

Before the
FOOD AND DRUG ADMINISTRATION
Washington, D.C.

In re: Petition for Health Claim:)
Vitamin E Dietary Supplements and) **Docket No. 99P-4375**
Heart Disease)
)

OCT 19 2000

MOTION FOR LEAVE TO
SUPPLEMENT COMMENTS WITH NEWLY PUBLISHED SCIENTIFIC
LITERATURE

Julian M. Whitaker, M.D.; Pure Encapsulations, Inc.; Durk Pearson and Sandy Shaw; and the American Preventive Medical Association (collectively, "Health Claim Petitioners") hereby move to supplement the record in the above-referenced proceeding with the attached scientific journal articles and studies. The articles and studies are submitted over a month in advance of the November 24, 2000 deadline set by the agency (and ordered by the U.S. District Court) for completion of a further evaluation of the above-referenced claim following FDA's January 11, 2000 denial of the claim. The articles and studies are submitted after the date set by the agency for filing additional science in this docket; nevertheless, good cause exists for agency review of the submission after the agency's deadline.

Through diligence the Health Claim Petitioners could not have filed the studies and articles in advance of the comment deadline because they have only recently been published. Moreover, the studies and articles are weighty evidence, in the form of peer-reviewed scientific literature, that, if omitted from agency analysis, could result in findings of fact or conclusions of law that are contradicted by the studies and articles. The studies and articles reveal that oral supplementation with Vitamin E results in a reduction in cardiovascular disease risk, and they corroborate the role that lipid oxidation

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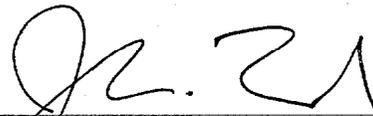
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and peroxidation modification play in cardiovascular disease etiology and progression. If FDA elects not to review this scientific literature and suppresses the proposed health claim based in whole or part on a perceived absence of evidence supporting the claim, that action would violate the First Amendment. Such an election would ignore proof of the veracity of the proposed claim at a time when that evidence was available and could have been reviewed. The agency has more than a month from the date of this filing before its self-imposed deadline for decision, ample time for it to review the studies and articles and evaluate their significance. For the foregoing reasons, the Health Claim Petitioners respectfully request that the agency accept this supplement and the attached studies and articles for review.

Sincerely,

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In re: Petition for Health Claim:)
Vitamin E Dietary Supplements and) **Docket No. 99P-4375**
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MEMORANDUM IN SUPPORT OF MOTION

The Health Claim Petitioners respectfully request that FDA review scientific articles and studies described below in connection with its evaluation of the proposed health claim that is the subject of this rulemaking proceeding.

The scientific articles and studies described below and contained in Attachment 1 recently appeared in peer reviewed scientific journals. They provide evidence of cardiovascular disease risk reduction resulting from oral Vitamin E supplementation and they corroborate the role that lipid oxidation and peroxidation modification play in cardiovascular disease etiology and progression. In addition, the Food and Nutrition Board (FNB) and the Institute of Medicine (IOM) published its review and analysis of publicly available scientific evidence citing extensive data supporting the cardiovascular protective role of vitamin E only this year. Food Nutrition Board and Institute of Medicine, Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium and Carotenoids, Washington, DC, National Academy Press: 2000. (FNB/IOM Report). The FNB and the IOM found credible scientific evidence supporting the role of vitamin E in reducing the risk of chronic diseases including cardiovascular disease and made the following assessments:

Vitamin E enrichment of endothelial cells down-regulates the expression of intercellular adhesion molecular (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), thereby decreasing the adhesion of blood cell components to the

endothelium (Cominacini et al., 1997). Vitamin E also up-regulates the expression of cytosolic phospholipase A2 (Chan et al., 1998; Tran et al., 1996) and cyclooxygenase-1 (Chan et al., 1998). The enhanced expression of these two rate limiting enzymes in the arachidonic acid cascade explains the observation that vitamin E, in a dose-dependent fashion, enhanced the release of prostacyclin, a potent vasodilator and inhibitor of platelet aggregation in humans (Szceklik et al., 1985; Tran and Chan, 1990).

FNB/IOM Report at 196.

Vitamin E does inhibit LDL oxidation whether induced by cells in culture or by copper ion in vitro. In addition, vitamin E could affect atherogenesis at a number of steps, based upon the following in vitro observations:

Vitamin E inhibits smooth muscle cell proliferation through the inhibition of protein kinase... Vitamin E inhibits platelet adhesion, aggregation, and platelet release reactions... Vitamin E inhibits plasma generation of thrombin, a potent endogenous hormone that binds to platelet receptors and induces aggregation... Vitamin E decreases monocyte adhesion to the endothelium by down regulating expression of adhesion molecules.

FNB/IOM Report at 211.

The hypothesis that reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a role in atherosclerosis rests on a solid basic science foundation and is strongly supported by studies in animal models. At the clinical level, a variety of correlational studies and studies of biochemical markers are consistent with the hypothesis. However, only four published, large-scale, randomized, double-blind clinical intervention studies have tested the ability of vitamin E to prevent myocardial infarction. One of these, a secondary prevention trial supplementing with 400 or 800 IU (268 or 567 mg)/day of *RRR- α -tocopherol*, was strongly positive (Stephens et al., 1996). The other three, one carried out in a group of high-risk smokers using 50 mg/day of *all rac- α -tocopherol* (ATBC Cancer Prevention Study Group, 1994) and two carried out in high-risk cardiovascular patients supplemented with 300 mg/day of *all rac- α -tocopherol* (GISSI-Prevenzione Investigators, 1999)¹ and 400 IU (268 mg)/day of *RRR- α -tocopherol* (HOPE Study Investigators, 2000)², were neutral.

FNB/IOM report at 216.

The available data strongly suggests that individuals with diabetes are subject to increased oxidative stress. However, no clinical intervention trials have tested directly whether vitamin E can ameliorate the complications of diabetes mellitus. A

¹ The GISSI-Prevenzione Trial did show a 21% decrease in risk of CHD and 35% decrease in risk of sudden death, and a 14% decrease in overall mortality with 300 mg vitamin E supplementation.

² HOPE has been criticized for not accounting for the influence of multiple cardiac and anti-platelet medications and non-trial use of vitamin E supplementation by both treatment and control groups.

gap remains between the effects of vitamin E treatment on biochemical markers oxidative stress, clinical efficacy, and validation of a relationship between biomarkers and clinical outcomes. Studies in humans show that lipid and lipoprotein oxidation proceed more rapidly in patients with diabetes than in nondiabetic people and that treatment with vitamin E can partially reverse this process... In theory then, intervention with vitamin E therapy to inhibit atherogenesis might be more effective in individual diabetics than in nondiabetics. However, as of this date there are insufficient data on which to base a recommendation of supplemental vitamin E in diabetics.

FNB/IOM report at 218.

The FNB/IOM recommendations were focused and based on prevention of classic deficiency disease models and they did not include an evaluation of the following recently published scientific studies:

1. Boaz M, et al., Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease (SPACE): randomised placebo-controlled trial. *Lancet*, 2000, 356, 1213-1218. Researchers examined the effect of oral supplementation with 800 IU of vitamin E on the incidence of cardiovascular events in end stage renal disease (ESRD) patients. This population is especially prone to cardiovascular disease. ESRD patients have greatly increased oxidative stress and a high rate of mortality due to cardiovascular events. Their age adjusted mortality rate is 3.5-4 times higher than the general population with an up to 20 times greater risk of cardiovascular disease. The double blind placebo controlled study (SPACE) was conducted over a two-year period. The patients who received the vitamin E supplements had a 46% reduction in the primary cardiovascular endpoint compared to placebo. The vitamin E supplementation group had a 70% lower incidence of myocardial infarction (fatal and non-fatal). The authors explain how the results of this study are consistent with those found in CHAOS. In addition, they explain the shortcomings of the GISSI and HOPE studies that may account

for differing results. The results of SPACE support the cardiovascular protective role of Vitamin E supplementation. This study was previously filed by the Joint Health Claim Petitioners on October 17, 2000, and is not resubmitted here.

2. Islam KN, O'Byrne D, Devaraj S, Palmer B, Grundy SM, Jialal I. Alpha-tocopherol supplementation decreases the oxidative susceptibility of LDL in renal failure patients on dialysis therapy. *Atherosclerosis*. 2000 May;150(1):217-24. End stage renal disease (ESRD) patients are under increased oxidative stress and at high risk of cardiovascular disease. Researchers examined whether daily dietary supplementation with oral vitamin E (800 IU) can reduce the oxidative susceptibility of LDL in chronic renal failure patients, both peritoneal dialysis and hemodialysis patients and controls. The controlled clinical trial lasted for 12 weeks. The subjects who received vitamin E had increased oxidation lag times indicating increased resistance to LDL oxidation and peroxidation. The authors conclude that daily oral supplementation with 800 IU of vitamin E for 12 weeks provides significant protection against LDL oxidation.

3. Jialal I and Abate N, Therapy and Clinical Trials, *Current Opin Lipidol*. 2000; 11: 93-97. In a review of clinical trials that affect lipid profiles and cardiovascular outcomes, the authors state "much epidemiological data, which appears to be supported by clinical trials to date, has suggested that tocopherols, especially alpha-tocopherol, might be beneficial with respect to cardiovascular disease." Thus, the authors conclude that the evidence, while not conclusive, supports the proposition that vitamin E may reduce the risk of cardiovascular disease.

4. Jialal I and Devaraj S. Vitamin E Supplementation and cardiovascular events in high-risk patients. *N Eng J Med*. 2000 Jun 22;342(25): 1917-18. In a commentary on

the HOPE study, the authors strongly criticized the HOPE authors' conclusions and stated there is sufficient evidence to support cardioprotective benefits from vitamin E supplementation. As scientists, they note that in a majority of prospective studies a positive result from vitamin E supplementation was obtained for either a primary end point or a secondary end point.

5. Kugiyama K, et al., Improvement of endothelial vasomotor dysfunction by treatment with alpha-tocopherol in patients with high remnant lipoproteins levels. *J Am Coll Cardiol.* 1999 May;33(6):1512-8. In a double-blind randomized placebo controlled study, researchers examined the effects of oral alpha-tocopherol 300 IU/day for four weeks on endothelial vasomotor functions in brachial arteries of patients with high and low serum levels of remnants lipoproteins. The flow mediated dilation (FMD) in the brachial arteries was significantly lower in subjects who had high remnant lipoprotein levels than the FMD in those with low remnant levels. Treatment with alpha-tocopherol significantly increased the FMD as compared to placebo in subjects with high remnants lipoproteins levels. The vitamin E did not affect the vasodilatation in response to nitroglycerin. The authors concluded that four weeks of oral Vitamin E supplementation improves endothelial vasodilation in subjects who have high remnant lipoprotein levels. The results of this study support the cardiovascular protective role of Vitamin E supplementation.

6. Kummerow FA, Olinescu RM, Fleischer L, Handler B, Shinkareva SV. The relationship of oxidized lipids to coronary artery stenosis. *Atherosclerosis.* 2000 Mar;149(1):181-90. In this study conducted in a clinical setting, researchers examined the relationship between lipid peroxides (LPX), acute phase proteins, and total

antioxidant capacity (TOAC) and cardiovascular disease. The researchers examined the LPX, acute phase proteins and the TOAC in the plasma of angina patients with cardiac catheterized confirmed stenosis and compared them to those plasma values obtained from age and sex matched non-smoking, cardiac disease free patients. The analyses within different groups of categorical variables revealed that high levels of antioxidant capacity were associated with a low percentage of stenosis, while high levels of LPX, age and diabetes were all associated with a higher stenosis percentage. The researchers found LPX concentration to be a dominating factor. The results of this study corroborate the role that lipid oxidation and peroxidation modification play in cardiovascular disease etiology and progression.

7. Meraji S, Abuja PM, Hayn M, Kostner GM, Morris R, Oraii S, Tatzber F, Wonisch W, Zechner R, Gey KF. Relationship between classic risk factors, plasma antioxidants and indicators of oxidant stress in angina pectoris (AP) in Tehran. *Atherosclerosis*. 2000 Jun;150(2):403-12. In this case controlled study of men with angina pectoris (AP) and matched controls researchers examined the relationship between classic risk factors, antioxidant status and indicators related to lipoprotein oxidation and AP. The AP subjects exhibited a significantly higher index of lipid peroxidation than controls. The authors concluded that lipid peroxidation independently contributes to progression of AP. The results of this study corroborate the role that lipid oxidation and peroxidation modification play in cardiovascular disease etiology and progression.

8. Neunteufl T, et al., Effects of vitamin E on chronic and acute endothelial dysfunction in smokers. *J Am Coll Cardiol*, 2000, 35: 277-283. In a placebo controlled randomized study of adult male smokers, researchers examined the effect of oral vitamin

E supplementation on impaired endothelium-dependent vasodilation. The researchers designed and conducted the trial after previous placebo controlled studies showed that oral intake of vitamin E improved vascular reactivity in hypercholesteremic men. The intervention group subjects received daily supplementation of 600IU of all racemic alpha-tocopherol for four weeks. Compared to placebo, the vitamin E group subjects had significantly less decline in endothelium-dependent flow-mediated dilation (FMD) after acute cigarette smoking. While four weeks of supplementation was not long enough to affect chronic FMD values, the results of the study demonstrate a beneficial effect of vitamin E. In addition the attenuation of transient endothelial dysfunction after acute smoking correlates with an improvement of the antioxidant status under vitamin E supplementation. The authors conclude that vitamin E exerts a direct tissue effect which may account for a cardioprotective effect independent of its ability to inhibit formation of oxidized LDL-cholesterol.

9. Ohmura H, Watanabe Y, Hatsumi C, Sato H, Daida H, Mokuno H, Yamaguchi H. Possible role of high susceptibility of high-density lipoprotein to lipid peroxidative modification and oxidized high-density lipoprotein in genesis of coronary artery spasm. *Atherosclerosis*. 1999; 142(1):179-84. In a controlled clinical trial, researchers examined the relationship between oxidized lipoproteins and the genesis of coronary artery spasm. The subjects with resting angina and atypical chest pain exhibited lower levels of plasma HDL and higher susceptibility of HDL to lipid peroxidative modification as compared to controls. The results of this study corroborate the role that lipid oxidation and peroxidation modification play in cardiovascular disease etiology and progression.

10. Russo C, Olivieri O, Girelli D, Faccini G, Zenari ML, Lombardi S, Corrocher R. Anti-oxidant status and lipid peroxidation in patients with essential hypertension. *J Hypertens*. 1998 Sep;16(9):1267-71. In this controlled clinical trial, researchers examined both the extent of lipoperoxidation in a group of essential hypertensive subjects compared to matched controls and the status of enzymatic and non-enzymatic anti-oxidants that potentially are able to modify that condition. In examining the characteristics of the groups, the researchers found that the hypertensive subjects exhibited lower levels of vitamins A and E, and copper and higher levels of zinc and plasma malonaldehyde (MDA) and higher GSHPx activity. Other parameters (LDL, HDL, triglycerides), lifestyle, dietary intake, and alcohol consumption did not differ between the groups. The authors concluded that the statistical analyses revealed several significant differences between subjects with essential hypertension and those normotensive subjects. Essential hypertension is associated with a greater than normal lipid peroxidation. Impaired antioxidant status or a greater than normal response to oxidative stress or both are important factors in the pathogenesis of essential hypertension and/or in arterial damage related to essential hypertension. The results of this study corroborate the role that lipid oxidation and peroxidation modification play in cardiovascular disease etiology and progression.

11. van de Vijver LP, et al., Oxidation of LDL and extent of peripheral atherosclerosis. *Free Radic Res*. 1999 Aug;31(2):129-39. In a controlled study of the relationship between peripheral atherosclerosis and LDL oxidation, researchers found that increased susceptibility of LDL to oxidation is associated with peripheral atherosclerosis. That association is demonstrated by a decreased oxidation resistance

time in subjects with peripheral atherosclerosis compared to controls. The results of this study corroborate the role that lipid oxidation and peroxidation modification play in cardiovascular disease etiology and progression.

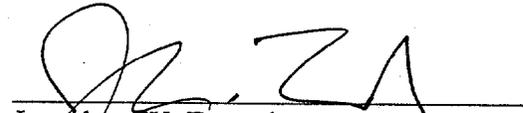
12. Watkins ML, et al., Multivitamin use and mortality in a large prospective study. *Am J Epidemiology* 2000 Jul 15; 152 (2): 149-62. In a large prospective study of 1,063,023 adult Americans over a seven year period, those study participants who had used oral vitamin supplements had lower death rates from ischemic heart diseases than those who took no vitamins. The rates of death were even lower for those who consumed multivitamins in combination with vitamins A, C or E. The authors conclude that the study results provide evidence that vitamin supplementation, especially when combined with vitamins A, C and /or E, may reduce death rates from ischemic heart disease in the general population. The possibility that there are differences other than the use of vitamin supplements in the self-selected group cannot, however, be ruled out.

