undercarboxylated osteocalcin.

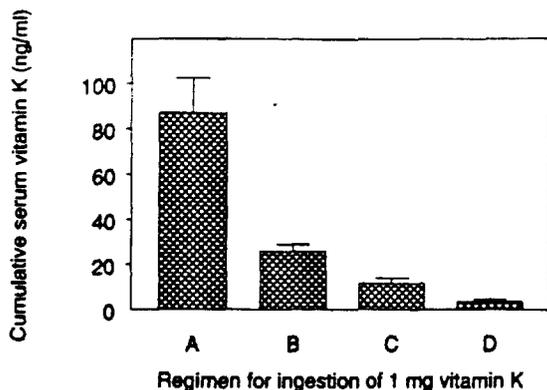


FIGURE 1 Effect of diet composition on circulating vitamin K. Five volunteers received 1 mg of phylloquinone after an overnight fast. The vitamin was given either in the form of Konaktion® (wk 1, bar A), butter enriched with vitamin K (wk 2, bar B), spinach (227 g) + 25 g butter (wk 3) or 227 g of spinach without additives (bar C). Serum vitamin K was measured at 1-h intervals during the first 10 h after consumption of these preparations and corrected for the starting value in each subject. For each individual the cumulative serum vitamin K concentration was calculated from the sum of these 10 measurements. All data are expressed as the means of five volunteers \pm SEM. Circulating phylloquinone was determined as described earlier (Gijsbers et al. 1996).

Factors determining the bioavailability of nutritional vitamin K

All K vitamers are fat-soluble compounds requiring bile salts for their intestinal absorption. In the small intestine vitamin K is extracted from the food and incorporated into micelles consisting of bile salts, free fatty acids and monoglycerides. These micelles are subsequently taken up by the intestinal mucosa, from which the vitamin—bound to chylomicrons—is set free in the lymphatic system and in the circulation.

Phylloquinone is a component of the photosynthetic electron transport system in plants, and it occurs exclusively in the thylakoid membranes of the chloroplasts (Lichtenthaler 1993). This binding is very tight, which may be the reason for the fact that the intestinal absorption of phylloquinone from vegetables is poor. In a recent experiment among human volunteers it was shown by Gijsbers et al. (1996) that even after induction of bile secretion by the simultaneous ingestion of fat, <10% of the vitamin was absorbed. Nutritional menaquinones are generally dissolved in fats, which may facilitate their intestinal absorption. In Figure 1 we compared the cumulative amounts of vitamin K found in serum during the first 10 h after ingestion of 1 mg of the vitamin, either in the form of spinach, spinach + fat, butter enriched with vitamin K or a detergent-solubilized pharmaceutical preparation. From this figure it appears that the absorption of vitamin K is strongly dependent on the source from which it is obtained.

A second factor that may influence the absorption of vitamin K is the length of the aliphatic side chain in the menaquinones. This was recently found by Groenen-van Dooren et al. (1995) and Crăciun et al. (unpublished results) who compared the extent to which the oral administration of various forms of vitamin were capable of restoring plasma prothrombin concentrations in vitamin K-deficient and in warfarin-treated rats. In the vitamin K-deficient animals the vitamin K cycle is fully operational, and relatively low amounts of vitamin K were required to normalize plasma prothrombin. In this model system the long-chain menaquinone-9 had a much higher biological activity than had menaquinone-4 (Groenen-van Dooren et al. 1995). In warfarin-treated rats, on the other hand, the vitamin K cycle is blocked resulting in a >100-fold higher vitamin K requirement for maintaining normal prothrombin synthesis. In this model menaquinone-4 was perfectly capable of counteracting the effect of warfarin, whereas menaquinone-9 had no detectable effect (Crăciun et al. unpublished results). We explain these results by assuming that, in contrast to menaquinone-4, the absorption of menaquinone-9 is bound to a certain maximum, which is too low to overcome the effects of vitamin K antagonists.

