Combined effects of lipid peroxidation and antioxidant status on carotid atherosclerosis in a population aged 59–71 y: The EVA Study

Claire Bonithon-Kopp, Charles Coudray, Claudine Berr, Pierre-Jean Touboul, Jean Marc Fève, Alain Favier, and Pierre Ducimetière

ABSTRACT There are few epidemiologic studies of the effects of lipid peroxidation and antioxidant status on atherosclerosis. The relation of lipid peroxidation evaluated by thiobarbituric acid-reactive substances (TBARS) and biological markers of antioxidant status to ultrasonographically assessed carotid atherosclerosis was examined from baseline data of a longitudinal study on cognitive and vascular aging (Étude sur le Vieillissement Artériel, the EVA Study). The study sample was composed of 1187 men and women aged 59–71 y without any history of coronary artery disease or stroke. Ultrasound examination included measurements of intima-media thickness (IMT) on the common carotid arteries (CCAs) and at the site of plaques. After adjustment for conventional cardiovascular risk factors, erythrocyte vitamin E was significantly and negatively associated with CCA-IMT in both men and women whereas plasma selenium and carotenoids were not. No association was found between TBARS and CCA-IMT in either sex. However, TBARS were significantly higher in men with carotid plaques than in those without. This association was strengthened in men with concentrations of erythrocyte vitamin E, plasma selenium, and carotenoids below the lowest quartile. Our findings give some epidemiologic support to the hypothesis that lipid peroxidation and low antioxidant status are involved in the early phases of atherosclerosis. Am J Clin Nutr 1997;65:121–7.

KEY WORDS Lipid peroxidation, antioxidants, vitamin E, selenium, carotid atherosclerosis, epidemiology

INTRODUCTION

There is a large body of experimental evidence that oxidative processes, especially lipid peroxidation, may be involved in the pathogenesis of atherosclerosis (1). Oxidative modification of low-density lipoprotein (LDL) by lipoperoxide products leads to enhanced uptake by macrophages via the “scavenger” receptor and to cellular accumulation of cholesterol (foam cells) generating fatty streaks, generally considered the early step of plaque formation. As reviewed by Witzum and Steinberg (2), many other mechanisms may explain the increased atherogenicity of oxidized LDL cholesterol. It is chemotactic for circulating monocytes and inhibits the mobility of tissue macrophages. In addition, it is cytotoxic for endothelial cells and immunogenic and can adversely affect coagulation pathways and vasomotor properties of arteries. Only a few clinical studies have reported associations between some indexes of lipid peroxidation or oxidative modification of LDL and myocardial infarction (3, 4), angina pectoris (5), peripheral arterial disease (4, 6, 7) and coronary, femoral, or carotid atherosclerosis (3, 8–11). Until now, epidemiologic evidence for an association between lipid peroxidation and atherosclerosis or related diseases is lacking. On the other hand, epidemiologic data regarding the preventive role of dietary or serum antioxidants such as selenium, vitamin E, and β-carotene or carotenoids are much more extensive as reviewed by several authors (12–15). However, these studies have yielded conflicting results, possibly because a large majority of them have considered as endpoints clinical complications of atherosclerosis and not atherosclerosis per se. The oxidation hypothesis of atherosclerosis emphasizes the prime importance of oxidation damage in the early phases of atherosclerosis and thus suggests that antioxidant protection may be more effective in the formation of early lesions (16).

Thus, the present analysis performed on baseline data of the Étude sur le Vieillissement Artériel (EVA Study) aimed to examine the cross-sectional associations between biological markers of lipid peroxidation (thiobarbituric acid–reactive substances, TBARS) and antioxidant defenses (plasma selenium and carotenoids, and erythrocyte vitamin E) and ultrasound measurements of early carotid atherosclerosis in a population aged 59–71 y.

SUBJECTS AND METHODS

The EVA Study is a 4-y longitudinal study on cognitive and vascular aging (17). The study population was composed of volunteers aged 59–71 y recruited from the electoral rolls of the City of Nantes (western France) and, to a lesser extent, via information campaigns. When a subject was recruited, his or her spouse was systematically asked to participate in the study.