13. Yla-Herttuala S. Oxidized LDL and atherogenesis. *Ann N Y Acad Sci.* 1999 Jun 30;874:134-7. The author presents a comprehensive review of scientific literature and concludes that research has provided strong evidence that LDL oxidation plays an important role in the pathogenesis of atherosclerosis and cardiovascular diseases. The author states "there seems to be no doubt that lipid peroxidation and oxidative damage to LDL resemble chronic inflammation, which causes various alterations in arterial gene expression and promotes lesion development."

Sincerely,

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and the AMERICAN PREVENTIVE
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ATTACHMENT 1

Alpha-tocopherol supplementation decreases the oxidative susceptibility of LDL in renal failure patients on dialysis therapy

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Abstract

Atherosclerotic cardiovascular disease is the leading cause of death in patients with end stage renal disease (ESRD) who have undergone dialysis treatment. The oxidation of low density lipoprotein (LDL) appears to be a crucial step in the pathogenesis of atherosclerosis. The increased oxidative stress and attendant increased oxidizability of lipoproteins, such as LDL could contribute to the accelerated atherosclerosis in dialysis patients. Since α -tocopherol (AT) is the major antioxidant in LDL, the aim of the present study was to test the effectiveness of RRR-AT supplementation (800 I.U. per day) for 12 weeks on the susceptibility of LDL to oxidation. The study subjects comprised patients with chronic renal failure on hemodialysis (HD), peritoneal dialysis (PD), and age and sex matched controls (C). Plasma fatty acids, lipoproteins and AT levels were measured in these subjects before and after supplementation. Also, LDL AT and oxidizability was studied. LDL was isolated by ultracentrifugation at baseline and after 12 weeks of supplementation, and subjected to a 5-h time course of copper catalyzed oxidation. Oxidation was measured by the formation of conjugated dienes (CD) and lipid peroxides (LP). Supplementation with AT did not alter the plasma lipid or lipoprotein profile of these subjects. Plasma lipid-standardized AT and LDL AT concentrations were not different among the groups at baseline. AT supplementation significantly increased plasma lipid-standardized AT (C = 150%, HD = 149%, PD = 217%, $P < 0.001$) and LDL AT concentrations (C = 94%, HD = 94%, PD = 135%, $P < 0.003$). AT enrichment of LDL resulted in a significant prolongation in conjugated diene lag phase in all groups (C = 34%, HD = 21%, PD = 54%, $P < 0.02$). Lipid peroxide lag phase was also increased significantly in C (27%,) and PD (40%) groups after AT supplementation ($P < 0.01$). There was a significant positive correlation between plasma lipid standardized AT and lag phase ($r = 0.54$, $P = 0.0003$). Overall, AT decreased the susceptibility of LDL to oxidation in patients with chronic renal failure but the benefit appears to be greater in patients on PD. Therefore, AT supplementation may also provide a measure of protection against CAD in patients with chronic renal failure on dialysis therapy. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Hemodialysis; Peritoneal dialysis; Low-density lipoprotein; Lipid peroxidation; α -Tocopherol; Atherosclerosis; Renal failure

1. Introduction

ESRD is a common final complication of systemic disorders including diabetes, and hypertension, and various primary chronic glomerulonephritides, etc. While there has been a steady increase in the number of patients developing ESRD, the advent of kidney transplantation and dialysis has prolonged the life span of ESRD patients considerably. However, the long-term

Abbreviations: CAPD, continuous ambulatory peritoneal dialysis; ESRD, end stage renal disease; FOX, ferrous oxide–xylenol orange; HD, hemodialysis; HDL, high-density lipoprotein; LDL, low-density lipoprotein; OX-LDL, oxidized LDL; PD, peritoneal dialysis; RLP, remnant lipoprotein; VLDL, very low-density lipoprotein.

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effectiveness of these therapies has been hampered by the increased incidence of accelerated atherosclerosis in patients with ESRD. Atherosclerotic cardiovascular disease is the most common cause of disability and death in patients maintained on long-term dialysis [1–3]. It has been reported that the cardiovascular disease in such patients is 7-fold greater than in the normal population [1–3]. It has also been observed that more than one half of the deaths in dialysis patients could be attributed to atherosclerotic complications [1–3]. Major risk factors that may potentially promote atherosclerosis in dialysis patients include dyslipidemia, hypertension and smoking [4]. Patients with chronic renal failure on dialysis have been reported to have an abnormal lipoprotein profile characterized by reduced HDL-cholesterol, moderate hypertriglyceridemia, and increased levels of remnants and lipoprotein(a) [5–10]. Oxidative stress has also been incriminated in the increased cardiovascular morbidity and mortality in patients with ESRD [11–19]. Evidence for increased oxidative stress in renal failure include the following: activation of oxidative metabolism in leukocytes by dialysis membrane; depletion of antioxidants such as ascorbic acid, vitamin E, glutathione, glutathione peroxidase and superoxide dismutase [11], increased MDA levels in serum, RBC, platelets and peripheral blood mononuclear cells [12–16]; increased formation of protein carbonyl and glycation products; and increased oxidation of VLDL, LDL and increased titer of autoantibodies to oxidized LDL in the plasma of patients on hemodialysis [17–19]. However, with regard to lipoprotein oxidation other groups have failed to confirm this increase [20–23].

Oxidation of LDL is a lipid peroxidation process in which polyunsaturated fatty acids (PUFAs), mainly linoleic and arachidonic acid are transformed into lipid hydroperoxides and then converted to aldehydes [24]. A wide range of antioxidants, including enzymes (such as superoxide dismutase and glutathione peroxidase) and chain-breaking antioxidants (such as ascorbate and α -tocopherol (AT)) help to prevent LDL oxidation [25].

AT is the most abundant and potent lipid soluble antioxidant present in LDL and tissues. It is a chain-breaking antioxidant, trapping peroxy free radicals. Several studies have associated low AT levels with development of atherosclerosis and a significant inverse correlation has been found between AT levels and mortality from coronary artery disease [25–29]. Vitamin E supplementation has been found to be associated with a decreased risk of coronary artery disease in men and women. A recent prospective study of 2002 patients with coronary artery disease showed that vitamin E supplementation resulted in a significant reduction in cardiovascular death plus non-fatal myocardial infarction. Various studies have shown the effectiveness of AT in decreasing LDL oxidation *in vitro* [25–29]. *In vivo*

supplementation with at least 400 I.U. per day AT has been shown to reduce LDL oxidation in healthy men and women [29]. However, there is a paucity of data on the effect of AT supplementation on LDL oxidation in patients on hemodialysis or continuous ambulatory peritoneal dialysis. Panzetta et al. [18] showed that 50 I.U. per day AT supplementation reduced the susceptibility of LDL to oxidation in hemodialysis patients. There appears to be no reports on the effect of AT supplementation in PD patients. Thus, there is insufficient evidence to conclude that AT supplementation reduces oxidative susceptibility in chronic renal failure patients on dialysis. Therefore, the aim of the present study was to investigate whether dietary supplementation with AT can reduce the oxidative susceptibility of LDL in chronic renal failure patients on hemodialysis and peritoneal dialysis and matched controls.

2. Subjects and methods

2.1. Subjects

The subjects were chosen from a group of chronic renal failure patients on HD and PD attending the Renal Dialysis Unit at Parkland Memorial Hospital, Dallas, Gambro Dialysis Unit, Dallas and Dallas Nephrology Associates (Fersenius), Dallas. The exclusion criteria were: (1) smoking, (2) ingestion of vitamin supplements or fish oil, hypolipidemic drugs, prednisone, anticoagulant therapy, thyroxine, oral contraceptives, antioxidant vitamins for the past 6 months except for nephrovite (vitamin B₁ 1.5 mg, B₂ 1.7 mg, B₆ 10 mg, B₁₂ 6 mg, biotin 300 mg, nicotinamide 20 mg, pantothenic acid 10 mg, folate 1 mg, and vitamin C 60 mg); (3) alcohol consumption > 1 oz. per day; and (4) clinical evidence for cardiovascular disease. No more than one third of the dialysis patients were diagnosed with diabetes. The PD group included 17 patients undergoing peritoneal dialysis. The HD group included 16 patients undergoing hemodialysis and the C group included 17 healthy controls. The three groups were matched for age, sex, racial background and body mass index. In the HD group, the causes of ESRD included the following: hypertension ($n=5$), diabetes ($n=1$), systemic lupus erythematosus ($n=2$), glomerulonephritis ($n=2$), reflux nephropathy ($n=1$), and diabetes and hypertension ($n=5$). In the PD group, the causes of ESRD included the following: hypertension ($n=8$), diabetes ($n=1$), diabetes and hypertension ($n=3$), glomerulonephritis ($n=3$), IgA nephropathy ($n=1$), and chronic pyelonephritis ($n=1$). The majority of the time the matched C, HD, and PD patients were studied together as a group.

Fasting blood samples at baseline for isolation of LDL, the plasma lipoprotein profile, plasma fatty acid and plasma and LDL AT were collected in tubes

containing EDTA (1 mg/ml). Blood samples of hemodialysis patients were drawn immediately prior to initiation of dialysis. All blood samples were collected on ice and the plasma was separated by low-speed centrifugation at 4°C. Thereafter, the participants were assigned to receive RRR-AT capsules at a dose of 800 I.U. per day for 12 weeks. They were advised to maintain their usual diet and activities during the 12 weeks and to report any side effects immediately to the investigators. The subjects returned to the clinic at 12 weeks. They continued to take the AT capsules until the day on which blood samples were obtained.

The plasma lipid and lipoprotein levels were assayed by using Lipid Research Clinic's methodology, except that cholesterol and triglyceride levels were determined enzymatically as previously described [29]. Plasma fatty acids at baseline and after 12 weeks of supplementation were measured by gas liquid chromatography after extraction and transmethylation as previously described [29]. An internal standard (C17:0) was added to all samples. Fatty acid standards were obtained from NuChek Prep. Data are expressed in mmol/l for 14:0, 16:0, 18:0, 18:1, 18:2, 18:3, and 20:4.

The concentration of AT was measured in plasma and LDL by reverse-phase high-performance liquid chromatography following ethanol precipitation and hexane extraction as previously described [29]. The plasma levels of AT were standardized to total plasma lipids as previously described [29].

LDL ($d = 1.019\text{--}1.063$ g/ml) was isolated by preparative ultracentrifugation in NaBr–NaCl solution containing 1 mg/ml EDTA as previously described [29]. The isolated LDL was extensively dialyzed against three exchanges (2, 2, and 1 l) of saline EDTA (150 mmol/l NaCl, 1 mmol/l EDTA, pH 7.4) at 4°C over 24 h. Thereafter the LDL was filtered and stored at 4°C under nitrogen until protein was measured by the method of Lowry et al. [29] on the same day using bovine serum albumin as the standard. LDL oxidation was undertaken after an overnight dialysis against 1 l phosphate-buffered saline (PBS), pH 7.4, at 4°C. Thus, oxidation studies were performed within 48 h of LDL isolation by ultracentrifugation. LDL (100 µg/ml) was oxidized in a cell free system using 5 µmol/l copper in PBS at 37°C as previously described [29]. The time course of oxidation was studied over a 5-h period. Duplicates samples were obtained at 0, 0.5, 1, 1.5, 2, 3, 4, and 5 h. Oxidation was stopped by the addition of 200 µmol/l EDTA and 40 µmol BHT to the samples, followed by refrigeration.

Two indices of oxidation were used in this study. Lipid hydroperoxides were quantitated by the FOX assay [30]. The amount of conjugated dienes formed during LDL oxidation was determined by measuring the absorbance of LDL against a PBS blank at 234 nm following 1:4 dilution of the samples in PBS as previ-

ously described [29]. The data are expressed as the increase in conjugated dienes over baseline (ΔA_{234}) [29]. The rate of LDL oxidation was determined from the propagation phase of the time–course curve as previously reported. The lag phase was obtained by drawing a tangent to the slope of the propagation phase and extrapolating it to the horizontal axis. The lag time constitutes the interval from zero time to the intersection point.

2.2. Statistics

Not all analyses were performed on all subjects entered into the study for a variety of reasons including patients not showing up, insufficient samples, etc. The number of subjects in whom data was analyzed is provided in the Tables and Figures. Results are expressed as mean \pm S.D. The Kruskal–Wallis non-parametric test was used to assess overall differences between the three groups for baseline values and responses to supplementation (post-supplementation–baseline differences). Follow-up comparisons of differences between C, HD, and PD were determined by Wilcoxon rank sum tests, if the Kruskal–Wallis test was significant. Differences within groups were determined by Wilcoxon signed rank tests. Pair-wise correlations between variables of interest were determined a priori and Spearman rank correlation coefficients were used to assess these relationships. The level of significance was set at $\alpha \leq 0.05$. Analyses were performed using SAS (SAS Institute, Cary, NC).

3. Results

Table 1 shows the characteristics of the subjects at baseline. Both age and body mass index were similar across the three groups. There were no significant changes in diet, activity level, or weight while the subjects were on supplementation. In addition, none of the subjects reported any adverse effects of supplementation, nor did routine laboratory measurements change. Table 1 also shows the plasma lipid and lipoprotein profiles of the study participants at baseline and after AT supplementation at 12 weeks. Both dialysis groups (HD and PD) had significantly higher plasma triglycerides and significantly lower plasma HDL-cholesterol levels at baseline compared to controls. Furthermore, the HD group had significantly lower LDL-cholesterol levels relative to controls. Supplementation with AT had no effect on plasma lipids or lipoprotein concentrations in any group.

The plasma fatty acid profiles for the subjects are presented in Table 2. The concentrations of fatty acids were not significantly different between groups at baseline, except for significantly higher levels of 18:1 in the

HD and PD groups. Plasma 18:2 was similar at baseline in all groups but after AT supplementation the change in 18:2 was significantly different in HD versus PD ($P < 0.05$).

Baseline lipid standardized AT levels were not significantly different among the three groups. Supplementation with AT 800 I.U. per day for 12 weeks resulted in a significant increase in plasma lipid standardized AT levels in all three groups (Table 3). Similarly, LDL AT was not different between groups at baseline and increased significantly in the C, HD, and PD groups (Table 3). Although the PD group appeared to have greater increases in LDL AT after supplementation, there were no significant differences in responses between groups ($P = 0.52$).

The ratio of plasma AT to plasma polyunsaturated fatty acids (linoleic and arachidonic acid) was also calculated. The ratios of AT to PUFA at baseline were 6.6 ± 3.2 , 4.6 ± 1.4 , and 6.1 ± 1.6 , for the C, HD and PD groups, respectively. The hemodialysis patients had a significantly lower AT/PUFA ratio compared to the other groups ($P = 0.04$) at baseline. After vitamin E supplementation, the ratios were increased similarly in all groups to 12.7 ± 3.7 , 12.0 ± 3.7 , and 15.6 ± 6.3 in the C, HD and PD groups, respectively. There was no significant difference in this ratio between the HD and PD groups.

The kinetics of LDL oxidation (lag phase, maximum oxidation and oxidation rate) were quantified from the time-course curves (Table 4). At baseline the susceptibility of LDL to copper-catalyzed oxidation was similar

Table 1
Group characteristics and lipid and lipoprotein profile^a

	C (n = 17)	HD (n = 16)	PD (n = 17)
Age	39.4 ± 11.6	38.4 ± 11.2	37.2 ± 10.0
Body mass index (kg/m ²)	27.3 ± 5.8	24.0 ± 5.5	26.8 ± 5.9
<i>Total cholesterol (mg/dl)</i>			
Week 0	193.0 ± 29.5	166.6 ± 44.8	192.6 ± 39.6
Week 12	203.2 ± 33.9	173.5 ± 37.2	184.5 ± 45.3
<i>Triglyceride (mg/dl)</i>			
Week 0*	84.2 ± 33.7	199.1 ± 203.5	202.2 ± 139.4
Week 12	97.7 ± 36.2	202.2 ± 259.9	183.9 ± 88.9
<i>LDL cholesterol (mg/dl)</i>			
Week 0**	131.2 ± 28.5	99.3 ± 32.8	121.7 ± 39.5
Week 12	138.7 ± 34.0	109.8 ± 33.9	116.1 ± 45.8
<i>HDL cholesterol (mg/dl)</i>			
Week 0*	50.9 ± 12.5	35.8 ± 12.3	37.9 ± 20.0
Week 12	51.4 ± 13.9	36.2 ± 14.2	35.1 ± 15.3

^a Data are presented as mean ± S.D.