The chylomicrons in which vitamin K is set free in the circulation are composed of a hydrophobic core of triglycerides and cholesterylesters, surrounded by an amphipathic shell of apolipoproteins, phospholipids and free cholesterol. Many kinds of apolipoproteins are known. They are generally classified in apo A, B, C, D, and E, whereas each class consists of various subtypes. It has recently been claimed that the genetically determined subtypes of apolipoprotein E play a crucial role in the transport of chylomicrons and thus of vitamin K to the liver and other target tissues (Kohlmeier et al. 1993, Saupe et al. 1993). The mechanism underlying this hypothesis is that the various apolipoprotein subtypes have different affinities for the tissue apolipoprotein receptors. As a consequence, they differ with respect to their plasma clearance rate. Because vitamin K is associated with these apolipoproteins, the serum vitamin K concentrations are low in subjects with apolipoprotein E4/4 or E3/4 (high clearance rate), and they are high in subjects with a low clearance rate (apolipoprotein E2/2 or E2/3). Obviously, this hypothesis raises serious questions concerning the value of circulating vitamin K as a marker for tissue vitamin K status.

Undercarboxylated Gla-proteins

In normal, healthy adults undercarboxylated clotting factors are rarely seen. Even with the most sensitive tests presently available, descarboxyprothrombin was found in only a limited number of newborns (Widdershoven et al. 1987), a group well known to be prone of developing vitamin K deficiency (Von Kries et al. 1993). It may be concluded, therefore, that the liver

capable of efficiently extracting the required amount of vitamin K from the bloodstream, even at very low circulating vitamin K concentrations. This is probably less so for extrahepatic tissues, notably bone. Because direct tests for undercarboxylated osteocalcin (ucOC) are not available, an indirect test has been developed, which is based on the differential affinity of normal and undercarboxylated osteocalcin for hydroxyapatite (HA) (Knapen et al. 1989, Knapen et al. 1993). In this test the serum immunoreactive osteocalcin (irOC) is fractionated into irOC_{bound} (with high affinity for HA) and irOC_{free} with low affinity. Both fractions may be established using an immunoradiometric assay, preferably one with which no osteocalcin degradation products are detected. It is generally assumed that irOC_{bound} consists of the fully carboxylated protein, whereas irOC_{free} is thought to be an undercarboxylated form of osteocalcin. Indeed the fraction of irOC_{bound} is strongly reduced during oral anticoagulant treatment (Jie et al. 1993, Menon et al. 1987). It was recently found that at low anticoagulant dosages the synthesis and carboxylation of osteocalcin were much more strongly affected than was prothrombin (Knapen, M. H. J., unpublished data). It must be concluded, therefore, that in the same subject bone tissue may be vitamin K deficient, whereas at the same time the liver is vitamin K sufficient. Hence, circulating osteocalcin is a far more sensitive marker for vitamin K status than are the Gla-containing blood coagulation factors (Knapen et al. 1991).

It is at least surprising that irOC_{free} has been detectable in all subjects tested. In two separate trials postmenopausal women were supplemented with pharmacological doses of vitamin K, which invariably caused an increase of irOC_{bound} and a decrease of irOC_{free}, but in all cases the latter variable remained detectable even after 3 mo of vitamin K treatment (1 mg/d). On the other hand, it was found that in coumarin-treated patients the level of serum irOC_{bound} decreased by 60–70% (Knapen, M. H. J., unpublished results), but that in the same patients the level of irOC_{free} remained nearly constant. This raises the question whether ucOC has a physiological role and whether its secretion is regulated by the osteoblast. Two domains have been described for osteocalcin: the Gla domain, which is required for the very tight binding of the protein to the hydroxyapatite crystals in the bone (Hauschka and Carr 1982), and the C-terminal domain, which has chemotactic properties for osteoclast-like cells (Glowaki et al. 1991, Liggett et al. 1994). A general phenomenon in chemotaxis is that a gradient of the stimulus is formed against which the attracted cells move to their target, and it is clear that ucOC, with its low affinity for hydroxyapatite, is a much more plausible candidate for building up this concentration gradient than is normal osteocalcin. By regulating the cellular secretion of ucOC, osteoblasts would thus be able to regulate the capture of osteoclasts and thus the rate of bone degradation.