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if he or she was in the desirable age range. During the baseline visit, which took place between June 1991 and July 1993, 1389 subjects were recruited and high-resolution ultrasound examinations of their carotid arteries were performed in 1384 of them. The study protocol was approved by the Comité d’éthique du Centre Hospitalier Universitaire de Kremlin-Bicêtre and written informed consent was obtained from all participants.

**Ultrasonography**

Ultrasound examinations were performed by four sonographers using the Aloka SSD-650, (Aloka, Co, Tokyo) with a transducer frequency of 7.5 MHz. This system provides an axial resolution of 0.30 mm. Acquisition, processing, and storage of B-mode images were computer-assisted with a software program specially designed for longitudinal studies (EUREQUA; TSI, Champs sur Marne, France) (18).

Details of the protocol were described elsewhere (17). All measurements were made at the time of examination. Briefly, it involved scanning of the common carotid arteries (CCAs), the carotid bifurcations, and the origin (first 2 cm) of the internal carotid arteries. The intima-media thickness (IMT) was measured on the far wall of the mid and distal CCA as the distance between the lumen-intima interface and the media-adventitia interface by using an automated edge detection algorithm. Two longitudinal measurements of IMT were completed on both the right and left CCAs at a site free of any discrete plaques. Optimal images were stored on an optical disk. The mean of the right and left CCA-IMT was used in the analysis.

Both near and far walls of all arterial segments were scanned longitudinally and transversally to assess the occurrence of plaques defined as localized echo structures encroaching into the vessel lumen for which the IMT was ≥ 1 mm. The quantification of plaque thickness was made by measuring the IMT at the site of the maximal encroachment perpendicularly to the vessel wall and the sonographer could be computer-assisted in the identification of interfaces and placement of electronic calipers by examining the inflections of the density profile curve taken at the site of plaque. When several plaques were present on the same arterial segment (ie, CCA or bifurcation-origin of the internal carotid artery), the examination was focused on the plaque showing the greatest encroachment into the lumen. When a plaque was mineralized, the sonographer had to obtain the best incidence so that the plaque could be visualized on the far wall and an estimation of the IMT was made by extrapolating the adjacent media-adventitia interface. For all arterial segments, optimal longitudinal and transversal images were stored on an optical disk.

For quality assessment, a rereading study was made on random subsamples of images of both CCAs and bifurcation-internal carotid arteries as described elsewhere (17). Mean absolute differences and correlation coefficients between repeated readings were 0.06 mm and 0.82, respectively, for longitudinal CCA-IMT (n = 81), and 0.40 mm and 0.78 for plaque thickness (n = 52). In a previous study by our group, it was shown that the inter- and intraobserver variabilities of CCA-IMT associated with the scanning procedure were substantially reduced after using the repositioning functions of the EUREQUA software. The aforementioned variabilities (expressed as absolute differences and correlation coefficients) were 0.10 mm (r = 0.58) and 0.10 mm (r = 0.62), respectively, with standard procedures whereas corresponding values obtained with repositioning procedures were 0.07 mm (r = 0.71) and 0.06 mm (r = 0.77), respectively (18).

Ultrasound exams performed early in the study (June-October 1991) were considered unreliable and were excluded from the analysis (n = 77). Subjects with missing data in IMT or plaque assessment (n = 14) and those who reported any history of coronary artery disease (myocardial infarction and/or angina pectoris) or stroke (n = 106) were also excluded. Thus, the study sample was composed of 1187 subjects (476 men and 711 women).

**Medical history**

All participants were administered a standardized questionnaire that gave information about demographic background, occupation, medical history, drug use, and personal habits such as smoking and alcohol consumption. With respect to smoking behavior, subjects were classified as current smokers, former smokers, or never smokers. Life-long exposure to smoking was estimated by the number of pack-years obtained by multiplying the average number of packs of cigarettes smoked per day by the number of years of smoking (1 pack = 20 cigarettes). Alcohol consumption was determined from the subject’s estimate of the average amount of alcohol ingested weekly and expressed as mL alcohol/d. Two independent measurements of systolic and diastolic blood pressure were made with a digital electronic tensiometer (SP9; Spengler, Paris) after a 10-min rest and the mean was used in the analysis. Self-reported history of myocardial infarction, angina pectoris, or stroke was also recorded.