* $P < 0.005$, significance in baseline comparisons via Kruskal-Wallis non-parametric test.

** $P < 0.05$, significance in baseline comparisons via Kruskal-Wallis non-parametric test.

Table 2
Plasma fatty acid profiles for study participants^a

	C (n = 16)	HD (n = 15)	PD (n = 16)
<i>14:0</i>			
Week 0	0.10 ± 0.03	0.16 ± 0.27	0.16 ± 0.09
Week 12	0.11 ± 0.05	0.15 ± 0.21	0.14 ± 0.07
<i>16:0</i>			
Week 0	2.39 ± 0.39	3.39 ± 2.84	3.14 ± 1.06
Week 12	2.50 ± 0.64	3.03 ± 2.16	3.14 ± 0.92
<i>16:1</i>			
Week 0	0.22 ± 0.11	0.36 ± 0.41	0.33 ± 0.22
Week 12	0.26 ± 0.14	0.32 ± 0.39	0.33 ± 0.22
<i>18:0</i>			
Week 0	0.76 ± 0.22	0.94 ± 0.57	0.96 ± 0.39
Week 12	0.73 ± 0.14	0.93 ± 0.76	0.87 ± 0.26
<i>18:1</i>			
Week 0*	2.05 ± 0.58	3.54 ± 2.65	3.50 ± 2.05
Week 12	2.19 ± 0.79	3.53 ± 4.18	3.41 ± 1.60
<i>18:2</i>			
Week 0	3.02 ± 0.61	3.96 ± 2.85	3.42 ± 0.86
Week 12	3.22 ± 0.82	3.20 ± 1.72	3.69 ± 1.04**
<i>18:3</i>			
Week 0	0.06 ± 0.05	0.09 ± 0.10	0.09 ± 0.07
Week 12	0.06 ± 0.04	0.09 ± 0.09	0.08 ± 0.05
<i>20:4</i>			
Week 0	0.64 ± 0.16	0.77 ± 0.38	0.71 ± 0.27
Week 12	0.66 ± 0.19	0.70 ± 0.25	0.71 ± 0.27

^a Values are mean ± S.D. and are given in mmol/l.

* $P < 0.03$, significantly different in baseline comparisons via non-parametric ANOVA.

** $P < 0.05$ change in concentration is significantly different between HD vs. PD, via Kruskal-Wallis test; all other comparisons were non significant.

Table 3
Levels of plasma and LDL AT^a

	C (n = 15)	HD (n = 15)	PD (n = 15)
<i>Plasma lipid-standardized AT (μmol/mmol lipid)</i>			
Week 0	3.1 ± 1.1	2.9 ± 0.9	3.6 ± 1.1
Week 12	7.6 ± 2.7**	7.3 ± 2.9*	11.3 ± 9.3*
<i>LDL-AT (nmol/mg protein)</i>			
Week 0	21.4 ± 10.1	17.9 ± 11.2	21.8 ± 10.5
Week 12	41.6 ± 22.4**	34.7 ± 24.1**	51.2 ± 35.6*

^a Data are presented as mean ± S.D.

* $P < 0.0001$, week 0 vs. week 12, comparisons were made by Wilcoxon signed rank test.

** $P < 0.003$, week 0 vs. week 12, comparisons were made by Wilcoxon signed rank test.

among the three groups. As shown in Table 4, supplementation with 800 I.U. of AT for 12 weeks resulted in a significant prolongation of the conjugated diene lag phase in all groups: C, 34%, $P < 0.005$; HD, 21%, $P = 0.0166$; and PD, 54%, $P = 0.0001$. The increase in

oxidative resistance was greater in the PD group compared with the HD group ($P = 0.026$). A significant increase in the LP lag phase was also observed in the C (27%, $P = 0.0079$) and PD (40%, $P = 0.0009$) but not in the HD group (20%, $P = 0.1937$). Supplementation of control subjects with AT also resulted in a decrease in the maximum amount of conjugated dienes formed ($P < 0.05$) and in the rate of formation of both conjugated dienes and lipid peroxides ($P < 0.05$).

Because all three groups had increases in the LDL lag phase after AT supplementation, the data was pooled in order to determine correlations. The strongest

association observed was between changes in conjugated diene lag phase and plasma lipid standardized AT ($r = 0.542$, $P = 0.0003$, $n = 40$). Changes in lipid standardized AT also correlated significantly with the change in the lag phase of lipid peroxides (Table 5). The correlation between LDL AT and lag phase for either conjugated dienes or lipid peroxides were not significant.

4. Discussion

Premature atherosclerosis is a major cause of morbidity and mortality in patients undergoing long-term dialysis. LDL oxidation might be a major mechanism that results in this premature atherosclerosis. There is widespread acceptance of the hypothesis that oxidation of LDL within the arterial wall is a key early event in the development of atherosclerosis. Increased susceptibility of LDL to in vitro oxidation has been demonstrated in several groups of patients known to be at increased risk of developing atherosclerosis [31,32]. To date, the increased oxidative susceptibility of LDL has been documented with smoking, hypertension, hypercholesterolemia, diabetes and coronary artery disease [33]. Some studies have suggested that AT supplementation can reduce the progression of atherosclerosis in animal models [25–29]. Since supplementation with AT can decrease the susceptibility of LDL to oxidation in human volunteers [25,29] and reduce cardiovascular end points [34,35], the aim of the present study was to determine whether dietary supplementation with AT can reduce the oxidative susceptibility of LDL in patients on HD and PD. In this study, all subjects were given AT 800 I.U. per day for 12 weeks. None of the subjects experienced any side effects as determined by clinical examination, routine laboratory analysis or direct questioning. Furthermore, in none of the groups receiving AT was there a deleterious effect on the plasma lipid and lipoprotein profile. In the present study we show that supplementation with AT (800 I.U. per day) for 12 weeks significantly increased plasma and LDL AT, leading to a significant increase in conjugated diene lag time in all groups and lipid peroxide lag time in the C and PD groups.

The pathogenic mechanisms responsible for the progression of atherosclerosis in the dialysis population have not been completely elucidated. Dyslipidemia often present in patients on long-term dialysis include hypertriglyceridemia, decreased HDL-cholesterol, increased levels of RLP and Lp(a) [5–10]. An important plasma lipid abnormality associated with renal insufficiency is hypertriglyceridemia. Several reports have shown that there is a preponderance of small dense LDL particles in hypertriglyceridemia patients [36–38].

Table 4
Effect of AT on LDL oxidation kinetics^a

	C (n = 15)	HD (n = 15)	PD (n = 15)
<i>Conjugated dienes lag phase (min)</i>			
Week 0	70.74 ± 17.91	74.15 ± 12.24	66.94 ± 13.33
Week 12	94.63 ± 17.20**	89.61 ± 14.35***	103.12 ± 26.40*†
<i>Maximum oxidation (nmol/mg protein)</i>			
Week 0	394.01 ± 68.68	393.75 ± 92.36	380.89 ± 51.42
Week 12	356.48 ± 97.85***	395.49 ± 91.38	395.97 ± 112.16
<i>Oxidation rate (nmol/mg protein/min)</i>			
Week 0	7.24 ± 2.11	7.37 ± 2.10	6.72 ± 2.27
Week 12	5.59 ± 1.85***	7.18 ± 2.66	5.82 ± 1.67
<i>Lipid peroxides lag phase (min)</i>			
Week 0	79.21 ± 20.13	82.45 ± 27.91	80.58 ± 17.13
Week 12	100.32 ± 18.55***	98.85 ± 18.17	112.55 ± 29.42*
<i>Maximum oxidation (nmol/mg protein)</i>			
Week 0*	568.84 ± 105.24	549.13 ± 139.78	557.39 ± 114.70
Week 12	540.64 ± 196.11	607.88 ± 156.65	605.33 ± 136.53
<i>Oxidation rate (nmol/mg protein/min)</i>			
Week 0	8.63 ± 2.25	8.94 ± 2.06	8.38 ± 2.56
Week 12	6.86 ± 2.24***	9.00 ± 2.99	7.86 ± 1.80

^a Data are presented as mean ± S.D.

* $P < 0.001$, week 0 versus week 12, comparisons were made by Wilcoxon signed rank test.

** $P < 0.005$, week 0 versus week 12, comparisons were made by Wilcoxon signed rank test.

*** $P < 0.05$, week 0 versus week 12, comparisons were made by Wilcoxon signed rank test.

† $P < 0.03$, significant difference in delta lag time between HD and PD, comparison was made by Kruskal-Wallis test.

Table 5
Spearman rank correlation coefficients of AT levels and LDL lag phase

ΔAT	ΔLag conjugated dienes	ΔLag lipid peroxides
Plasma lipid standardized AT	0.54*	0.40**
LDL AT	0.23	0.24

* $P < 0.0005$.

** $P < 0.01$.

Small dense LDL is more prone to oxidation than large buoyant LDL [37,38]. This might explain the increased LDL oxidation reported by some previous investigators [18,19]. McEneny et al. [17] and Daerr et al. [39] have also reported increased peroxidative modification of VLDL in chronic hemodialysis patients. However, in the present report, in accordance with most investigators [21–23], we did not demonstrate an increased susceptibility of LDL to oxidation. Possible reasons for this include: most of the dialysis subjects were taking Nephrovite, they were not severely hypertriglyceridemic, plasma 18:1 fatty acids were increased and the dialysis membrane was biocompatible polysulphane (high flux) rather than cuprophane. It should be noted that a nonbiocompatible dialysis membrane may activate polymorphonuclear leukocyte resulting in ROS production which leads to an increased susceptibility of LDL to oxidation in dialysis patients [40,41]. After hemodialysis, RBC show increased membrane lipid peroxidation, reduced membrane fluidity, and increased osmotic fragility [42–44]. However, a recent study failed to show increased lipid peroxidation with cuprophane dialysis compared to cellulose acetate or polysulfone membrane dialysis [20].

In patients on chronic hemodialysis therapy, decreased levels of water-soluble vitamins have been mainly attributed to substrate losses induced by dialysis. Ascorbate is generally considered to be a key aqueous-phase antioxidant, and ascorbate deficiency may contribute significantly to oxidative stress in these patients. In contrast, fat-soluble vitamins are bound in plasma to specific plasma proteins and/or to lipoproteins and no elimination is to be expected during dialysis. Decreased, normal and even increased plasma concentrations of fat-soluble vitamins, such as vitamin E, have been described in patients on regular hemodialysis therapy [11,18,45–47]. In our study we found that the HD group tended to have lower plasma AT levels at baseline, but there were no significant differences between the various groups when AT was standardized for plasma lipids. Furthermore, LDL AT levels were not significantly decreased. Supplementation with AT for 12 weeks resulted in a significant increase in plasma AT in the C, HD, and PD groups, respectively. Similarly, we found LDL AT was not different between groups at baseline, and increased significantly in all groups. Although the PD group appeared to have greater increases in LDL AT after supplementation, there were no significant differences in responses between groups.

The susceptibility of LDL to oxidation *in vitro* is widely interpreted as an indicator of its atherogenic potential and AT is the most abundant antioxidant present in LDL.[24–26,28] Vitamin E supplementation (oral or intramuscular) has been shown to decrease levels of MDA in plasma, RBC, mononuclear cells and

platelets [48–51]. AT supplementation prevents oxidative stress by increasing RBC vitamin E and appears to improve the efficacy of erythropoietin in hemodialysis patients [52]. However, these studies appear to be confined to HD patients. There is scanty data on the effect of AT supplementation on LDL oxidation in patients on PD. The lag phase correlates inversely with the severity of clinical atherosclerosis; thus, prolongation of the lag phase with AT could prove beneficial. Panzetta et al [18] reported that vitamin E (50 I.U. per day) supplementation in HD patients for 30 days significantly increased the lag phase. However, they studied HD patients only and used a fluorescence method to measure LDL oxidation. Also, this group administered a low dose of Vitamin E (50 I.U. per day) for a shorter period (30 days). In our study, to obtain a better appreciation of the effect of AT on LDL oxidation we monitored LDL oxidation by using two different indices of oxidative modification, the formation of conjugated dienes and lipid peroxides and we studied both HD and PD patients for a longer duration (3 months). Supplementation with 800 I.U. per day of AT for 12 weeks resulted in a significant prolongation of the conjugated diene lag phase in the C, HD, and PD groups. The increase in oxidative resistance was greater in the PD group compared with HD group. An increase in the LP lag phase was also observed in the C and PD but not in the HD group. Since the increment in conjugated diene lag phase was greater in the PD group than the HD group and the prolongation of the lipid peroxide lag phase was only significant in the PD group, one could speculate that the PD group responds better to AT therapy. Since LDL AT and the ratio of AT/PUFA were not significantly different between the HD and PD groups, we cannot readily explain the greater benefit seen in the PD group. Our findings also show significant positive correlations between plasma lipid standardized AT and the lag phase of oxidation but not with LDL AT. It is unclear why a better correlation was seen with plasma AT than LDL AT. However, we have observed this previously [29,53]. It is possible that enrichment of plasma LDL and HDL with AT reduces preformed lipid peroxides which appear to be the substrate for copper-catalyzed LDL oxidation, thus explaining the better correlation with the LDL lag phase. Also, with a larger sample size these correlations could become significant.

Our study confirms a significant effect of AT on plasma and LDL AT, whereas the plasma lipid or lipoprotein profile remained unchanged after AT administration for 12 weeks. AT enrichment of LDL resulted in a significant increase in conjugated diene lag time in all groups and lipid peroxide lag time was also increased significantly in the C and PD groups after AT supplementation. In conclusion, the results of the present study show that the supplementation with AT

increased its level in the plasma and LDL of subjects on dialysis and decreased LDL oxidative susceptibility. The major novel observation as it relates to antioxidants and LDL oxidation is that this is the first study to show that there is a significant protection of LDL oxidation with 800 I.U. per day of AT in both HD and PD patients. Also, the benefit appears to be greater in the PD patients. Future studies will be directed at examining the effect of AT on surrogates of clinical atherosclerosis.

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Editorial comment

Therapy and clinical trials Ishwarial Jialal^{a,b} and Nicola Abate^b

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Despite serum cholesterol being a strong risk factor for coronary artery disease (CAD), its predictive power for stroke is weak. In the Cholesterol and Recurrent Events (CARE) trial [1•], the investigators tested the effect of lipid lowering with the use of pravastatin on cerebrovascular events in patients with pre-existing CAD. In a large study undertaken in 4159 subjects with a mean total and LDL cholesterol of 209 and 139 mg/dl, respectively, stroke was a pre-specified secondary endpoint. Subjects were assessed over a median 5 year follow-up period. Patients were well matched for stroke risk factors and the use of antiplatelet agents (85% of subjects in each group). Compared with placebo, pravastatin lowered LDL cholesterol 32%, triglycerides 14% and raised HDL cholesterol 5% over the course of the trial. Pravastatin therapy resulted in a 32% reduction ($P=0.03$) in all-cause stroke and a 27% reduction ($P=0.02$) in stroke or transient ischemic attack (TIA). All categories of stroke were reduced and treatment effects were similar when adjusted for age, sex, history of hypertension, smoking, diabetes, left ventricular ejection fraction and a baseline total, LDL and HDL cholesterol and triglyceride levels. No increase was observed in the incidence of hemorrhagic stroke in patients on pravastatin compared with placebo. This observation is very significant, because the CARE study [1•] is the first to show clearly, as a defined secondary endpoint, that pravastatin therapy significantly reduces both stroke and stroke and TIA after myocardial infarction in patients with average serum cholesterol levels, despite the high concurrent use of antiplatelet therapy. Because patients who have already experienced myocardial infarction are at an increased risk of subsequent stroke, this is a major observation in the field of clinical lipidology, and thus gives greater impetus to lipid lowering in such patients, not only for reducing cardiovascular events but also cerebrovascular disease. However, one should emphasize that the CARE study [1•], like the Scandinavian Simvastatin Survival Study, which also showed a benefit of simvastatin on stroke and TIA, were both secondary prevention trials. No primary prevention study to date

has shown a significant reduction in stroke and TIA with the use of lipid-lowering therapy. Another important observation in the study is that the reduction of stroke and TIA with pravastatin therapy was observed despite the high background use of platelet inhibitor therapy, which has been documented to protect against cerebrovascular disease. To date, no plausible mechanism can be offered to explain this benefit. However, pravastatin has effects on the vasculature that have been well documented, including plaque stability, suppression of inflammation, improvement of endothelial function and platelet anti-aggregant activity. One could speculate that one or more of these mechanisms accounted for the reduction in stroke and TIA.