Vitamin K status and bone metabolism

There are several indications that postmenopausal loss of bone mass is associated with a long-lasting poor vitamin K status. First, nutritional vitamin K intake decreases substantially with age (Jie et al. 1995). Second, patients with hip fractures or spinal crush fractures were reported to have very low concentrations of circulating phylloquinone (Hart et al. 1984, Hart et al. 1985, Hodges et al. 1993) and menaquinone (Hodges et al. 1993). Third, circulating ucOC was reported to be inversely correlated with hip bone mineral density (Szulc et al. 1994) and subjects with increased concentrations of ucOC had a sixfold increased risk of hip fracture (Szulc et al. 1993). Finally, it has been shown that vitamin K supplementation increases the serum markers for bone formation (including osteocalcin and bone alkaline phosphatase) and may reduce urinary calcium and hydroxyproline excretion (a well-known marker for bone resorption) (Knapen et al. 1989, Knapen et al. 1993, Plantalech et al. 1990). It is not surprising, therefore, that clinical trials have been initiated in which the effect of long-term vitamin K supplementation on bone mass is investigated. The results of the first trial (in Japan) have been published and show a marked reduction of postmenopausal bone loss by vitamin K treatment (Orimo et al. 1992). At this time the data have not yet been confirmed in the Western population, however.

If vitamin K has a positive effect on net bone formation, it might be expected that vitamin K antagonists (coumarin derivatives, also known as oral anticoagulants) have an opposite effect. Indeed, serious bone deformations were reported to develop in human fetuses during oral anticoagulant treatment of pregnant women (Pettifor and Benson 1975). Animal models were developed in which this so-called fetal warfarin syndrome could be mimicked. It was shown that treatment of young rats with sublethal doses of warfarin induced excessive calcification with growth plate closure and reduced growth of the long bones (Howe and Webster 1992, Price et al. 1982), maxillofacial hypoplasia, reduced length of the nasal bones and massive calcification of the cartilage of the nasal septum (Howe and Webster 1992). The reported effects of warfarin on bone in lambs were even more prominent and included osteopenia with 30% lower bone mass in 3 mo (relative to control animals), mildly decreased bone resorption, strongly decreased bone formation, irregular calcium deposition and remodeling abnormalities (Pastoreau et al. 1993). It is striking that both in humans and in animals substantial effects of warfarin on young rapidly growing bone have been found, whereas in adults the effects are less clear. No placebo-controlled prospective trials have been published in which the potential effects of coumarins on bone were investigated. Conflicting data were obtained from cross-sectional studies among patients on long-

term oral anticoagulant therapy, with two studies showing a reduced bone mass (Fiore et al. 1990, Resch et al. 1991), whereas in two others no significant differences were found between the bone mass in patients and that in age- and sex-matched controls (Piro et al. 1982, Rosen et al. 1993). The fact that no profound effects of oral anticoagulant therapy on bone metabolism were observed suggests that the mode of action of vitamin K on bone cells may be different from that in the liver.

Without doubt, vitamin K acts as a coenzyme for bone carboxylase in the formation of bone Gla-proteins. In this respect it is understandable that during periods of vitamin K deficiency or during coumarin treatment, the production of bone Gla-proteins is decreased. It is less clear why also vitamin K-independent bone markers, such as serum alkaline phosphatase or urinary hydroxyproline are affected by nutritional vitamin K intake. Recently, it was reported by Hara et al. (1995) that menaquinone-4 inhibited the calcium release from mouse calvaria, as well as the formation of osteoclast-like multinucleated cell formation in a co-culture of spleen cells and stromal cells. A similar effect was not found for phylloquinone, and neither were the effects counteracted by warfarin. In a second series of experiments these investigators used only the aliphatic side chains instead of the complete vitamins, and it turned out that only geranylgeraniol, the side chain of menaquinone-4, was capable of inducing the inhibitory effects described above. These data indicate that at least part of the effects of menaquinone-4 on bone resorption are not due to γ -carboxylation and that the side chain of menaquinone-4 may regulate bone cell activity via an as yet unidentified mechanism.