**Laboratory procedures**

Blood samples were drawn between 0800 and 0900 after a 12-h fast. Ten-millimeter volumes of peripheral blood collected in evacuated containers with heparin were obtained for > 98% of the participants. Erythrocyte pellets were obtained by centrifuging blood at 500 × g for 15 min at room temperature. Plasma and buffy coats were then removed and the erythrocytes were washed in sterile 9 g NaCl/L solution. Plasma and erythrocyte preparations were stored at −80 °C until assayed. Standard laboratory determinations were performed daily and other determinations within 2 mo.

**Assay of plasma selenium**

Plasma selenium was determined by electrothermal atomic absorption spectrometry (EAAS) (Perkin Elmer 5100; Perkin Elmer, Norwalk, CT) using Zeeman background correction (19). Plasma samples were diluted in 0.2% Triton X100 (dissolved in 0.1 mol HNO₃). Nickel (10 μg) was added in the plateform graphite furnace as a modifier. Selenium concentration was obtained by using standard addition calibration. Intra- and inter assay CVs were 1.4% and 1.8%, respectively.

**Spectrophotometric assay of plasma carotenoids**

After precipitation of proteins with ethanol, carotenoids were extracted with hexane and the absorbance of the hexane phase was measured with a spectrophotometer at 350, 450, and 550 nm (Uvikon 860; Kontron, Rotkreuz, Switzerland). Concentrations were calculated on the basis of a molecular extinction
factor at 450 nm of 134 000 L·mol⁻¹·cm⁻¹. Absorbances at 350 and 550 nm were used to correct the obtained absorbance at 450 nm by applying an adequate equation. Intra- and inter-assay CVs were 5.4% and 4.9%, respectively.

**HPLC assay of erythrocyte vitamin E (α-tocopherol)**

The sample volume of 400 μL red blood cells was diluted in saline solution combined with 1% pyrogallol (20). After red blood cell proteins were precipitated with ethanol, vitamin E was extracted with hexane. The hexane phase was dried under a nitrogen stream and quantification at 290 nm was performed by HPLC coupled to a spectrophotometer detector. An external standard of α-tocopherol and an internal standard of α-tocopherol acetate were used. We excluded 59 subjects from the analysis, for whom quantity determination revealed the possible presence of artificial vitamin E components. Intra- and interassay CVs were 8% and 9%, respectively.

**TBARS assay using fluorometry**

Plasma concentrations of TBARS were determined according to the method of Richard et al (21). Briefly, the aldehyde groups react with two molecules of thiobarbituric acid at pH 3 and elevated temperature (95 °C) to form a pink-colored complex. The colored complex was then extracted by using 1-butanol. The test tubes were stirred vigorously and centrifuged at 1000 × g before the fluorescence was read at excitation and emission wavelengths of 532 and 553 nm, respectively. 1,1,3,3-Tetraethoxypropane was used as an external standard. Intra- and interassay CVs were 2.1% and 3.2%, respectively.

**Standard biochemical indexes**

Total cholesterol and triacylglycerol assays were performed by enzymatic methods using the PAP enzymatic cholesterol kit (reference 61227; Boehringer Mannheim, Mannheim, Germany) and the PAP enzymatic triacylglycerol kit (reference 759350), respectively, supplied by Biomérieux (Paris). Glucose concentrations were determined by the enzymatic glucose oxidase method (reference 61274; Boehringer Mannheim). High-density-lipoprotein (HDL) cholesterol was measured enzymatically after precipitation of apolipoprotein B-containing lipoproteins with phosphotungstic acid and Mg²⁺ (precipitant, reference 543004; Boehringer Mannheim). LDL cholesterol was computed with the Friedewald formula (22).