It is widely appreciated that inflammation is crucial in the pathogenesis of the atherosclerotic lesion. C-reactive protein (CRP) appears to be an independent predictor of cardiovascular events. In a preliminary study [2•], the investigators showed, in 66 hyperlipidemic patients after therapy with either simvastatin (20 mg/day) or atorvastatin (20 mg/day), a significant reduction in CRP levels. Although these results are clearly preliminary, they point to the added benefit of statin therapy in being anti-inflammatory. A major weakness of the report is that the investigators could not compare the effects of the two statins on CRP levels. It has previously been reported that pravastatin reduces the risk of recurrent myocardial infarction in patients with signs of inflammation at baseline, including elevated levels of CRP. The study, although preliminary, is provocative in suggesting that the statin group of drugs, pravastatin, simvastatin, and atorvastatin have anti-inflammatory effects in addition to their well-described beneficial effects on the plasma lipid profile. The superiority of the effects of any of these drugs on inflammation will only be settled by future studies.

Tocopherols and tocotrienols are two classes of compounds with vitamin E activity. Each group comprises four different isomers indicated α , β , γ and δ , which have different biological activity. Tocopherols are present in most vegetable oils and are more common in the diet than tocotrienols, which are found at relatively high concentrations in palm oil and rice bran oil. Much epidemiological data, which appears to be supported by clinical trials to date, has suggested that tocopherols, especially α -tocopherol, might be beneficial with respect to cardiovascular disease. The effects of α -tocopherol

could be through its effects as an antioxidant (decreasing LDL oxidation), its anti-inflammatory effects such as decreasing monocyte activity, the inhibition of platelet aggregation, improving endothelial function and inhibiting smooth muscle cell proliferation. There is no data to support a beneficial effect of α -tocopherol on the plasma lipid profile. However, tocotrienols in various animal species appear to lower LDL cholesterol, in addition to their antioxidant activity. This has not been supported by all investigators. In addition, tocotrienols have been suggested to have effects on platelet aggregation, which has also not been confirmed by all investigators. Mensink *et al.* [3**] examined in detail, in a randomized, double-blind placebo controlled trial, the effects of vitamin E concentrate rich in tocotrienols on the serum lipoprotein profile and platelet function in men at risk of cardiovascular disease. The study included 20 men receiving four capsules a day for 6 weeks, each capsule containing 35 mg tocotrienol and 20 mg α -tocopherol; and 20 other men receiving four capsules a day of 20 mg α -tocopherol only. All men had elevated concentrations of serum cholesterol (251–309 mg/dl). Compliance was confirmed by changes in serum tocopherol and tocotrienol concentrations after the 6 week study. No significant effect of the tocotrienol-enriched vitamin E concentrate was observed versus the so-called placebo (the tocopherol group) with regard to plasma lipid profile. The study also failed to show a beneficial effect of tocotrienol on platelet function. The well-conducted study suggested that, although it had previously been suggested that tocotrienols might have a beneficial effect on the plasma lipid profile, especially lowering total and LDL cholesterol, this did not appear to be the case. A study examining the effect of the purified α , β , γ , and δ -tocotrienols on the lipid profile in hypercholesterolemic individuals is urgently needed to settle the question of whether tocotrienols have a beneficial effect on the lipid profile.

The recent 'statin trials' have provided strong evidence of a beneficial effect of cholesterol-lowering therapy on both the primary and secondary prevention of cardiovascular disease morbidity and mortality. An important observation from those trials is that the reduction in cardiovascular events is evident in the first 6 months of treatment, a time period probably insufficient to induce significant anatomical changes of the atherosclerotic artery. On the basis of these and other experimental observations, some investigators have proposed that lipid-lowering therapy has an acute effect on the stabilization of the plaque, followed by a chronic remodelling of the atherosclerotic plaque and diseased artery. Several studies now support the view that lipid-lowering therapy acutely resolves endothelial dysfunction and later stabilizes the fibrous cap of the atherosclerotic plaque. Dupuis *et al.* [4**] evaluated the effects

of cholesterol reduction on endothelial function in humans. A group of patients with acute myocardial infarction or unstable angina, who also had hypercholesterolemia, were randomly selected to receive either placebo or pravastatin 40 mg a day. Thirty patients per group were studied at baseline and at the end of the 6 week treatment period. A reduction in LDL cholesterol of 33% in the patients on pravastatin was associated with a significant increase in the endothelium-dependent dilation evaluation of the brachial artery, measured using post-ischemic ultrasound. The short-term study emphasized an acute effect of lipid-lowering therapy that may contribute to improved event rate, and support the notion that early lipid-lowering intervention should be used for patients with myocardial infarction or unstable angina. The study of Dupuis *et al.* [4**] did not evaluate the possible mechanisms involved. A plausible mechanistic explanation could be found in a restoration of endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) production in the endothelium promoted by treatment with a lipid-lowering agent. eNOS is the enzyme that synthesizes NO from arginine in the endothelial cell. It has been observed that the loss of NO production is an early manifestation of an atherosclerotic lesion. This abnormality may be secondary to a direct effect of LDL cholesterol. Therefore, acute LDL cholesterol lowering may account for an acute amelioration of eNOS activity and vascular function, which will result in vasodilation. On the other hand, some investigators suggested that statins may ameliorate eNOS function directly and independently of LDL cholesterol lowering.

The hypothesis that statins could directly improve eNOS was recently explored by Kaesemeyer *et al.* [5**] using pravastatin and simvastatin in a model of cultured bovine aortic endothelial cells. These investigators utilized the thoracic aorta of male Sprague-Dawley rats to study the effect of two statins, simvastatin and pravastatin, on vasorelaxation function. The authors found that pravastatin and simvastatin induced vasodilation in the isolated aorta. The elimination of the endothelium abolished the effects of statins. Because the study model did not involve changes in cholesterol levels, the vasodilation effect of statins was attributed to a direct amelioration of endothelial function. NO production was found to be reduced in cultured bovine aortic cells perfused with statins. eNOS messenger RNA was found to be increased by statins, particularly pravastatin.

Other agents may reverse endothelial dysfunction in atherosclerotic arteries through the same mechanism of eNOS activation. Gokce *et al.* [6**] recently reported a study on the effects of ascorbic acid administration on endothelial function in humans with CAD. Forty-six

patients with angiographically established CAD were randomly assigned to receive single dose (2 g, by mouth) and long-term (500 mg/day) ascorbic acid treatment or placebo. The study was double-masked. The patients were studied at baseline, acutely after administration of the 2 g dose and after 30 days of chronic ascorbic acid administration. Flow-mediated vasodilation was studied by post-ischemic ultrasound evaluation of the brachial artery. The study showed that flow-mediated vasodilation improves within 2 h of ascorbic acid administration, and the effect persists unchanged with chronic administration of ascorbic acid in patients with CAD. Although the mechanism was not evaluated in the study of Gokce *et al.* [6**], improved NO production may again be responsible for the observed effects. The effects of the therapeutic intervention were independent of changes in plasma lipid concentrations.

The studies discussed above would suggest that an acute effect of lipid-lowering therapy and antioxidants is the regression of abnormalities in endothelial NO production observed in atherosclerotic arteries. This effect has been related to LDL lowering. However, a direct pharmacological activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors or antioxidants, i.e. ascorbic acid, may also play a significant role. Whatever the mechanism, the reversal of endothelial dysfunction can be obtained very quickly and can result in an acute improvement of event risk. Therefore, a prompt initiation of lipid-lowering therapy should be advocated in all patients diagnosed with CAD.

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- 3 Mensink RP, van Houwelingen AC, Kromhout D, Hornstra G. A vitamin E concentrate rich in tocotrienols had no effect on serum lipids, lipoproteins, or platelet function in men with mildly elevated serum lipid concentrations. *Am J Clin Nutr* 1999; 69:213-219.
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- 5 Kaesemeyer WH, Caldwell RB, Huang JZ, Caldwell RW. Pravastatin sodium activates endothelial nitric oxide synthase independent of its cholesterol-lowering actions. *J Am Coll Cardiol* 1999; 33:234-241.
- 6 Gokce N, Keaney JF, Frei D, Holbrook M, Olcslak M, Zachariah BJ, et al. Long-term ascorbic acid administration reverses endothelial vasomotor dysfunction in patients with coronary artery disease. *Circulation* 1999; 99:3234-3240.

Recommended reading

- Ballantyne CM, Herd JA, Ferlic LL, Dunn JK, Farmer JA, Jones PH, et al. Influence of low HDL on progression of coronary artery disease and response to fluvastatin therapy. *Circulation* 1999; 99:736-743.
- In this study, referred to as the Lipoprotein and Coronary Atherosclerosis Study, in patients with mildly to moderately elevated LDL cholesterol and low levels of HDL cholesterol, the investigators compared angiographic progression and the benefits of the statin, fluvastatin, in patients with low versus high HDL cholesterol. In patients on placebo with low HDL cholesterol (<35 mg/dl), significantly greater angiographic progression was observed than in those with higher HDL cholesterol. Fluvastatin significantly reduced progression among low HDL cholesterol patients compared with placebo. The treatment effect of fluvastatin on the change in minimal lumen diameter was significantly greater among low than among high HDL patients. Also, among low HDL cholesterol patients, fluvastatin improved event-free survival compared with placebo. Although the predominant lipid-modifying effect of fluvastatin is to decrease LDL cholesterol, patients with low HDL cholesterol thus received the greatest angiographic and clinical benefit. This study should prompt further studies examining combined therapy with both a LDL-lowering agent with a drug known to raise HDL to see if one obtains additional benefits with regard to cardiovascular events.
- de Longier M, Salen P, Paillard F, Lacon P, Richard G. Lipid-lowering drugs and homocysteine. *Lancet* 1999; 353:209-210.
- In this study, the investigators tested the effect of two hyperlipidemic drugs, simvastatin and fenofibrate, on sulphur-containing amino acid levels in 57 clinically stable men. The interesting observation in the study is that after 12 weeks of therapy, fenofibrate caused a significant increase in sulphur-containing amino acids (methionine, 20%, cysteine, 60% and homocysteine, 46%). However, simvastatin had no significant effect on the homocysteine level and, in fact, had the additional effect of increasing arginine levels, which is the precursor of NO, a major mediator of coronary vasodilation. It thus appears that the improvement in endothelial function with simvastatin therapy might be caused by increasing the precursor of NO in addition to its lipid-lowering effects. Whether the elevation of homocysteine seen with fenofibrates attenuates its benefits with regards to cardiovascular disease will only be settled by future studies.
- Dupuis J, Tardif JC, Cernacek P, Theroux P. Cholesterol reduction rapidly improves endothelial function after acute coronary syndromes—the RECIFE (Reduction of Cholesterol in Ischemic and Function of the Endothelium trial). *Circulation* 1999; 99:3227-3233.
- This clinical study evaluated the acute effect of pravastatin on endothelial dysfunction in patients with CAD and hypercholesterolemia, soon after an acute event (myocardial infarction or unstable angina). Although other studies have previously shown a role of lipid-lowering therapy on the amelioration of endothelial dysfunction in patients with CAD, the above investigation has demonstrated a specific and rapid improvement of endothelial-dependent vasodilation in a unique group of patients characterized by an unstable plaque and at very high risk of event recurrence.
- Gokce N, Keaney JF, Frei D, Holbrook M, Olcslak M, Zachariah BJ, et al. Long-term ascorbic acid administration reverses endothelial vasomotor dysfunction in patients with coronary artery disease. *Circulation* 1999; 99:3234-3240.
- This study demonstrated that ascorbic acid can improve the arterial function of patients with CAD both acutely and chronically. Although the mechanism of improved vasodilation was not investigated, it was not associated with changes in lipid levels and may be the result of an independent effect of ascorbic acid on the amelioration of endothelial dysfunction.
- Kaesemeyer WH, Caldwell RB, Huang JZ, Caldwell RW. Pravastatin sodium activates endothelial nitric oxide synthase independent of its cholesterol-lowering actions. *J Am Coll Cardiol* 1999; 33:234-241.
- This study identifies a mechanism for improved endothelial-dependent vasodilation with lipid-lowering therapy. The increased production of eNOS observed in cultured bovine cells perfused with statins supports the hypothesis that statins may 'directly' induce the restoration of NO production. This mechanism could complement the beneficial effects of statins on arterial function mediated by their lipid-lowering action.
- Kano H, Hayashi T, Sumi T, Esaki T, Asai Y, Takur KN, et al. A HMG-CoA reductase inhibitor improved regression of atherosclerosis in the rabbit aorta without affecting serum lipid levels: possible relevance of up-regulation of endothelial NO synthase mRNA. *Biochem Biophys Res Commun* 1999; 259:414-419.
- This study examined the question of a 'direct' effect of statins on vascular function. The effects of fluvastatin on vascular tone, NO production and eNOS mRNA expression were evaluated in the thoracic aortas of rabbits. This study included three groups of male rabbits with hypercholesterolemia who were either treated with atherogenic diet only (group A1), or received a 12 week regular diet after the atherogenic diet (group A2), or received 12 weeks of regular diet and fluvastatin after the atherogenic diet (group A3). eNOS mRNA was measured using competitive reverse transcriptase polymerase chain reaction assays on the thoracic aorta of the animals. The rabbits treated with regular diet after the atherogenic diet period (group A2) had lower eNOS mRNA levels, compared with the rabbits of group A1 that were fed an atherogenic diet only, thus indicating progression of endothelial damage. However, despite no reduction in cholesterol, eNOS mRNA levels did not decrease in the rabbits treated with fluvastatin. These data would suggest that HMG-CoA reductase inhibitors may have a direct effect on eNOS mRNA levels, and may therefore maintain normal NO production and vasodilation response of the atherosclerotic arteries.
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- This is an interesting observation with a dietary supplement (tocotrienol-enriched vitamin E) on the lipid profile. It had previously been suggested that tocotrienols reduced LDL cholesterol. However, in this carefully performed study, no significant effect of tocotrienol-enriched palm oil was observed on the lipid profile or on platelet function.
- Plehn J, Davis BR, Sacks FM, Rouleau JL, Pfeffer MA, Bernstein V, et al. Reduction of stroke incidence after myocardial infarction with pravastatin: the Cholesterol and Recurrent Events (CARE) study. *Circulation* 1999; 99:216-223.
- This is the first clear demonstration that pravastatin therapy in patients with pre-existing coronary disease with average LDL cholesterol reduces stroke and stroke/TIA (32 and 20% reduction, respectively). This benefit persisted after adjustment for known risk factors for stroke. This effect was seen despite the high background use of platelet inhibitor therapy.
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- This study evaluated the effects of therapy with lipid-lowering agents and insulin sensitizers on the collagen content in plaques of Watanabe heritable hyperlipidaemic rabbits. The study included four groups of rabbits with similar plasma cholesterol levels. One group was of untreated rabbits (controls), a second group was treated with pravastatin, a third group was treated with troglitazone and a fourth group was treated with the combination of pravastatin and troglitazone. The collagen content in the atherosclerotic plaques of the rabbits treated with troglitazone was significantly decreased compared with the control group, whereas the extracellular lipid deposition was significantly decreased in the pravastatin- and combination-treated groups. The evaluation of the atherosclerotic surface area in the thoracic aorta and coronaries demonstrated a synergistic effect of pravastatin and troglitazone on atherosclerosis regression. The two different mechanisms of action of the two drugs may explain the observed synergisms. The results of this study support a direct effect of insulin resistance on atherogenesis, reversed with an insulin sensitizer. If confirmed in humans, these findings would favor an additional target of therapy for cardiovascular disease prevention in type 2 diabetes with the use of insulin sensitizers.
- Strandberg TE, Vanhanen H, Tikkanen MJ. Effect of statins on C-reactive protein in patients with coronary artery disease. *Lancet* 1999; 353:118-119.
- This is a very provocative paper that suggests that statin therapy (simvastatin and atorvastatin) reduces CRP. This adds to the literature on the non-lipid effects of the statin group of drugs, especially with relevance to inflammation.

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Vitamin E Supplementation and Cardiovascular Events in High-Risk Patients

To the Editor: We would like to comment on the design and interpretation of the recent study by the Heart Outcomes Prevention Evaluation (HOPE) investigators on vitamin E supplementation and cardiovascular events (Jan. 20 issue).¹ Although this study was undertaken in many geographic areas (the United States, Canada, western Europe, and South America) with different dietary intakes, dietary data (especially on antioxidants) were not reported. In addition, for no subgroup were plasma levels of vitamin E provided to confirm supplementation. Whereas the investigators in the Cambridge Heart Antioxidant Study (CHAOS), the Alpha-Tocopherol, Beta Carotene Cancer Prevention (ATBC) study, and the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI) study used alpha-tocopherol, the HOPE investigators used natural-source vitamin E, which consists of tocopherols and tocotrienols. Since alpha-tocopherol is the most potent member of the vitamin E family, this could have a bearing on their findings, since there is scanty information on the other forms of vitamin E.