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EXHIBIT EE

Symposium: Nutritional Advances in Human Bone Metabolism

Chemistry, Nutritional Sources, Tissue Distribution and Metabolism of Vitamin K with Special Reference to Bone Health¹

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ABSTRACT Vitamin K occurs in nature as a series of compounds with a common 2-methyl-1,4 naphthoquinone nucleus and differing isoprenoid side chains at the 3 position. They comprise a single major plant form, phyloquinone with a phytyl side chain and a family of bacterially synthesized menaquinones (MKs) with multiprenyl side chains.

The major dietary source to humans is phyloquinone for which the chief food contributors are green, leafy vegetables followed by certain vegetable oils (soybean, rapeseed and olive oils). Recent analyses by high pressure liquid chromatography are now providing a wide-ranging database of phyloquinone in foods. Menaquinones are found in moderate concentrations in only a few foods such as cheeses (MK-8 and MK-9). A wider spectrum of MKs is synthesized by the gut microflora, and their intestinal absorption probably accounts for most of the hepatic stores, particularly those with very long side chains (MKs-10-13) synthesized by members of the genus *Bacteroides*. The site of absorption of floral MKs is not known, but reasonable concentrations are found in the terminal ileum where bile salt-mediated absorption is possible.

Both phyloquinone and menaquinones are bioactive in hepatic gamma-carboxylation but long-chain MKs are less well absorbed. Liver stores of vitamin K are relatively small and predominantly MKs-7-13. The hepatic reserves of phyloquinone (~10% of the total) are labile and turn over at a faster rate than menaquinones. Trabecular and cortical bone appear to contain substantial concentrations of both phyloquinone and menaquinones. A majority (~60-70%) of the daily dietary intake of phyloquinone is lost to the body by excretion, which emphasizes the need for a continuous dietary supply to maintain tissue reserves. *J. Nutr.* 126: 1181S-1186S, 1996.

INDEXING KEY WORDS:

• vitamin K • phyloquinones • menaquinones

Chemistry and nomenclature

The parent structure of the vitamin K group of compounds is 2-methyl-1,4-naphthoquinone (common name menadione). This compound, as far as is known, does not occur in nature but does possess biological activity in vertebrates because of their ability to add on a geranylgeranyl side chain at the 3 position (thus converting it into MK-4): in this way menadione may be regarded as a provitamin. Although not a natural vitamin in the true sense, menadione, as water-soluble salts, is used as a feed supplement in animal husbandry and therefore may indirectly enter the human food chain, presumably as preformed MK-4 present in the tissues of animal meats.

Naturally occurring K vitamins all possess the same naphthoquinone ring structure as menadione, but differ in the structure of the side chain at the 3 position. They are traditionally classified into two groups according to whether they are synthesized by plants or bacteria, respectively. In plants the only major form is phyloquinone (vitamin K₁), which has the same phytyl side chain as chlorophyll. Bacteria, on the other hand, synthesize a family of compounds called menaquinones (vitamin K₂) with side chains based on a number of repeating prenyl units; this number being given as a suffix [i.e., menaquinone-n, (MK-n)]. Some bacteria also

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synthesize forms with partially saturated side chains; the additional hydrogen atoms are indicated by the prefix dihydro, tetrahydro and so forth and abbreviated to MK-n(H₂), MK-n(H₄) and so forth.

Dietary sources

Until recently there has been little impetus from regulatory or nutritional bodies to obtain accurate and comprehensive data of the vitamin K content of foods. In part, this has been due to the lack of any perceived threat to health or well-being from a dietary deficiency of vitamin K as manifested by its classical function in blood coagulation and partly due to the lack of suitable analytical techniques for measuring vitamin K in foods. Therefore, most food composition tables either do not include values for vitamin K, or the values cited are of questionable worth (Booth et al. 1993), many deriving from bioassays intended only as a qualitative guide to vitamin K content (Suttie 1992).