**Statistical analysis**

Standard procedures from the Statistical Analysis System (SAS Institute, Inc, Cary, NC) were used for univariate and multivariate analyses. Crude descriptive data are presented as means of CCA-IMT or percentages of subjects with carotid plaques by quartiles of antioxidant biomarkers and TBARS. Linear univariate and multivariate associations between carotid atherosclerosis and both antioxidant biomarkers and TBARS were tested by linear regression (CCA-IMT) or logistic regression (carotid plaques) where antioxidant biomarkers and TBARS were separately introduced as dependent variables. Because of sex-related differences in the relations between plaques and antioxidant biomarkers and TBARS, analysis was systematically performed in men and women separately. Multivariate analyses were performed by adjusting for demographic variables and conventional cardiovascular risk factors that were significantly associated with TBARS or biological markers of antioxidant status in at least one sex. They included age, body mass index, systolic blood pressure, pack-years, use of lipid-lowering drugs, years of education, alcohol consumption, HDL and LDL cholesterol, triacylglycerols, and glucose. All of these potential confounding variables were systematically added in the regression models. Adjusted odds ratios for carotid plaques were calculated either for 1 SD increase in antioxidant biomarkers and TBARS or in each higher quartile by reference to the first quartile. Interaction terms between each antioxidant biomarker considered as a dichotomous variable (below the first quartile or above the first quartile) and TBARS considered as a continuous variable were also tested.

**RESULTS**

The main characteristics of the EVA Study population are shown in Table 1. The mean CCA-IMT and the prevalence of carotid plaques were significantly higher in men than in women (P < 0.001). The means and distribution indexes of plasma selenium and carotenoids, erythrocyte vitamin E, and TBARS are given in Table 2. Significantly higher concentrations of each antioxidant biomarker and TBARS were observed in women than in men.

The mean concentrations of CCA-IMT by quartiles of antioxidant biomarkers and TBARS are shown in Table 3. There was a progressive decrease in CCA-IMT with increasing concentrations of plasma carotenoids in both men and women. Similarly, CCA-IMT was inversely related to erythrocyte vitamin E but this association was significant only in men. No association was found between CCA-IMT and both plasma

<table>
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<th>TABLE 1</th>
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<td>Main general and ultrasound characteristics of the 59–71-y-old population in the EVA Study</td>
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<tr>
<td><strong>Men</strong> (n = 476)</td>
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<tr>
<td>Age (y)</td>
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<tr>
<td>Carotid plaques (%)</td>
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<tr>
<td>Intima-media thickness (mm)</td>
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<td>Body mass index (kg/m²)</td>
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<td>Systolic blood pressure (mm Hg)</td>
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<td>Diastolic blood pressure (mm Hg)</td>
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<td>Total cholesterol (mmol/L)</td>
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<td>HDL cholesterol (mmol/L)</td>
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<tr>
<td>LDL cholesterol (mmol/L)</td>
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<td>Triacylglycerol (mmol/L)</td>
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<td>Alcohol use (%)</td>
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<td>40–80 (mL/d)</td>
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<td>&gt;80 (mL/d)</td>
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<td>Years of education (%)</td>
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<td>8–13 y</td>
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* x ± SD.
selenium and TBARS. After adjustment for demographic and conventional cardiovascular risk factors, plasma carotenoids were no longer associated with CCA-IMT in either sex. The main confounding variable appeared to be body mass index, which was significantly and negatively associated with plasma carotenoids in both sexes. Its introduction in the regression model reduced the regression coefficient of plasma carotenoids by 44% in men and 29% in women (the regression coefficient became insignificant in both sexes). After further adjustment for systolic blood pressure, the regression coefficient was reduced by 70% in men and 33% in women. On the other hand, erythrocyte vitamin E remained negatively associated with CCA-IMT in men and became significantly associated with CCA-IMT in women. There was no significant interaction term between antioxidant status and TBARS on CCA-IMT.

The prevalence of carotid plaques by quartiles of antioxidant biomarkers and TBARS is shown in Table 4. In men, the prevalence of plaques decreased with increasing plasma carotenoids whereas it increased with increasing concentrations of TBARS. A similar but not significant trend was observed in women. No association was found between carotid plaques and either plasma selenium or erythrocyte vitamin E in men or in women. When conventional risk factors were taken into account, the risk of carotid plaques tended to be lower with increasing plasma carotenoids in men but this association was not significant. On the other hand, the positive association

TABLE 3
Common carotid intima-media thickness by quartiles of antioxidant biomarkers and thiobarbituric acid–reactive substances (TBARS) in men and women"
between TBARS and the risk of carotid plaques remained significant after risk factor adjustment in men. The adjusted odds ratio associated with a 1-SD increase in TBARS (0.39 and 0.40 μmol/L in men and women, respectively) was 1.36 (95% CI: 1.04, 1.76) in men and 1.02 (95% CI: 0.81, 1.30) in women.