We disagree with the HOPE investigators' interpretation of the prospective studies as they related to alpha-tocopherol supplementation. In the majority of the studies, a positive result was obtained for either a primary end point or a secondary end point. In CHAOS, there was a significant benefit of alpha-tocopherol with regard to the primary end point of cardiovascular death or nonfatal myocardial infarction (a 47 percent reduction). Steiner and coworkers² showed that in patients with transient ischemic attacks, minor strokes, or residual neurologic deficits, the combination of alpha-tocopherol and aspirin as compared with aspirin

alone resulted in a significant reduction in ischemic strokes and recurrent transient ischemic attacks. The ATBC investigators showed that alpha-tocopherol supplementation was associated with a decrease in the incidence of angina pectoris.³ In the GISSI study, alpha-tocopherol supplementation resulted in significant reductions in cardiovascular events, deaths from cardiac causes, deaths from coronary causes, and sudden deaths, even though the primary end point was not significantly affected.⁴ Thus, we believe that the conclusions of the HOPE investigators — that the prospective studies of alpha-tocopherol do not appear to suggest a benefit — is inappropriate.

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Dr. Yusuf replies:

To the Editor: The comments by Jialal and Devaraj do not alter the primary conclusion of the HOPE study and two other large trials — that vitamin E has not been shown to reduce cardiovascular events. First, epidemiologic studies conducted in North America and western Europe suggested that intake of vitamin E at doses larger than 100 IU per day for more than two years was associated with lower rates of cardiovascular disease.¹ In populations recruited from these areas, the HOPE study evaluated vitamin E at doses of 400 IU per day and indicated no evidence of benefit in preventing cardiovascular events over a period of approximately five years. Second, the bioavailability and antioxidant activity of the natural vitamin E preparation used in the HOPE study have been documented to be at least as good

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as those of synthetic vitamin E.² Third, Table 4 of our article shows results for the most important composite cardiovascular end point in four major trials involving more than 50,000 subjects. No benefit is evident.

Jialal and Devaraj emphasize differences in selected end points (which are variable among the trials) after a post hoc analysis of several secondary outcomes. CHAOS³ was relatively small, had imbalances in numerous base-line characteristics, and was relatively short (with a median follow-up of 1.3 years, a period during which it is unlikely that atherosclerotic clinical events could be prevented). The differences observed in preventing nonfatal myocardial infarction in CHAOS were based on very few events and could reflect a type I error. Furthermore, Steiner et al.⁴ did not report a reduction in strokes or myocardial infarction, and the claim of a reduction in ischemic events is based on few events and a selective emphasis on the composite end point of ischemic (not all) strokes and transient ischemic attacks in the "final" (not entire) phase of the study. Both GISSI-Prevenzione,⁵ based on a predefined analysis of all randomly assigned patients, and the ATBC study⁶ showed no effect on the end points used in CHAOS or in HOPE. Therefore, the overall data from all the studies show no benefit associated with vitamin E in preventing myocardial infarction, stroke, or death during five years of treatment. We await the results of other ongoing trials as well as data from longer-term follow-up in our trial.

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Tuberculosis in a Child in North Dakota

To the Editor: In an informative report, Curtis et al. describe extensive transmission of tuberculosis by an adopted nine-year-old child (Nov. 11 issue).¹ However, I think the authors of the paper and the author of the accompanying editorial² overlooked a factor that probably was involved in the development of tuberculosis in this child. It seems clear that the child was not infected in North Dakota, where the tuberculosis case rate is about 2 per 100,000 population, but rather in his native Marshall Islands, where the case rate is 104 per 100,000 population. A tuberculin skin test was performed shortly after his arrival in North Dakota in 1996, but unfortunately the result was not assessed. He appar-

ently was well at that time, and his tuberculosis developed two years later.

Tuberculosis is common among persons from the Indian subcontinent who immigrate to the United Kingdom,³ in part because in the winter months dark-skinned people may be deficient in 1,25-dihydroxyvitamin D₃, an important immunoregulatory hormone.⁴⁻⁶ The Marshall Islands lie in the Pacific Ocean at a latitude of only 9 degrees north, where it is never cold enough to require staying indoors or dressing warmly. North Dakota is at about 46 degrees north, so there is a great difference in sunlight between the two locations for several months each year.

Finally, Douglas et al.⁷ have shown that the peak incidence of tuberculosis among persons with dark pigmentation who immigrate to the United Kingdom is in July (the month in which the tuberculosis of the source case was found), in contrast to the winter peak for other respiratory diseases. Perhaps the reason for the delayed development of tuberculosis after the winter nadir of vitamin D₃ is that it takes several months for a dormant tuberculosis infection to reactivate and produce sufficient symptoms for the illness to be diagnosed.

This experience should teach us that when dark-skinned people immigrate to northern states or Canada from areas where tuberculosis is common and sunshine abundant year round, we should be sure that they have adequate vitamin D to enable their immune system to function properly year round.

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The authors reply:

To the Editor: Stead suggests a causal relation between 1,25-dihydroxyvitamin D₃ deficiency and the development of tuberculosis, on the basis of the temporal peak in the diagnosis of tuberculosis among dark-skinned immigrants from the Indian subcontinent and on the basis of other data suggesting impaired host defenses.¹ However, this hypothesis of seasonal vitamin D deficiency is unlikely to account for the development of tuberculosis in the source case described in our report. Tuberculosis was diagnosed in this child in July, and the disease probably developed before the winter months. The boy was the source of the tuberculous osteomyelitis in his mother, whose symptoms started in

Improvement of Endothelial Vasomotor Dysfunction by Treatment With Alpha-Tocopherol in Patients With High Remnant Lipoproteins Levels

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- OBJECTIVES** This study sought to examine whether oral intake of alpha-tocopherol, an antioxidant, could improve endothelium-dependent vasorelaxation in patients with high remnant lipoproteins levels.
- BACKGROUND** Remnant lipoproteins are known to be atherogenic and impair endothelium-dependent arterial relaxation, but the underlying mechanisms remain unclear. Oxidative stress is a common feature of various risk factors for atherosclerosis.
- METHODS** Flow-mediated vasodilation of the brachial artery during reactive hyperemia was examined by high resolution ultrasound technique before and at the end of 4 weeks treatment with oral administration of alpha-tocopherol acetate (300 IU/day) or placebo, which was randomly assigned, in 40 patients with high serum levels of remnants and in 30 patients with low remnant levels in the fasting state (>75th percentile and <25th percentile, respectively, of the distribution of remnant levels in 150 consecutive hospitalized patients).
- RESULTS** Before treatment, flow-mediated vasodilation was lower in patients with high remnant levels than in those with low levels ($4.1 \pm 0.3\%$ vs. $6.0 \pm 0.5\%$, $p < 0.01$). Treatment with alpha-tocopherol but not with placebo significantly increased flow-mediated dilation in patients with high remnant levels ($7.5 \pm 0.4\%$ after alpha-tocopherol vs. $4.2 \pm 0.4\%$ after placebo, $p < 0.01$). In patients with low remnant levels, alpha-tocopherol was not effective. The beneficial effect with alpha-tocopherol in high remnant patients was associated with decrease in plasma levels of thiobarbituric acid reactive substances, an indicator of lipid peroxidation (6.6 ± 0.3 nmol/ml before alpha-tocopherol vs. 4.6 ± 0.3 after alpha-tocopherol, $p < 0.05$).
- CONCLUSIONS** Alpha-tocopherol improved impairment of endothelium-dependent vasodilation in patients with high remnant levels. The increase in oxidative stress may at least partly contribute to endothelial vasomotor dysfunction in patients with high remnant levels. (J Am Coll Cardiol 1999;33:1512-8) © 1999 by the American College of Cardiology

There is increasing evidence that triglycerides and triglycerides-rich lipoproteins are atherogenic (1-3). We recently showed that remnant lipoproteins have an important and causative role in the impairment of endothelium-

dependent vasodilation, an early sign of atherosclerosis, in patients with postprandial hypertriglyceridemia (4,5). However, the underlying mechanisms for the remnant-induced endothelial dysfunction remain to be determined. Recent reports (6-10) showed that the increase in oxidative stress is a common feature of various risk factors for atherosclerosis and that oxidative stress mediated by reactive oxygen species has a causative role in endothelial dysfunction not only in patients with coronary artery disease but also in patients with coronary risk factors and free of obvious coronary atherosclerosis. Thus, we hypothesized that the increase in oxidative stress may also participate in endothelial dysfunction.

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Abbreviations and Acronyms

- apo = apolipoprotein
- LDL = low density lipoproteins
- TBARS = thiobarbituric acid reactive substances
- VLDL = very low density lipoproteins

tion in patients with high levels of remnant lipoproteins. Flow-mediated vasodilation in brachial artery has been used as a noninvasive method to assess endothelial function (7,8,11,12).

Thus, this study examined effects of oral administration of alpha-tocopherol, an antioxidant, for four weeks on endothelial vasomotor functions in brachial artery of patients with high and low serum levels of remnant lipoproteins in a randomized and placebo-controlled manner.

METHODS

Study population. The study population included 40 consecutive patients with high serum levels of remnant lipoproteins and 30 consecutive patients with low remnant levels, all of whom underwent diagnostic coronary angiography for atypical chest pain in Kumamoto University Hospital. This study excluded patients treated with lipids-lowering drugs, pharmacologic doses of other antioxidants, angiotensin-converting enzyme inhibitors or calcium channel blocking agents at least a month before and during this study. Clinical features of the study patients are shown in Table 1. Beta-adrenergic blocking agents, nitrates or diuretics were used in some of the study subjects with coronary artery disease or hypertension before and throughout this study, but there was no significant difference in the rates of each of these medications used among the study subgroups. Type III hyperlipoproteinemia was found in two of the patients

with high remnants levels. Diabetes mellitus was complicated in 14 of the patients with high remnants levels. The exact cause of high remnants levels was unknown in the remaining patients. Patients with previous myocardial infarction, congestive heart failure, valvular heart disease or other serious diseases were also excluded from this study. High and low serum levels of remnants lipoproteins were determined as levels >75th percentile (>5.1 mg cholesterol/dl) and <25th percentile (<2.4 mg/dl), respectively, on the basis of the distribution of serum remnants levels in 150 consecutive patients hospitalized in the Cardiology Section of this hospital, as described in our previous report (4). Written informed consent was obtained from all patients before the study. This study was in agreement with the guidelines approved by the ethics committee at our institution.

Study protocol. The study patients were randomly assigned to have oral intake for 4 weeks of alpha-tocopherol acetate (300 IU/day, 20 and 15 patients with high and low remnants levels, respectively) or placebo (similar-appearing placebo tablet, 20 and 15 patients with high and low remnants levels, respectively) using a random number table generated by a computer. All of the patients were blinded to the content of the tablet. Our preliminary data showed that this dose of alpha-tocopherol increased its content in the isolated remnants by two- or threefold. They were advised to adhere to their usual diet and lifestyle throughout four weeks. All of the study patients completed the trial. Measurements of vasoactivity in the brachial artery and blood sampling were performed in overnight fasting state on the same morning before and at the end of the treatment, in exactly the same manner before and at the end of the treatment. All medications were withdrawn 12 h before the measurements.

Table 1. Clinical Characteristics of Alpha-Tocopherol- and Placebo-Treated Groups in Patients With High and Low Remnants Levels

	High Remnants (n = 40)		Low Remnants (n = 30)	
	Alpha-Tocopherol (n = 20)	Placebo (n = 20)	Alpha-Tocopherol (n = 15)	Placebo (n = 15)
Age (yr)	58.3 ± 2.1	59.6 ± 2.1	61.7 ± 2.3	60.8 ± 1.9
Men/women	8/12	9/11	6/9	7/8
Smokers	8 (40.0)	7 (35.0)	6 (40.0)	6 (40.0)
Hypercholesterolemia	7 (35.0)	8 (40)	3 (20.0)	3 (20.0)
Hypertension	6 (30.0)	7 (35.0)	4 (26.7)	5 (33.3)
Diabetes mellitus	7 (35.0)	7 (35.0)	3 (20.0)	3 (20.0)
Coronary artery disease	3 (15.0)	3 (15.0)	2 (13.3)	2 (13.3)
One-vessel disease	1 (5.0)	2 (10.0)	2 (13.3)	1 (6.7)
Two-vessel disease	2 (10.0)	1 (5.0)	0	1 (6.7)
Body mass index (kg/m ²)	23.3 ± 2.9	23.4 ± 2.8	22.9 ± 1.7	22.8 ± 1.9

Values are expressed as mean value ± SE (in age and body mass index) and number of patients. Values in parentheses indicate % of total patients. Smoker, ≥10 cigarettes/day for 10 years; hypercholesterolemia ≥240 mg/dl of serum cholesterol levels; hypertension, blood pressure ≥140/90 mm Hg or on an antihypertensive medication; diabetes mellitus, fasting blood glucose ≥140 mg/dl or on an antidiabetic medication.

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Measurements of flow-mediated dilation in brachial artery. Vasodilator responses in the brachial arteries were measured by use of B-mode ultrasound images with a 7.5-MHz linear array transducer (SSH-160A ultrasound system, Toshiba Corp., Tokyo, Japan) as validated previously by our and other studies (7,8,11,12). Measurements were performed by two observers who were blinded to the protocols of the study and the subject grouping. The brachial artery was scanned in the antecubital fossa in a longitudinal fashion. Optimal brachial artery images were obtained between 1 and 5 cm above the antecubital crease. This location was marked, and all subsequent images were obtained at the same location. The exact distance of the measured point of the skin surface from the antecubital crease was recorded in each study subject to ensure that the same point of the brachial artery was measured 4 weeks later. Gain setting was optimized at the beginning of the study and was kept constant throughout the recording period. After baseline measurements of the diameter and flow velocity in the brachial artery, a blood pressure cuff placed around the forearm was inflated with a pressure of 250 to 300 mm Hg for 5 min, and then the cuff was released. Diameter measurements during the reactive hyperemia were taken 45 to 90 s after the cuff deflation. Then, sublingual nitroglycerin (300 μ g) was administered, and 3 min later the measurements were repeated. Images were recorded on a super-VHS videocassette recorder (model BR-S601M, Victor Corp., Tokyo, Japan), and brachial arterial diameters were measured from the tape with ultrasonic calipers, as described previously (8,11,12). Responses of the vessel diameters to the reactive hyperemia and nitroglycerin were expressed as a percentage increase in the diameter from the baseline value. The diameter responses were assessed at three points along a 10-mm length of the artery, and the diameter responses were averaged. Blood flow was calculated by multiplying the velocity-time integral of the Doppler flow signal by heart rate and the vessel cross-sectional area. Increase in brachial blood flow was calculated as a maximum flow recorded in the first 15 s after the cuff deflation and was expressed as a percentage increase in the flow from the baseline value.

Measurements of remnant lipoproteins levels. Remnant lipoproteins levels were measured in overnight fasting serum. Remnant lipoproteins were isolated by the application of the fasting serum to the immunoaffinity mixed gel that contained anti-apolipoprotein (apo)A-1 and anti-apoB-100 monoclonal antibodies (Japan Immunoresearch Laboratories, Takasaki, Japan) as validated in our previous reports (4,5,13). Cholesterol concentrations in the isolated fraction were measured by the enzymatic method (4,5,13). According to the analyses with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, elution profiles with high performance liquid chromatography, agarose gel electrophoretograms, electron photomicrographs and compositions of lipids and apolipoproteins, the lipoproteins isolated

by this method mainly consisted of remnants of very low density lipoproteins (VLDL) (data not shown), as demonstrated in our previous reports (4,5,13). The fasting remnants levels, measured in this study, were found to be well correlated with the postprandial levels, as shown in our previous study (4).

Plasma levels of alpha-tocopherol and thiobarbituric acid reactive substances (TBARS) and measurement of susceptibility of remnants to oxidative modification. Blood sampling was performed just before the ultrasound study. The plasma levels of alpha-tocopherol were determined by high performance liquid chromatography (14). The plasma level of TBARS, an indicator of lipid peroxides in plasma, was determined as previously described (15,16). Briefly, 2.0 ml of trichloroacetic acid-thiobarbituric acid-HCl reagent was added to 1.0 ml of sample and vortexed. To minimize peroxidation during the assay procedure, butylated hydroxytoluene was added to the thiobarbituric acid reagent mixture. The results were expressed as malondialdehyde equivalent content (nmol malondialdehyde/ml plasma). The susceptibility of remnants to oxidative modification was determined by measuring Cu^{2+} -induced formation of conjugated dienes. The conjugated dienes formation in remnants was assayed by monitoring the change in absorbance at 234 nm in a spectrophotometer as described previously (17).