The evidence that has been obtained in the last 5 years, or so, that a subclinical deficiency of vitamin K, while having no impact on hemostasis, may have an effect on bone health has altered our perception of the function and health effects of vitamin K. In doing so it has provided a new impetus to obtain a reliable database for the vitamin K content of foods. Another area where dietary information for vitamin K is needed is to provide information for physicians and their patients during treatment with coumarin oral anticoagulant drugs. With such data, the goal of improving anticoagulant control by giving patients dietary advice based on a detailed knowledge of the vitamin K content of foods would become more than a theoretical possibility. To date, the possibility of improved stability by avoiding large fluctuations in dietary intakes of vitamin K has been a neglected area of the therapeutic control of vitamin K antagonists.

Phylloquinone in foods. Using high performance liquid chromatography (HPLC), the determination of the major dietary form of vitamin K, phylloquinone, in all foods is now possible with a high degree of specificity and accuracy (Booth et al. 1994, Shearer et al. 1980). In the United States a systematic approach to the expressed need for a database of vitamin K in foods is evident from recent publications from the U.S. Department of Agriculture's Human Nutrition Research Center on Aging in Boston (Booth et al. 1993, 1994), which includes a recent collaboration with the Food and Drug Administration to obtain values for phylloquinone in the latter's Total Diet Study (Booth et al. 1995).

It is clear from the food analyses so far carried out that, although phylloquinone has a ubiquitous distribution in the diet, the range of concentrations in different food categories is very wide. Table 1 shows some values of the phylloquinone content of various (mainly raw) foods determined in the author's laboratory and ar-

ranged into four concentration ranges. The results in brackets show the actual values obtained for each item. These actual values should at the present time be treated with caution for several reasons. First, many of the values represent analyses of a single sample of that particular food and therefore do not take into account known sampling variations. The analysis of leafy vegetables is a good example of this problem. Thus, not only do phylloquinone concentrations reflect the degree of plant maturation and geographical location (Ferland and Sadowski 1992a) but also the different tissue distribution in the same plant. Thus for cabbage, the outer (greener) leaves contain 3-6 times more phylloquinone than the inner leaves (Ferland and Sadowski 1992a, Shearer et al. 1980). The phylloquinone content of edible oils too, varies with brand or batch, but just as important is the decline on exposure to light when oils are stored in transparent containers (Ferland and Sadowski 1992b, Shearer et al. unpublished results).

The good overall agreement between phylloquinone values obtained for many foods analyzed in Boston and London suggest that the analytical problems of measuring phylloquinone in foods have now been largely overcome. In the near future this should enable a wide-ranging phylloquinone food database to be established. This will require extending the types of foods analyzed including the analysis of cooked and composite foods as has been started in the U.S. Food and Drug Administration Total Diet Study (Booth et al. 1995). The phylloquinone content of composite foods can also be calculated from a core knowledge of basic food items and calculations according to standard recipes. The latter approach is at present underway in the United Kingdom. Further studies on the sources of variation of phylloquinone in foods are also needed, including how different storage and cooking conditions influence food phylloquinone values. More extensive knowledge of batch variations, particularly for manufactured foods, is also warranted.

Despite the above caveats, some reasonably accurate estimations of the relative importance of different foods to phylloquinone intakes and requirements can already be made. In general, the relative values in vegetables confirm the known association of phylloquinone with photosynthetic tissues with the highest values (normally in the range 400-700 µg/100 g) being found in green, leafy vegetables. The next best sources are certain vegetable oils (e.g., soybean, rapeseed and olive oils) that contain amounts varying from 50 to 200 µg/100 g and make significant contributions to the diet when these oils are incorporated into composite foods or are otherwise used for culinary purposes. On the other hand, some vegetable oils such as peanut, corn, sunflower and safflower oils have a relatively lower phylloquinone content (1-10 µg/100 g) and will contribute much less to daily phylloquinone intakes. The great differences between vegetable oils obviously presents problems to the calculation of the phylloquinone con-

TABLE 1
Phylloquinone content of common foods determined by high pressure liquid chromatography¹