Finally, we examined whether the concentrations of antioxidant biomarkers could modify the association between TBARS and carotid plaques. Participants were classified depending on whether their antioxidant concentrations were below or above the first quartile. As indicated in Table 5, male subjects having low plasma selenium or low erythrocyte vitamin E showed a two- to threefold higher risk of carotid plaques associated with a 1-SD increase in TBARS than were subjects with high values of antioxidant biomarkers (tests of interaction between TBARS and antioxidant status: \( P < 0.059 \) for selenium, \( P < 0.051 \) for vitamin E). A similar trend was seen in men with low concentrations of carotenoids compared with those with high concentrations. This pattern was not observed in women (data not shown).

**DISCUSSION**

In the EVA Study population, aged 59–71 y, erythrocyte vitamin E was inversely associated with CCA-IMT independently of conventional cardiovascular risk factors in both men and women. Plasma selenium and carotenoids did not show any association with CCA-IMT nor with the presence of carotid plaques. TBARS, a global index of lipid peroxidation, was positively and independently related to carotid plaques only in men. This association was strengthened in male subjects with low plasma selenium concentrations and low erythrocyte vitamin E and, to a lesser extent, low plasma carotenoids.

Vitamin E is known as the major essential chain-breaking antioxidant in the lipid phase, thus protecting polyunsaturated fatty acids against peroxidation (1, 23), whereas β-carotene is the next most common lipophilic antioxidant (1). Selenium is an essential component of the enzyme glutathione peroxidase, which catalyzes the reduction of hydrogen peroxide in several tissues and also has an important role in the removal of lipid peroxides (24). Evidence from cross-cultural, case-control, or prospective epidemiologic studies for a link between dietary or serum antioxidants and cardiovascular disease has been equivocal (12–15). Similarly, results from randomized trials of antioxidant supplementation are contradictory and raise some caution about the beneficial effect of vitamin E and β-carotene (25–29). Few epidemiologic studies have examined the relation of dietary or serum antioxidants to atherosclerosis. An inverse association was found between serum selenium and the severity of coronary atherosclerosis (30) whereas supplementary vitamin E has been associated with a reduction in the progression of coronary atherosclerosis (31). Interestingly, the beneficial effect of supplementary vitamin E was observed in subjects with mild to moderate coronary lesions at baseline and not in subjects with severe lesions. With regard to carotid atherosclerosis, only two ultrasound studies have provided information. In a subsample of the Kuopio Ischemic Heart Disease Risk Factors Study, low serum selenium was associated with accelerated progression of CCA-IMT (32). In the Atherosclerosis Risk In Communities (ARIC) Study, carotid IMT was inversely related to dietary vitamin E in women and to dietary β-carotene in men (33). These associations were only observed in participants aged > 55 y.

The present study was performed in an elderly population of volunteers with low levels of atherosclerosis (only two subjects had stenoses > 40%), and our results cannot be generalized to other populations. They emphasize the primary protective role of vitamin E in atherogenesis. Its inverse association with increased IMT but not with confirmed atherosclerotic plaques suggests that, even in elderly subjects, it may be more effective in the early phases of atherosclerosis. There was also a crude negative association between plasma carotenoids and increased IMT in both sexes and with carotid plaques in men, but these associations did not remain significant after adjustment for cardiovascular risk factors, especially body mass index and systolic blood pressure. Furthermore, erythrocyte vitamin E was the only antioxidant biomarker to be weakly associated with TBARS in both sexes. Erythrocyte determinations reflect long-term dietary intake and bioavailability than do plasma determinations. It may partly explain the stronger associations observed between lipid peroxidation, early atherosclerosis, and erythrocyte vitamin E than those observed with plasma carotenoids and selenium. However, differences in antioxidant capacity may represent another explanation, as suggested by two recent studies showing that supplementation with vitamin E may be more effective than supplementation with β-carotene in the protection of LDL from lipid peroxidation (34, 35).