Statistical analysis. Data are expressed as mean \pm SEM. The effects of the treatment on lipids profiles, hemodynamic parameters, brachial arterial parameters and plasma alpha-tocopherol and TBARS levels were compared by two-way analysis of variance with repeated measures followed by post hoc testing with Scheffé test by computer statistical software package SAS, version 6.12 (Cary, North Carolina). Difference between two means among the subgroups were performed by two-tailed paired or unpaired Student *t* test, as appropriate. Difference in frequencies of risk factors among the subgroups was compared using chi-square test. Statistical significance was defined as $p < 0.05$.

RESULTS

Clinical characteristics of study patients. Clinical features and lipids profiles before treatment were comparable between alpha-tocopherol-treated patients and placebo-treated patients in either patients with high or low remnants levels (Tables 1 to 3). Levels of total cholesterol and triglycerides were significantly higher in patients with high remnants levels than in those with low remnants levels (total cholesterol, 212 ± 15 mg/dl in high remnants patients vs. 186 ± 11 mg/dl in low remnants patients, $p < 0.05$; triglycerides, 201 ± 19 mg/dl in high remnants patients vs. 101 ± 16 mg/dl in low remnants patients, $p < 0.01$). After treatments, levels of remnants cholesterol, total cholesterol, high density lipoprotein cholesterol, and triglycerides were

Table 2. Effects of Treatments in Patients With High Remnants Levels

	Alpha-Tocopherol (n = 20)		Placebo (n = 20)		Two-Way Analysis of Variance p Value
	Before	After Treatment	Before	After Treatment	
Remnants cholesterol (mg/dl)	6.5 ± 1.2	6.2 ± 1.1	6.4 ± 0.5	6.3 ± 0.3	NS
Total cholesterol (mg/dl)	214 ± 9.9	213 ± 9.3	210 ± 10	206 ± 23	NS
HDL cholesterol (mg/dl)	49 ± 4	50 ± 5	49 ± 4	51 ± 7	NS
Triglyceride (mg/dl)	196 ± 14	189 ± 21	205 ± 22	200 ± 19	NS
Alpha-tocopherol (μmol/liter)	23 ± 1.2	51 ± 4.2*	25 ± 1.5	23 ± 1.9	< 0.01
TBARS (nmol MDA/ml)	6.6 ± 0.3	4.6 ± 0.3†	6.3 ± 0.3	6.3 ± 0.4	< 0.05
Heart rate (beats/min)	61 ± 5.3	63 ± 4.3	62 ± 5.1	62 ± 6.5	NS
Mean blood pressure (mm Hg)	88 ± 5.2	90 ± 7.2	88 ± 7.1	90 ± 6.4	NS
Resting arterial diameter (mm)	3.90 ± 0.19	3.91 ± 0.18	3.88 ± 0.16	3.89 ± 0.21	NS
Resting arterial blood flow (ml/min)	188 ± 10	190 ± 9	192 ± 9	193 ± 12	NS
Increase in arterial blood flow (%)	236 ± 15	237 ± 20	234 ± 20	234 ± 17	NS
Dilator response to nitroglycerine (%)	17.3 ± 5.4	17.4 ± 5.7	17.6 ± 5.9	17.9 ± 7.1	NS

*p < 0.01, †p < 0.05 vs. before alpha-tocopherol using two-way analysis of variance followed by post hoc testing with Scheffé test. Values are expressed as mean value ± SE. HDL = high density lipoprotein; MDA = malondialdehyde equivalent content; TBARS = thiobarbituric acid reactive substances.

not significantly changed as compared with those before treatments in each of the subgroups (Tables 2 and 3).

Flow-mediated dilation in brachial artery. Before treatment, heart rates, mean blood pressure, baseline arterial diameter of brachial artery, baseline blood flow of brachial artery and percentage increase in the blood flow during reactive hyperemia were comparable between alpha-tocopherol-treated patients and placebo-treated patients in either patients with high or those with low remnants levels (Tables 2 and 3). Flow-mediated dilation of the brachial artery before treatment was significantly lower in patients with high remnant lipoproteins levels as compared with that in those with low remnants levels, but it was comparable between alpha-tocopherol and placebo groups in patients with high remnants levels as well as those with low remnants levels (percentage increase in arterial diameter from baseline, 4.1 ± 0.2% in high remnants patients with

alpha-tocopherol vs. 4.2 ± 0.4% in high remnants patients with placebo, p = 0.67; 6.2 ± 0.7%* in low remnants patients with alpha-tocopherol vs. 5.8 ± 0.3%† in low remnants patients with placebo, p = 0.21; *p < 0.01 vs. high remnants patients with alpha-tocopherol, †p < 0.01 vs. high remnants patients with placebo). The dilator response to sublingual nitroglycerin before treatment was comparable among the subgroups (Tables 2 and 3).

After treatment, neither alpha-tocopherol treatment nor placebo treatment affected heart rates, mean blood pressure, baseline arterial diameter of brachial artery, baseline blood flow of brachial artery and percentage increase in the blood flow during reactive hyperemia in both patients with high and those with low remnants levels (Tables 2 and 3). Treatment with alpha-tocopherol significantly increased the flow-mediated dilation as compared with placebo treatment in patients with high remnants lipoproteins levels (Fig. 1).

Table 3. Effects of Treatments in Patients With Low Remnants Levels

	Alpha-Tocopherol (n = 15)		Placebo (n = 15)		Two-Way Analysis of Variance p Value
	Before	After Treatment	Before	After Treatment	
Remnants cholesterol (mg/dl)	1.8 ± 0.4	1.9 ± 0.5	1.7 ± 0.9	1.7 ± 0.1	NS
Total cholesterol (mg/dl)	186 ± 11	187 ± 15	185 ± 12	182 ± 14	NS
HDL cholesterol (mg/dl)	53 ± 6	54 ± 9	52 ± 3	52 ± 3	NS
Triglyceride (mg/dl)	101 ± 19	95 ± 22	103 ± 13	101 ± 16	NS
Alpha-tocopherol (μmol/liter)	21 ± 1.9	47 ± 5.5*	23 ± 1.7	20 ± 1.1	< 0.01
TBARS (nmol MDA/ml)	5.9 ± 0.4	4.4 ± 0.3†	6.0 ± 0.2	6.2 ± 0.5	< 0.05
Heart rate (beats/min)	61 ± 6	62 ± 5	60 ± 5	60 ± 9	NS
Mean blood pressure (mm Hg)	87 ± 6	87 ± 7	87 ± 7	90 ± 8	NS
Resting arterial diameter (mm)	3.90 ± 0.22	3.91 ± 0.21	3.91 ± 0.18	3.90 ± 0.20	NS
Resting arterial blood flow (ml/min)	191 ± 14	191 ± 11	192 ± 13	191 ± 15	NS
Increase in arterial blood flow (%)	232 ± 16	232 ± 16	230 ± 18	230 ± 20	NS
Dilator response to nitroglycerin (%)	17.9 ± 6.2	18.1 ± 5.9	17.6 ± 6.1	17.9 ± 7.7	NS

*p < 0.01, †p < 0.05 vs. before alpha-tocopherol using two-way analysis of variance followed by post hoc testing with Scheffé test. Values are expressed as mean value ± SE. Abbreviations as in Table 2.

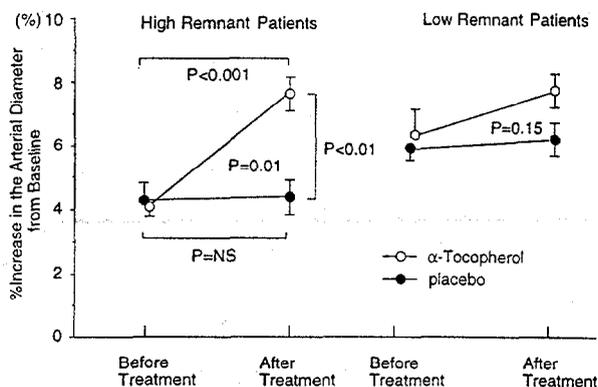


Figure 1. Percentage increase in brachial arterial diameter from baseline during reactive hyperemia before and after treatment with alpha-tocopherol (open circles) or placebo (closed circles) in patients with high remnant lipoproteins levels ($n = 20$ patients in each treatment) and in those with low remnants levels ($n = 15$ patients in each treatment). Significant effect of alpha-tocopherol treatment was observed in patients with high remnant lipoproteins levels as compared with that of placebo but not in those with low remnants levels (by two-way analysis of variance followed by post hoc testing with Scheffé test).

In patients with low remnants levels, alpha-tocopherol did not significantly increase the flow-mediated dilation as compared with placebo treatment (Fig. 1). Alpha-tocopherol treatment also improved flow-mediated dilation in a subgroup of normocholesterolemic and nondiabetic patients (<240 mg/dl of serum cholesterol levels, <140 mg/dl of fasting blood glucose, no lipids-lowering medication and no antidiabetic medication) with high remnant lipoproteins levels (percentage increase in the arterial diameter, $4.6 \pm 0.3\%$ before treatment vs. $7.8 \pm 0.8\%$ after treatment, $n = 10$, $p < 0.01$). Neither alpha-tocopherol nor placebo treatment changed the dilator response to nitroglycerin in either patients with high or those with low remnants levels (Tables 2 and 3).

Levels of alpha-tocopherol and TBARS and oxidative susceptibility of remnants. Plasma levels of alpha-tocopherol were significantly increased after alpha-tocopherol treatment in both patients with high and those with low remnant lipoproteins levels, whereas the levels were not significantly changed after placebo treatment (Tables 2 and 3). Treatment with alpha-tocopherol but not with placebo significantly decreased plasma levels of TBARS in both patients with high and those with low remnants levels (Tables 2 and 3). The lag time for Cu^{2+} -induced conjugated dienes formation of remnant lipoproteins was longer after treatment with alpha-tocopherol as compared with that after placebo (480 ± 58 min after alpha-tocopherol vs. 230 ± 18 min after placebo, $n = 5$, $p < 0.01$).

DISCUSSION

The present study demonstrated that flow-mediated vasodilation in brachial arteries was impaired in patients with

high remnant lipoproteins levels, a result which is consistent with data observed in human coronary arteries as shown in our previous report (4). The present study further showed that oral administration of alpha-tocopherol for four weeks increased flow-mediated vasodilation in brachial artery of patients with high remnant lipoproteins levels as compared with placebo treatment. The present study also showed that alpha-tocopherol treatment did not affect vasodilation in response to nitroglycerin, an endothelium-independent vasodilator, in either group of patients. Thus, the present study indicates that alpha-tocopherol improved endothelium-dependent arterial dilation in patients with high remnant lipoproteins levels. The improvement of the endothelial dysfunction is unlikely to be due to changes of lifestyle and habits, since profiles of plasma lipids, blood pressure and habits were not altered after treatment as compared with those before treatment, and placebo treatment in the otherwise identical protocol as alpha-tocopherol treatment did not affect the endothelial vasomotor function.

Oxidative stress and remnant lipoproteins. Alpha-tocopherol, a lipid soluble antioxidant, has been shown to protect endothelial cells against oxidative damage (18-21). Previous reports showed that alpha-tocopherol supplementation restored endothelial function in parallel with suppression of lipid peroxidation in low density lipoproteins (LDL) in experimental hypercholesterolemic animals (19,21). In this regard, the present study also showed that the improvement of endothelium-dependent vasodilation was associated with decrease in plasma TBARS levels, an indicator of lipid peroxidation, after alpha-tocopherol treatment in patients with high remnants levels. This strongly suggests that the beneficial effect of alpha-tocopherol, as shown in the present study, was at least partly mediated through prevention of endothelial function from oxidative stress-induced injury in patients with high remnant lipoproteins levels. We have previously shown that oxidatively modified LDL causes endothelial dysfunction and has an important role in atherosclerotic impairment of various endothelial functions (15). It has recently been reported that oxidatively modified remnant lipoproteins caused greater formation of foam cells in *in vitro* experiments than native remnant lipoproteins (22), suggesting that oxidized remnant lipoproteins may also play an important role in atherogenesis in hyperlipidemic patients. Our preliminary study showed that oxidized remnant lipoproteins exhibited greater impairment of endothelium-dependent relaxation of isolated rabbit aortas than native remnants (unpublished data). In patients with high remnant lipoproteins levels, the prolonged retention of remnants in the circulation, as a result of the delayed hepatic uptake and/or the increased hepatic secretion of VLDL (23,24), may augment susceptibility of remnants to oxidative modification in the arterial intima, leading to increase in injurious effects of remnants on endothelial function. Among antioxidants, alpha-tocopherol is the major antioxidant contained in lipoproteins (25). Thus, the improve-

ment of endothelial function by alpha-tocopherol in the present study may at least partly occur as a result of protection of remnant lipoproteins against oxidative modification in the arterial intima.

In patients with low levels of remnant lipoproteins, the decrease in TBARS after alpha-tocopherol treatment was not associated with an increase in the endothelium-dependent vasodilation as compared with placebo, though endothelium-dependent dilation in these patients was not impaired before treatment on the basis of our data in healthy adults. The results are in agreement with those in the previous reports (7-10) showing that the beneficial effect of ascorbic acid, another antioxidant, on vasomotor function was exerted in only patients with impaired endothelium-dependent dilation. This presumably may be due to less oxidative damage on endothelial function in patients with low levels of remnant lipoproteins. However, it cannot be completely excluded that alpha-tocopherol exerted the beneficial effects on endothelial functions in high levels of remnant lipoproteins independent of its antioxidant action. In this context, it is interesting that alpha-tocopherol is capable of suppressing activity of protein kinase C, which is suggested to play a role in the impairment of endothelium-dependent vasorelaxation in atherosclerotic arterial walls (26,27).

Hypercholesterolemia and diabetes mellitus, independent factors of endothelial dysfunction, coexisted with high levels of remnant lipoproteins, as shown in the present and previous studies (4,28). Thus, there is a possibility that the beneficial effect of alpha-tocopherol on endothelial function in patients with high remnants levels may result from the improvement of endothelial function in patients with hypercholesterolemia and/or diabetes mellitus. However, this possibility cannot explain all of the present data, since beneficial effect of alpha-tocopherol was observed also in normocholesterolemic and nondiabetic patients with high remnants levels, as shown in the present study.

Measurement of remnant lipoproteins levels. Remnant lipoproteins are known to have heterogeneous properties (4,29). Thus, simple and reliable methods to separate remnant lipoproteins had not been established. The current method with immunoaffinity mixed gel containing anti-apoA-1 and anti-apoB-100 monoclonal antibodies has been reported to be capable of isolating apoE-rich VLDL particles containing apoB-100 together with chylomicron remnants containing apoB-48, neither of which binds to the immunoaffinity gel (4,5,13,29). This unique anti-apoB-100 monoclonal antibody has been shown to recognize apoB-100 in LDL and most VLDL but not in apoE-enriched VLDL (13). Remnants levels in the postprandial state may be a better predictor (2,30). However, we have recently shown that fasting levels of remnant lipoproteins are an independent predictor for the presence of endothelial dysfunction in human coronary arteries (4). Thus, the measurement of fasting levels of remnant lipoproteins using the

current immunoaffinity gel seems to have a good theoretical basis and could be useful to quantify levels of the atherogenic triglyceride-rich lipoproteins.

Previous studies. The results of the present study are compatible with those of previous studies showing beneficial effects of either vitamin C (7-10), a water soluble antioxidant, or alpha-tocopherol (31) and probucol (32), lipid soluble antioxidants on the endothelial function. However, Gilligan and others (33,34) showed negative results on the effects of antioxidant vitamins. Different backgrounds, such as the associated risk factors in the study population, may possibly cause the incompatible results among the studies.

Study limitations. Many assays are available for measurement of lipid peroxidation, but no single assay accurately reflects free radical generation. Thiobarbituric acid reactive substances measurement is also susceptible to artifacts caused by variation in sample lipid content and iron contamination of reagents. In the present study, we prevented auto-oxidation of the samples by addition of butylated hydroxytoluene to the samples.

Conclusions. The present study showed that alpha-tocopherol improved impairment of endothelium-dependent vasodilation in patients with high remnant lipoproteins levels. Oxidative stress may at least partly play a role in the abnormal endothelial vasomotor function in patients with high remnant lipoproteins levels.