Concentration ranges			
0.1-1.0	1-10	10-100	100-1000
μg of phylloquinone per 100 g food			
Avocado (1.0)	Apples (6)	Beans, runner (26)	Broccoli tops (179)
Bananas (0.1)	Aubergines (6)	Beans, French (39)	Brussels sprouts (147)
Beef, steak (0.8)	Baked beans (3)	Beans, broad (19)	Cabbage, green (339)
Bread, white (0.4)	Barley (7)	Cabbage, red (19)	Kale (618)
Chicken, thigh (0.1)	Beef, corned (7)	Cauliflower (31)	Lettuce (129)
Coconut oil (0.5)	Beef, minced (2)	Chuck peas (21)	Parsley (548)
Cod, fresh, fillet (<0.1)	Bilberries (4)	Cucumber (21)	Rapeseed oil (123)
Cornflakes (<0.1)	Bran, wheat (10)	Greengages (15)	Soybean oil (173)
Flour, white (0.8)	Bread, wholemeal (2)	Mustard greens, cress (88)	Spinach (380)
Grapefruit (<0.1)	Butter (7)	Olive oil, extra virgin (80)	Water cress (315)
Ham, tinned (0.1)	Carrots (6)	Peas (34)	
Maize (0.3)	Cheeses, various (2-6)		
Mangoes (0.5)	Chocolate, plain (2)		
Melon, yellow (0.1)	Corn oil (3)		
Melon, water (0.3)	Courgettes (3)		
Milk, cows (0.6)	Cranberries (2)		
Mushrooms (0.3)	Cream, double (6)		
Oranges (<0.1)	Dates, fresh (6)		
Paranips (<0.1)	Egg yolk (2)		
Peanuts, roast (0.4)	Figs, fresh (3)		
Pilchards, brine (0.6)	Grapes, black (8)		
Pineapple (0.2)	Grapes, green (9)		
Pork, chop, lean (<0.1)	Leeks (10)		
Potatoes (0.9)	Liver, lamb (7)		
Rice white (0.1)	Liver, ox (4)		
Rice brown (0.8)	Nectarines (3)		
Salmon, tin, brine (0.1)	Oats (10)		
Sausage pork/beef (0.2)	Palm oil (8)		
Spaghetti (0.2)	Peaches, fresh (4)		
Tuna, tin, brine (0.3)	Pears (6)		
Turnips (0.2)	Peppers, green (6)		
Yoghurt (0.8)	Peppers, red (2)		
	Plums, red (8)		
	Raisins (4)		
	Rhubarb (4)		
	Safflower oil (3)		
	Strawberries (3)		
	Sunflower oil (6)		
	Swede (2)		
	Tomatoes (6)		
	Wheat (8)		

¹ Data are for raw foods except where cooked form is indicated.

tents of oil-containing foods when the type of oil (or its storage conditions) is not known. Sensitive HPLC assays have also identified poor contributors of phylloquinone to the diet such as root vegetables, lean fish and meats, fleshy parts of fruits and nearly all beverages.

Menaquinones in foods. Although there is now a significant and growing database for phylloquinone in foods, there is much less information about foods as a source of bacterially synthesized menaquinones. The livers of various animal species have long been known to be a good source of a wide variety of long-chain menaquinones with side chains ranging from MK-6 to

MK-13 (Duello and Matschiner 1971, Matschiner and Amelotti 1968). However, quantitative HPLC analysis (Hirauchi et al. 1989) suggests that only livers of ruminant species such as the cow contain high enough concentrations of some menaquinones (e.g., MKs-7, -11, -12, and -13) likely to be of possible nutritional significance (e.g., in the range of 10-20 $\mu\text{g}/100\text{ g}$). On the other hand, because liver is eaten only rarely by the majority of the population, the impact on human nutrition of these concentrations is likely to be slight. The concentrations of menaquinones in other animal organs (e.g., kidney, heart and muscle) are very low

(Hirauchi et al. 1989) and nutritionally insignificant. This is confirmed by our own analyses of skeletal meat items in the human diet. One food category analyzed in our laboratory that does contain significant quantities of two menaquinones (MKs-8 and -9) is cheese (Bach, A. and Shearer, M. J., unpublished results). In all cheeses analyzed the contents of these two forms were in the range of 5–10 $\mu\text{g}/100\text{ g}$ for MK-8 and 10–20 $\mu\text{g}/100\text{ g}$ for MK-9. Other MKs are also present but at concentrations of $\leq 1\ \mu\text{g}/100\text{ g}$. Similarly, very low concentrations of MKs are also present in yogurt and milk.