We found increased plasma lipid peroxides assessed by TBARS in men with carotid plaques compared with those without. Adjustment for cardiovascular risk factors, especially LDL cholesterol and triacylglycerol, which were the main correlates of TBARS, only slightly reduced this relation. On the other hand, there was no association between TBARS and increased IMT in either sex. Estimation of plasma lipid peroxide concentrations by TBARS is a sensitive, well-established method that measures the amount of malondialdehyde formed as a breakdown product of lipoperoxides. It is also a common product released during prostaglandin synthesis. However, this method needs to be viewed cautiously because of its lack of specificity due to interference from heme, proteins, purines, and iron (36, 37). There is no clear explanation for the sex differences in the association between TBARS and carotid plaques. Similar results were obtained when a more restrictive definition of plaques was retained (plaques ≥ 2.0 mm). In our

<table>
<thead>
<tr>
<th>Antioxidant biomarkers</th>
<th>Below first quartile of antioxidant</th>
<th>Above first quartile of antioxidant</th>
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<tbody>
<tr>
<td>Plasma selenium</td>
<td>2.57 (1.34–4.95) [103]</td>
<td>1.14 (0.83–1.55) [312]</td>
</tr>
<tr>
<td>Plasma carotenoids</td>
<td>1.89 (1.05–3.39) [104]</td>
<td>1.14 (0.84–1.56) [311]</td>
</tr>
<tr>
<td>Erythrocyte vitamin E</td>
<td>3.43 (1.58–7.43) [90]</td>
<td>1.21 (0.87–1.68) [303]</td>
</tr>
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1 OR; 95% CI in parentheses; \( n \) in brackets. The number of subjects is slightly reduced because of missing TBARS or antioxidant biomarker values in a few subjects.

2 \( P < 0.005 \).

3 \( P < 0.033 \).

4 \( P < 0.002 \).
laboratory, assay variabilities were quite satisfactory and we have no reasons to suspect greater biological or ultrasound measurement errors in women than in men. Furthermore, the power of the study was adequate to detect relatively small variations in TBARS associated with atherosclerosis. In men as well as in women, the power of a two-tailed t test ($\alpha = 5\%$) was >67% and 95% to detect differences of 0.10 and 0.15 $\mu$mol/L, respectively, with an SD of 0.40 mmol/L. However, we cannot exclude the possibility that the results observed in men may have been due to chance. On the other hand, the lack of association in women might be explained by their better antioxidant defenses. The fact that the association between TBARS and carotid plaques was more pronounced in men with low concentrations of erythrocyte vitamin E and of plasma carotenoids and selenium gives some support to this hypothesis and suggests that TBARS determination may be a better index of lipid peroxidation in subjects with a low antioxidant status.

Our failure to find any association between TBARS and CCA-IMT is intriguing. Measurements of IMT are more reliable at the CCA level than at the level of the plaque. In the present study (17), as well as in others (38, 39), CCA-IMT was related to the presence and severity of carotid plaques. It is also related to atherosclerosis in other localizations (40–42) and to incident myocardial infarction (43). Thus, it is generally considered to be an early marker of generalized atherosclerosis. Differences in ultrasound methodology or in populations may explain the discordance between our results and those observed in a previous study showing that the progression of carotid atherosclerosis based on CCA-IMT measurements was related to the tier of autoantibodies to malondialdehyde-LDL in Finnish men (10). In addition, the lack of association with TBARS in our study may have been due to the low specificity of TBARS measurements. Lasty, it may have also reflected the equivocal nature of increased IMT, resulting partially from the technical inability of ultrasound to differentiate the intima from the media layer. The fact that both atherogenic and nonatherogenic stimuli such as aging or hemodynamic stresses (44) are certainly involved in the genesis of intima-media thickening may obscure its relation to lipid peroxidation. Thus, only subjects who developed confirmed atherosclerosis are likely to show increased TBARS.

In conclusion, this study gives some epidemiologic support for the hypothesis that increased lipid peroxidation and low antioxidant status, especially vitamin E, are involved in the early phases of atherosclerosis even in the elderly. Whether vitamin E supplementation may reduce the progression of intima-media thickening and plaques in the carotid arteries needs to be assessed in controlled supplementation trials.

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26. The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study