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The relationship of oxidized lipids to coronary artery stenosis

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Abstract

A total of 1200 patients with angina were cardiac catheterized establishing that 63% had 70–100% stenosis, 12% had 10–69% stenosis of one or more of their coronary arteries and 25% had microvascular angina listed as 0% stenosis. Prior to catheterization 10 ml of blood was drawn and the plasma subjected to analysis for the concentration of cholesterol, lipid peroxides (LPX), total antioxidant capacity (TAOC), fibrinogen (FB), ceruloplasmin (CP) and activation of polymorphonuclear leukocytes (PMNLs). Comparisons were made to non-smoking controls without angina. Significant differences in LPX were found between the patients with 0 and 10–69% stenosis ($P < 0.001$), with 10–69 and 70–100% stenosis ($P < 0.001$), and with 0 and 70–100% stenosis ($P < 0.001$). Under 70 years of age there was a significant difference in LPX between patients with all levels of stenosis and age and sex matched controls ($P < 0.001$). Differences in the mean plasma cholesterol concentration for different levels in the degree of stenosis were not significant, indicating that LPX provided consistent data on the severity of stenosis while the plasma cholesterol concentration did not. Compared with controls an increase in activation of PMNLs ($P < 0.01$), an increase in concentration of both FB and CP ($P < 0.01$) and a decrease in total antioxidant capacity were noted in the plasma of catheterized patients. In summary the concentration of oxidation products rather than the concentration of cholesterol in the plasma identified stenosis in cardiac catheterized patients. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Oxidized lipids; Antioxidants; Cholesterol; Stenosis of the coronary arteries; Fibrinogen; Ceruloplasmin; Polymorphonuclear leukocytes

1. Introduction

Two recent reviewers [1,2] discussed the hypothesis that low density lipoprotein (LDL) must be oxidatively modified to oxidized LDL (ox-LDL) in order to trigger the pathological events leading to atherosclerosis. An increase in ox-LDL, found in patients with coronary artery disease [3,4] and in patients subjected to heart transplants [5], has been credited with an important role in the initiation and progression of atherosclerosis [6,7]. The increase in ox-LDL correlated not only with the extent of coronary artery stenosis but also with the development of the stenosis [5]. These oxidation

products induced damage of cellular membrane, caused loss of cell viability [8], inhibited wound-healing response of vascular endothelial cells [9] and produced an increase in cytosolic Ca^{2+} [10]. This increase was associated with irreversible morphological changes. Ox-LDL also induced activation of endothelial recruitment of leukocytes, enhancement of macrophage cytokine production and stimulation of smooth muscle cell proliferation [11]. The survival of cells was significantly reduced [12] when ox-LDL was added into a culture medium.

In vitro studies indicate that oxidation in the plasma is challenged by a host of defense mechanisms [13] including ceruloplasmin, fibrinogen, transferrin and albumin [14]. Albumin inhibits both metal and peroxy radical mediated lipid peroxidation of LDL [14,15]. We

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found that free and albumin-bound bilirubin (BR) at the physiological concentration of the bile pigment in blood plasma could greatly inhibit [16] metal-catalyzed oxidation of low density lipoprotein (LDL). This was shown by the reduced thiobarbituric acid reactivity, the smaller or lack of shifts in electrophoretic mobility, the less apo B fragmentation and the decreased amount of cholesterol oxidation products as detected by gas chromatography.

Sustained presence of lipid peroxides in the circulation is a controversial issue [17–22]. Yla-Hertuala [23] believes that clinical and epidemiological studies have provided circumstantial evidence that oxidized LDL as measured by serum autoantibody levels may be associated with the progression of atherosclerotic vascular disease. In the present study with cardiac catheterized patients with proven angina we analyzed the concentration of acute phase proteins, lipid oxidation products (LPX) and the total antioxidant capacity (TOAC) in their plasma. These values were compared with plasma obtained from age and sex matched non-smoking, non-cardiac catheterized individuals (controls) without apparent coronary heart disease (angina).

2. Methods

2.1. Patient source

Of the patients admitted to Carle Foundation Hospital with chest pains, those having an esophageal source of pain were eliminated during initial examination. Routine cholesterol screening was carried out on fasted blood in a Beckman CX-7 autoanalyzer as part of the initial examination. The autoanalyzer was checked for accuracy at least eight times daily against a control furnished by Beckman Inc. An electrocardiogram (ECG) was performed. A written informed consent for the cardiac catheterization and blood tests was obtained from each patient. Protocol approved by the research review boards of both Carle Foundation Hospital and the University of Illinois was followed.

The videotape of each cardiac catheterized patient was identified by patient clinic number and was viewed in a suite equipped with a video viewer which could be stopped to evaluate the percentage of stenosis as suggested by Bain and Grossman [24]. During our study 1203 patients were catheterized, revealing that 63% had 70–100% stenosis, 12% had 10–69% stenosis of one or more of the large coronary arteries and 25% had no stenosis of any of the large coronary arteries. However, they had microvascular angina also known as Syndrome X disease [25–28] which we listed as 0% stenosis.

2.2. Biochemical determinations

Prior to catheterization 10 ml of blood was drawn into tubes containing citrate or heparin but no antioxidant. The tube was immediately stored in chilled ice, transferred to the laboratory and centrifuged at 2800 rpm for 10 min at 4°C. The plasma was subjected to analyses within minutes after centrifugation for LPX, TOAC, FB, CP and for polymorphonuclear leukocytes (PMNLs), listed as the stimulatory index (SI). LPX analysis was determined according to the thiobarbituric acid reaction, as described by Duthie [29]. Thiobarbituric acid reactive species (TBARS) were expressed as μmol malondialdehyde/l plasma. A Medline search indicated that 240 studies since 1994 have relied on this method to measure the level of oxidation products in the plasma of animal models and in human diseases. As Gutteridge and Halliwell [30] have pointed out, lipids are not the only source for TBARS in the plasma. We were aware of their precaution. We measured TAOC by chemiluminescence and expressed the results in arbitrary units, using ascorbic acid as standard according to Popov's method [31] with one unit of TAOC equivalent to 4.1 μmol ascorbic acid/l.

The separation of cells from blood to obtain a suspension of PMNLs was accomplished by using a two step discontinuous Percoll kit (Sigma). The dilution of PMNLs to a concentration of 1×10^6 cells/ml was carried out with Hanks' balanced salt solution (Gibco, Life Technol, Grand Island, NY) The chemiluminescence technique recommended by Trush [32] was used for the measurement of PMNLs activation by using zymosan^A (Sigma) opsonized with serum [33]. The activation of leukocytes was expressed as the SI, the ratio of chemiluminescence value at 12 min after zymosan addition as compared with an identical sample without opsonized zymosan [33]. The chemiluminescence (SI) emission produced by the activated PMNLs was measured with a Beckman scintillation counter LS 3801 equipped with a single photon counting program and recorded as U/l.

The commonly used techniques for measuring fibrinogen (FB) and fibrinogen degradation products (FDP) in the plasma do not discriminate between FP and FDP as a result of both induce similar impairment of homeostatic functions [34–37]. In order to test the inhibitor properties of FB and FDP on oxidation, we purchased from Sigma (St. Louis, MO) FB type I from human plasma. This plasma contained $\approx 60\%$ protein composed of fibrin, fibrinopeptide A and FB degrading compounds X and Y. The FB was dialyzed against 50 mM Na–K phosphate buffer, pH 7.3., and the concentration was measured at 280 nm by using a conversion factor of $E(1\%)_{280} = 15$. The purified FB solution was adjusted to 5 mg/ml. FB was measured with Sigma kit No. 880-B, and FDP was measured with

a Sigma kit (Proc. No. 850) and recorded as mg/dl. For subjects with low FB levels a second technique was added based on calcium precipitation and subsequent protein measurement [38]. The FB level was reported as the total FB and FDP.

Ceruloplasmin (CP) is an acute phase protein that serves as a circulating scavenger of oxygen derived free radicals [39]. The CP concentration in the plasma was determined according to Lehmann et al. [40] and was expressed in international units U/l.

To determine if dietary intake of fatty acid differed between the patients and controls, we extracted 1 ml of plasma with 10 ml of chloroform/methanol (2:1). BHT was added as an antioxidant at a concentration of 0.005% (w/v) to all solvents and all operations were carried out under nitrogen. The lipid extract was saponified, acidified and converted to methyl esters. The composition of fatty acids in each sample was determined in a Packard Model 428 gas chromatograph equipped with an all-glass injection splitter and flame ionization detector. Retention time, peak areas and relative peak area percentages were determined electronically using a Hewlett Packard Model 3390A Reporting Integrator. Identification of methyl esters of fatty acids was accomplished by comparing relative retention times with authentic standards [41].

3. Statistical analysis

The statistical analysis was done with S-PLUS and SAS statistical software packages. A multivariate logistic regression model was fitted to the data to determine the significance of each covariate. Student's paired *t*-test and Welch modification of the *t*-test were used for testing significance. A *P*-value of less than 0.05 was taken as the level of significance. The results were reported as means \pm SD.

4. Results

A significant difference was found in the means of LPX between cardiac catheterized patients with 0 and 10–69% stenosis ($P < 0.001$), between patients with 10–69 and 70–100% stenosis ($P < 0.001$) and between patients with 0 and 70–100% stenosis or 2.87, 3.29 and 3.56 $\mu\text{mol/l}$ of LPX, respectively ($P < 0.001$). Relationships between the plasma cholesterol concentration and the lipid peroxide concentration (LPX) are provided in (Fig. 1). The means of plasma cholesterol concentration at 0, 10–69 and 70–100% stenosis were 201.9 ± 40.2 , 203.2 ± 41.9 and 207.0 ± 50.6 mg/dl, respectively. The differences between plasma cholesterol concentration at all levels of stenosis were not significant ($P = 0.94$, $P = 0.40$, $P = 0.25$). LPX, therefore, provided consistent data on the severity of the stenosis up to 70 years of age while the plasma cholesterol concentration did not. Two men age 52 and 54 and one woman age 49 had plasma cholesterol concentrations of more than 450 mg/dl (Fig. 1c). A total of ten catheterized patients had cholesterol concentrations below 100 mg/dl. Five men age 67–79 had plasma cholesterol concentrations ranging between 67 and 98 mg/dl and five women age 24–82 had plasma cholesterol concentrations ranging between 60 and 96 mg/dl, respectively. Three patients had plasma cholesterol concentrations below 100 mg/dl because of cancer [42].

Data on LPX and TAOC are summarized in Table 1. Up to 70 years of age the LPX concentration increased with the severity of the degree of stenosis. However, the severity of stenosis at 70–100% was as high for 75 patients less than 50 years of age as for 275 patients more than 70 years of age. The TAOC decreased as the degree of stenosis increased but was no longer a significant factor after 70 years of age. Differences in LPX and TAOC for 0 and 70–100% stenosis were all significant ($P < 0.001$) for age and sex matched cardiac

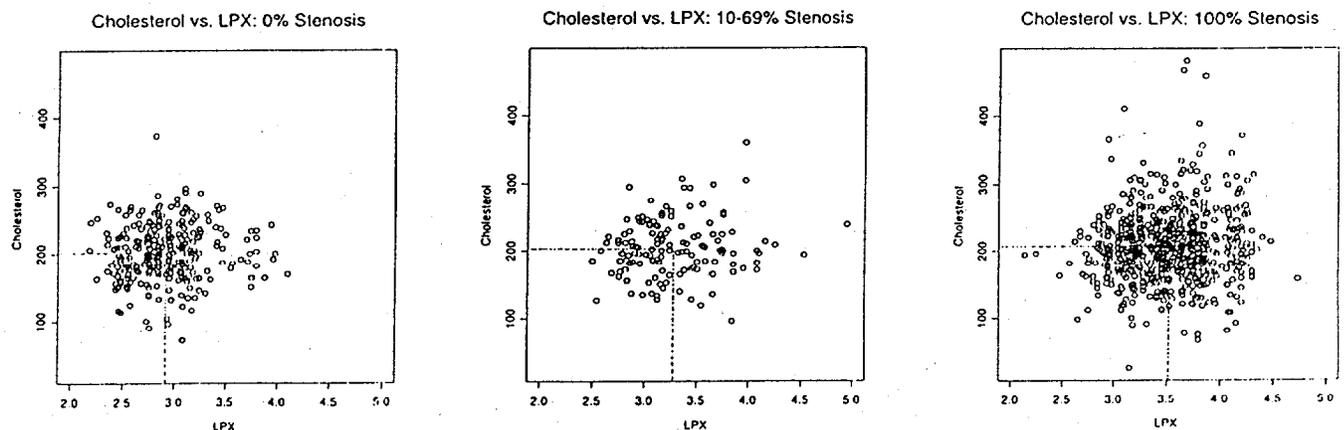


Fig. 1. The plasma cholesterol concentration vs. the concentration of oxidation products (LPX) at 0, 10–69 and 70–100% stenosis of 1200 cardiac catheterized patients with angina. At 51–69 years of age the LPX was 2.92 at 0% stenosis, 3.19 at 10–69% stenosis and 3.48 $\mu\text{mol/l}$ at 70–100% stenosis. The plasma cholesterol concentration was 201.9 at 10% stenosis, 203.2 at 10–69% stenosis and 207.5 mg/dl at 70–100% stenosis.

Table 1
The concentrations of lipid peroxides (LPX) and total antioxidant capacity (TAOC) in the plasma from the cardiac catheterized patients and controls^a

Degree of stenosis	Age (years)		
	< 50	51–69	> 70
<i>LPX (μmol/l)</i>			
Controls (%)	2.43 ± 0.39 (32)	2.43 ± 0.48 (21)	3.24 ± 0.78 (20)
0	2.87 ± 0.32 (89)**	2.92 ± 0.36 (150)**	2.98 ± 0.39 (63)
10–69	3.29 ± 0.47 (18)**	3.19 ± 0.37 (80)**	3.42 ± 0.48 (46)
70–100	3.56 ± 0.36 (75)**	3.48 ± 0.39 (400)**	3.54 ± 0.44 (275)
<i>TAOC (Units/l)</i>			
Controls (%)	21.76 ± 3.00 (32)	20.80 ± 4.05 (21)	17.96 ± 3.63 (20)
0	20.25 ± 2.50 (89)**	20.13 ± 2.23 (150)	19.20 ± 1.86 (63)
10–69	18.97 ± 1.64 (18)**	18.87 ± 1.68 (80)*	18.06 ± 1.49 (46)
70–100	17.21 ± 1.38 (75)**	17.73 ± 1.50 (400)**	17.31 ± 1.78 (275)

^a The number of people in each group is indicated in parenthesis. Values expressed as mean ± SD. Significantly different as compared with respective matched control for $P < 0.05$ (*); $P < 0.01$ (**). Tests of significance used the Welch modification of the *t*-test.

catheterized patients compared with the controls (Fig. 2). There was a significant difference in LPX between patients with 0, 10–69 and 70–100% stenosis and matching control groups younger than 70 years ($P < 0.001$).

Approximately 51% of the men with 70–100% stenosis of the coronary arteries had plasma cholesterol concentrations below 200 mg/dl, 34% had concentrations between 200 and 240 mg/dl, and only 15% of the men had plasma cholesterol levels above 240 mg/dl (Table 2). A similar situation occurred among the women with 70–100% stenosis of their coronary arteries although the differences were not as great. Approximately 35% of the women with 70–100% stenosis had plasma cholesterol values below 200 mg/dl, 32% between 200 and 240 mg/dl and 33% had plasma cholesterol values above 240 mg/dl. Therefore, the percentage of women with 70–100% stenosis with plasma concentration over 240 mg/dl was twice as high than for men or 32.8 and 14.7%, respectively. Cholesterol values were not available for three men and one woman and were not included in Table 2.

Of the 1199 patients listed in Table 2 data on smoking, diabetes and lipid lowering drugs were available for 1075 of them. Approximately 25% (24.70%) of the men and 17% (16.80%) of the women with 70–100% stenosis had been smokers. The incidence of diabetes was higher

among women than men or 29.9 and 18.97%, respectively ($P < 0.01$). It was higher for both women and men at 10–69 and 70–100% stenosis than at 0% stenosis ($P < 0.001$). At 70–100% stenosis the percentage of women taking lipid lowering drugs was slightly higher than for men or 16.8 and 15.8%, respectively. Eliminating the plasma cholesterol values for the patients taking lipid lowering drugs did not alter the pattern of the data presented in Fig. 2. The incidence of hypertension at 70–100% stenosis was higher among men than women or 17.1 and 8.7%, respectively ($P < 0.005$). The percentage of previous myocardial infarctions was also higher in men than women or 4.1 and 3.8%, respectively (Table 2). A logistic regression model was fit to the data. The main effects model suggested that cholesterol and smoking based on LPX values were insignificant in predicting percentage stenosis in the cardiac catheterized patients. The analyses within different groups of categorical variables revealed that for women diabetes was more strongly associated with stenosis than for men. High levels of total antioxidant capacity were associated with a low percentage of stenosis. High levels of LPX, age and diabetes were all associated with the higher percentage stenosis.