Questions of bioavailability and bioactivity

K vitamins taken in the diet are absorbed in the proximal intestine by the well-established bile salt-mediated pathway that operates for dietary lipids (Shearer et al. 1974). It has been estimated that healthy adults absorb ~80% of an oral dose of phylloquinone given in its free form (Shearer et al. 1974). As discussed by Vermeer in this volume the efficiency of absorption of phylloquinone from naturally occurring foods depends on the food source of phylloquinone and whether this is accompanied by other food. As expected, the bioavailability is lowest from the richest source, green leafy vegetables, presumably because of the tight association of the vitamin with the photosynthetic apparatus.

The long-disputed questions of whether intestinally synthesized menaquinones provide a useful source of vitamin K and the site and extent of any absorption still remain largely unanswered (Shearer 1992, 1995). Quantitative measurements of menaquinones at different sites of the human intestine and the bacteria producing them have been made by Conly and Stein (1992). Major forms of menaquinones present were MK-10 and MK-11 produced by *Bacteroides*, MK-8 by *Enterobacteria*, MK-7 by *Veillonella* and MK-6 by *Eubacterium lentum*. The most promising site of absorption would seem to be the terminal ileum that contains both reasonable concentrations of menaquinones and of bile salts. Recent animal experiments suggest that no or negligible absorption of a long-chain menaquinone (MK-9) occurs from the colon (Groenen-van Dooren et al. 1995, Ichihashi et al. 1992) and that the efficiency of absorption from the upper intestine is lower for MK-9 than for phylloquinone (Will and Suttie 1992). The intestinal absorption and bioactivity of a menaquinone mixture taken orally has recently been demonstrated indirectly in humans (Conly and Stein 1993). It is noteworthy that menaquinones with very long-chains (MKs-10–13) are known to be synthesized by members of the anaerobic genus *Bacteroides* and are major inhabitants of the intestinal tract but, to date, have not been detected in significant amounts in foods. The widespread presence of MKs-10–13 in human livers at high concentrations (Shearer et al. 1988, Usui et

al. 1990) would therefore suggest that these forms, at least, originate from intestinal synthesis.

Closely related to the question of the bioavailability of different K vitamins is the question of their relative bioactivity, which in turn is related to relative turnover rates. There is presently little information on these questions for humans except that the hepatic turnover of long-chain menaquinones is very much slower than that of phylloquinone (Usui et al. 1990). This is in line with the animal studies showing the slower turnover (Will and Suttie 1992) but longer biological response (Groenen-van Dooren et al. 1995) of MK-9 compared with phylloquinone.

Tissue stores and distribution

Until the 1970s, the liver was the only known site of synthesis of vitamin K-dependent proteins and hence was presumed to be the only significant storage site for the vitamin. However, the discovery that vitamin K-dependent processes and proteins occur in a number of extrahepatic tissues together with recent insights into its plasma transport (see Kohlmeier et al. in this volume) suggest that this may not be the case.

With respect to human liver stores, it is known that these normally comprise ~90% menaquinones and 10% phylloquinone (Shearer et al. 1988, Usui et al. 1990), that the phylloquinone stores are very labile and that under conditions of severe dietary depletion these may be reduced to ~25% of original concentration after only 3 d (Usui et al. 1990). As discussed in detail by Kohlmeier et al. (this volume), the liver receives dietary vitamin K as chylomicron remnants. Because these are the same lipoprotein vehicles responsible for the transport of dietary vitamin A (as retinyl esters) to the liver, it is of interest to compare the ability of the liver to store these two fat-soluble vitamins even though the requirements for vitamin A are greater than for vitamin K. Thus the median hepatic concentration of phylloquinone in adults is ~5 ng/g (Shearer et al. 1988), whereas the minimum adequate stores of vitamin A are 20 $\mu\text{g}/\text{g}$ of liver (Olson 1987) and normal replete stores may be tenfold higher. This vast difference in the hepatic stores between vitamins A and K, which for healthy individuals approaches a 40,000-fold difference is not matched by their relative daily intakes that are only ~10-fold higher for vitamin A (~1000 μg vs. 100 μg). It may be concluded that the evolution of specific mechanisms to store vitamin A in the liver (hepatic vitamin A accounts for >90% of the total body stores) have not evolved for vitamin K. For vitamin K the relationship between hepatic stores and total body stores is not known. In terms of its putative role in bone metabolism it is of obvious interest to determine the vitamin K levels in bone. To date, the only published study is that by Hodges et al. (1993) who examined the vitamin K content of trabecular and cortical

bone in six patients undergoing hip replacement. The results suggested that bone concentrations of phylloquinone and MKs-6, -7 and -8 are as high as those in liver. To explain the apparent paradox of these high bone concentrations in the elderly, who have a tendency for impaired gamma-carboxylation of osteocalcin (Plantalech et al. 1991), Hodges and co-workers suggested that the vitamin K stores may be in adipocytes and not readily available to active osteocalcin-synthesizing osteoblasts. Further work is needed to determine the extrahepatic distribution of vitamin K and the degree of mobilization of these stores, particularly with relation to the role of vitamin K in bone metabolism.

Unlike the preponderance of long-chain menaquinones found in the liver, the major circulating form of vitamin K is invariably phylloquinone. The menaquinones MK-7 and possibly MK-8 are also present, but the common hepatic forms MKs-9-13 are not detectable in plasma. This might be a consequence of a different route of absorption (e.g., the possibility of a portal route for long-chain MKs vs. a lymphatic route for phylloquinone) but might suggest that once in a target tissue the long-chain menaquinones, which are extremely lipophilic, are not easily mobilized.

Metabolism

The liver plays an exclusive role in the metabolic transformations that lead to the excretion of vitamin K from the body. Tracer experiments with labeled phylloquinone have shown that a sizable fraction of a single dose is rapidly catabolized and excreted in either the urine or bile (Shearer et al. 1974). Furthermore, the fraction of the vitamin excreted was not dependent on the administered dose. Thus, regardless of whether the injected dose was 1 mg or 45 μg , ~20% was excreted in the urine within 3 d, whereas ~40-50% was excreted in the feces via the bile (Shearer et al. 1974). These proportions are likely to be underestimates because of the possibility of incomplete collection of urine and stools. This extensive catabolism of phylloquinone by the liver explains the rapid turnover and depletion of hepatic reserves in patients on a low phylloquinone diet observed by Usui et al. (1990). It seems likely, therefore, that ~60-70% of the amounts of phylloquinone absorbed from each meal will ultimately be lost to the body by excretion. This alone suggests that the body stores of phylloquinone are being constantly replenished.

At the present time the human requirements for vitamin K are based solely on its classical function in coagulation being listed as a Recommended Dietary Allowance (RDA) in the United States (Suttie 1992) and a Safe and Adequate Intake in the United Kingdom (Department of Health Report 1991). In both cases these requirements were set at a value of 1 $\mu\text{g}/\text{kg}/\text{d}$. If, as argued by Vermeer et al. and Kohlmeier et al. in this

volume, vitamin K is important to bone health and its requirements for this bone function are greater than for its hepatic function, a great challenge to researchers and future committees alike is to determine whether these putative extra demands can be quantified more precisely. Finally, it should be noted that the concept of reexamining the optimal intake of a vitamin with respect to the extra health benefits, which may be conferred by giving amounts over and above those required to protect against the originally discovered deficiency disease, is not new. There is already a recognition of the newer and often unexpected roles played by several other vitamins including in some cases the beneficial effects of extra intakes (Sauberlich and Machlin 1992).

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