Except for patients at 25–45 years of age the activation of PMNLs, expressed as the SI in the plasma from the cardiac catheterized patients, was significantly higher at all levels of stenosis ($P < 0.01$) compared with the controls (Table 3). FB became less significant after age 70 at 0 and 10–69% stenosis and after age 45 at 70–100% stenosis ($P < 0.01$) (Table 3). However, all of the catheterized patients had higher concentrations of FB in the plasma than the controls. The concentration of ceroplasmin (CP) in the plasma was significantly higher at all age groups in the cardiac catheterized patients than in the respective age groups of the controls ($P < 0.01$).

Although there were no significant differences in the plasma FB concentration between the smoking and the non-smoking cardiac catheterized patients (Table 4), there was a significant difference in the CP concentration ($P < 0.05$) and SI ($P < 0.01$) in the smokers at 70–100% stenosis as compared with non-smoking patients. Four out of 23 men with 70–100% stenosis and four out of 23 men and three out of ten women with 0% stenosis below 40 years of age had been smokers. Except for the diabetics with LPX at 0% stenosis and FB at 10–69 and 70–100% stenosis ($P < 0.05$), there were no significant differences in SI, CP, plasma cholesterol concentration between non-diabetic and diabetic catheterized patients (Table 5). For patients with microvascular angina (0% stenosis), diabetes enhanced the LPX from 2.93 to 3.17 μmol/l. Also except for LPX at 0% stenosis, there were no significant differences ($P < 0.05$) in SI, FB, CP, plasma cholesterol concentration and LPX between catheterized patients taking lipid

lowering drugs and patients not taking lipid lowering drugs (Table 6). The analyses for the concentration of the fatty acids in the plasma lipid obtained from the cardiac catheterized patients and the controls showed that the mean \pm SD in the percentage of linoleic and arachidonic acid was not significant indicating that dietary lipid intake was not a significant factor in the development of angina in the cardiac catheterized patients (Table 7).

5. Discussion

Although Steinberg et al. [19] believe that oxidation products in the plasma 'would be swept up within minutes by the liver', we found oxidation products at significantly higher concentrations in the plasma of cardiac catheterized patients with proven stenosis of their coronary arteries than in age and sex matched controls. That the concentration of oxidation products provided consistent data on the severity of stenosis, while the plasma cholesterol concentration did not, indicates that a relationship exists between the concentration of oxidation products in the plasma and the development of atherosclerosis. Age was not a factor as 70–100% stenosis occurred at less than 50 as well as at

70 years of age. The suggestion of Steinberg et al. [19] that the liver would 'sweep up' oxidation products may be true in healthy persons. However, under pathological conditions where the rate of in vivo formation of oxidation products exceeds the rate of their removal by the liver, these oxidation products accumulate in the circulation. As Steinberg et al. have suggested, oxidation products are highly immunogenic and may be trapped in a space between cells where antioxidant levels may be low.

Both smoking cigarettes [43–46] and diabetes [47–49] have been recognized risk factors in persons suffering from coronary heart disease. However, we found that the concentration of LPX was such a dominating risk factor that smokers could not be identified by measuring only the LPX concentration of the plasma. Smoking could be identified as a risk factor in cardiac patients by comparing the concentration of CP and SI in smokers with 70–100% stenosis to non-smokers with 70–100% stenosis of the coronary arteries. A previous study by Hulea et al. [50] indicated that the LPX, FB, SI and CP were all significantly higher in smokers than in age and sex matched non-smokers ($P < 0.01$) in an apparently healthy population living in urban areas.

Except for patients with microvascular angina (0% stenosis), LPX was a more significant factor than dia-

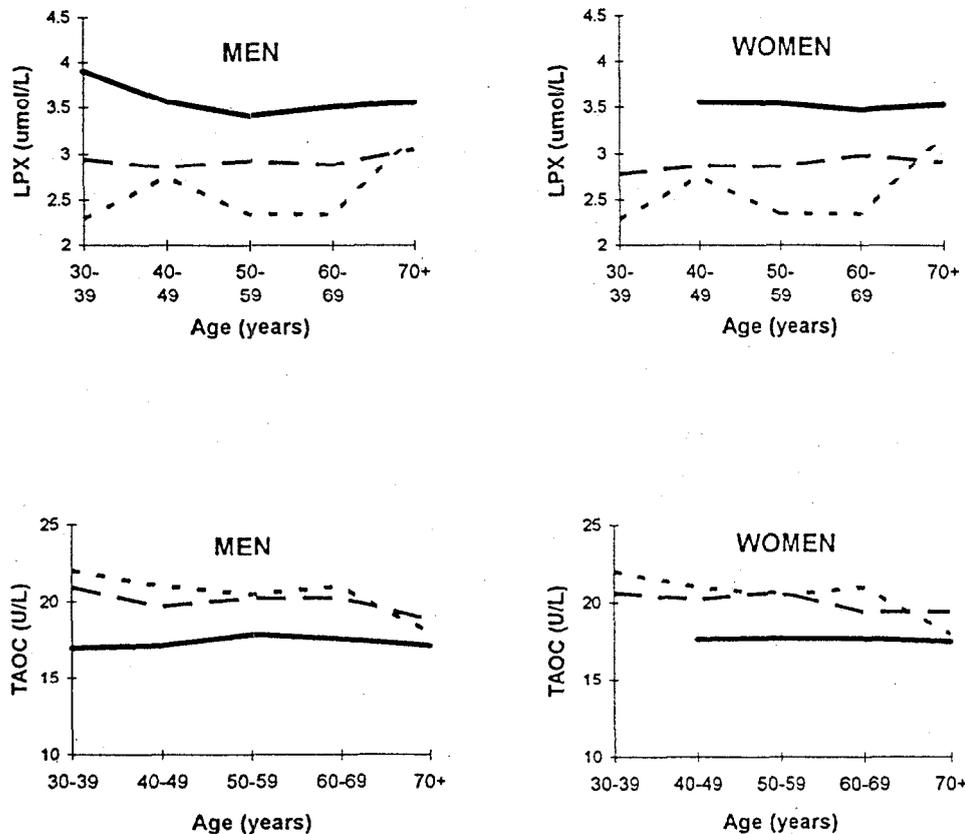


Fig. 2. The concentration of oxidation products (LPX μ mol/l) and total antioxidant capacity (TAOC u/l) at 70–100% stenosis — and 0% stenosis - - - in cardiac catheterized men and women compared with non-cardiac catheterized age and sex matched controls ----.

Table 2

The number of men and women with 0, 10–69 and 70–100% stenosis, percentage of smokers, diabetics, lipid lowering drugs, hypertensives, MI's and plasma cholesterol concentration below 200, 200–240 and above 240 mg/dl^a

Stenosis	Men (740)			Woman (459)		
	0%	10–69%	70–100%	0%	10–69%	70–100%
Number	149	82	506	152	62	244
<i>Cholesterol (%)</i>						
<200 mg/dl	55.41	56.10	50.89	43.42	41.94	34.84
200–240 mg/dl	30.41	29.27	34.45	34.87	35.48	32.38
>240 mg/dl	14.19	14.63	14.65	21.71	22.58	32.79
<i>Smokers (%)</i>						
Yes	24.16	26.83	24.70	14.47	12.90	16.80
No	67.11	62.20	66.21	74.34	75.80	70.08
Unavailable	8.72	10.98	9.09	11.18	11.29	13.11
<i>Diabetics (%)</i>						
Yes	6.70	17.00	18.97	7.89	19.35	29.9
No	85.20	73.17	73.12	82.24	67.74	59.00
Unavailable	8.05	9.76	7.91	9.87	12.9	29.92
<i>Lipid lowering drugs (%)</i>						
Yes	5.37	7.32	15.81	5.92	14.52	16.8
No	86.58	82.93	76.09	84.21	74.19	72.13
Unavailable	8.05	9.76	8.10	9.87	11.29	11.07
Hypertensive	2.28	2.05	17.16	6.08	3.42	8.74
Previous MI	0.22	0.22	4.11	1.52	2.28	3.80

^a These data were provided by Sylvia Lofrano from each patient's chart. Data on smoking, diabetics and lipid lowering drugs were not available for all patients and were recorded as the percentage unavailable from patients' records.

Table 3

The concentration of ceruloplasmin (CP), fibrinogen (FB), and stimulatory index (SI) in the plasma from cardiac catheterized patients and controls^a

Condition	Age (years)	<i>n</i>	SI (U/l)	FB (mg/dl)	CP (IU/l)
Control	25–45	24	25 ± 18	246 ± 69	116 ± 39
	46–69	22	28 ± 24	288 ± 80	120 ± 56
	70+	20	28 ± 31	295 ± 81	117 ± 67
0% Stenosis	25–45	40	40 ± 13**	307 ± 72**	167 ± 33**
	46–69	137	39 ± 12**	354 ± 77**	148 ± 18**
	70+	38	41 ± 10*	328 ± 80	173 ± 32**
10–69% Stenosis	25–45	7	39 ± 10	339 ± 95**	178 ± 20**
	46–69	73	53 ± 14**	394 ± 97**	204 ± 52**
	70+	27	52 ± 10**	333 ± 119	213 ± 18**
70–100% Stenosis	25–45	26	54 ± 10**	356 ± 80**	213 ± 39**
	45–69	332	35 ± 9**	326 ± 117	184 ± 26**
	70+	209	49 ± 11**	318 ± 92	203 ± 34**

^a Values expressed as mean ± SD. Significantly different as compared with respective matched control for $P < 0.05$ (*); $P < 0.01$ (**). Tests of significance used two-tailed Student's *t*-test (equal variance assumed).

betes in cardiac catheterized patients with stenosis of their coronary arteries. The significantly higher incidence of diabetes among women than men ($P < 0.01$) and both women and men at 10–69 and 70–100% stenosis than at 0% stenosis ($P < 0.001$) indicates that diabetes is a risk factor in its own right and is an added risk factor in the complex metabolic process that results in stenosis of the coronary arteries. That hypertension

among men is higher than among women is well known [51–54].

The favorable influence of lipid soluble antioxidants in the development of atherosclerosis in animal models [55–60] supports the hypothesis that lipid oxidation products are a risk factor for heart disease. In an in vivo study [61] we showed that oxidation products were an atherogenic factor in the development of atherosclerosis.

rosis in the arteries of cholesterol-fed rabbits. We compared the atherogenic effects of a diet enriched either with 0.5% oxidized cholesterol (OC) or with pure cholesterol (PC) or with PC plus vitamins E and C. Although the OC and PC diets were equally hyperlipidemic and hypercholesterolemic, the severity of atherosclerotic lesion was in the order: OC > PC > PC + antioxidants. These data indicated that atherogenesis can result from an excessive accumulation of oxidation products in the plasma which antioxidants can moderate.

We showed in a previous publication [62] that the hemolytic effect of homocysteine was higher in erythrocytes obtained from cardiac catheterized patients with either 0 or 100% stenosis of the coronary arteries than

in the controls. Homocysteine was able to increase the activation in vitro of PMNLs when triggered by opsonized zymosan. The hemolytic action of homocysteine was shown to depend on the ratio of PMNLs to erythrocytes. This relationship may help to explain the great individual variations in the hemolytic activity noticed in blood obtained from cardiac catheterized patients [63,64] and may also explain the mild anemia in some patients suffering from cardiovascular disease. Van der Griend et al. [65] have recently suggested a combination of low-dose folic acid and pyridoxine for treatment of hyperhomocysteinaemia in patients with premature arterial disease.

As a result of acute phase proteins in the plasma increase during inflammation and coronary heart dis-

Table 4

The concentration of ceruloplasmin (CP), fibrinogen (FB), and stimulatory index (SI) of non-smokers and smokers with stenosis^a

Condition	Group	n	FB (mg/dl)	CP (IU/l)	SI (U/l)
0% Stenosis	Non-smokers	150	330 ± 86	170 ± 34	39 ± 12
	Smokers	41	325 ± 78	165 ± 30	39 ± 12
10–69% Stenosis	Non-smokers	70	337 ± 80	188 ± 28	46 ± 12
	Smokers	24	338 ± 98	194 ± 30	45 ± 10
70–100% Stenosis	Non-smokers	385	343 ± 82	199 ± 32	51 ± 12
	Smokers	113	345 ± 94	208 ± 35*	54 ± 12**

^a Values expressed as mean ± SD. Significantly different as compared with respective matched control for $P < 0.05$ (*); $P < 0.01$ (**). Tests of significance used two-tailed Student's *t*-test (equal variance assumed).

Table 5

The concentration of ceruloplasmin (CP), fibrinogen (FB), stimulatory index (SI), cholesterol, and LPX in diabetics^a

Condition	Age	n	SI (U/l)	FB (mg/dl)	CP (IU/l)	Cholesterol (mg/%)	LPX (μmol/l)
0% Stenosis	Non-diabetics	178	39 ± 12	329 ± 84	169 ± 33	200 ± 40	2.93 ± 0.36
	Diabetics	13	36 ± 8	327 ± 90	176 ± 33	209 ± 43	3.17 ± 0.44*
10–69% Stenosis	Non-diabetics	74	46 ± 11	329 ± 80	189 ± 28	201 ± 40	3.25 ± 0.36
	Diabetics	20	44 ± 11	372 ± 104*	195 ± 27	214 ± 41	3.22 ± 0.46
70–100% Stenosis	Non-diabetics	380	51 ± 12	338 ± 85	201 ± 33	204 ± 46	3.50 ± 0.39
	Diabetics	122	53 ± 12	359 ± 83*	205 ± 34	212 ± 57	3.55 ± 0.47

^a Values expressed as mean ± SD. Significantly different as compared with non-diabetic of same degree of stenosis for $P < 0.05$ (*); $P < 0.01$ (**). Tests of significance used two-tailed Student's *t*-test (equal variance assumed).

Table 6

The concentration of ceruloplasmin (CP), fibrinogen (FB), stimulatory index (SI), cholesterol, and LPX in patients taking lipid-lowering drugs^a

Condition	Age	n	SI (U/l)	FB (mg/dl)	CP (IU/l)	Cholesterol (mg/%)	LPX (μmol/l)
0% Stenosis	No lipid drugs	177	39 ± 12	329 ± 84	169 ± 33	201 ± 41	2.93 ± 0.35
	Lipid drugs	14	40 ± 9	327 ± 87	171 ± 34	199 ± 36	3.15 ± 0.55*
10–69% Stenosis	No lipid drugs	82	46 ± 11	343 ± 88	188 ± 28	205 ± 42	3.25 ± 0.39
	Lipid drugs	12	44 ± 11	302 ± 77	202 ± 30	197 ± 26	3.25 ± 0.32
70–100% Stenosis	No lipid drugs	402	52 ± 12	343 ± 87	201 ± 33	204 ± 48	3.52 ± 0.41
	Lipid drugs	99	50 ± 12	340 ± 76	202 ± 31	212 ± 54	3.47 ± 0.39

^a Values expressed as mean ± SD. Significantly different as compared with non-user of lipid-lowering drugs of same degree of stenosis for $P < 0.05$ (*); $P < 0.01$ (**). Tests of significance used two-tailed Student's *t*-test (equal variance assumed).

Table 7
Fatty acid composition of plasma from 0 and 100% stenosis of cardiac catheterized patients

% Fatty acid	0% Stenosis	100% Stenosis	Controls
14:0	0.9 ± 0.3	0.9 ± 0.3	1.0 ± 0.3
16:0	21.5 ± 2.3	22.9 ± 2.5	22.2 ± 2.6
16:1 (n-7)	2.4 ± 0.9	3.1 ± 1.5	2.1 ± 0.8
18:0	6.2 ± 1.2	5.0 ± 1.8	6.8 ± 2.3
18:1 (n-4)	1.2 ± 0.3	1.2 ± 0.4	0.7 ± 0.3
18:1 (n-9)	16.6 ± 1.4	18.4 ± 1.0	15.1 ± 1.4
18:1 (n-7)	1.6 ± 0.3	1.73 ± 0.3	1.5 ± 0.2
18:1 (n-5)	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.1
18:2 (n-6)	29.6 ± 4.3	27.4 ± 4.6	30.8 ± 8.5
18:3 (n-6)	0.7 ± 0.3	0.7 ± 0.3	0.5 ± 0.1
18:3 (n-3)	–	–	–
20:2 (n-6)	–	–	–
20:3 (n-6)	1.8 ± 0.2	2.1 ± 0.5	2.0 ± 0.5
20:4 (n-6)	9.8 ± 2.9	9.1 ± 2.1	8.8 ± 1.6
22:2 (n-6)	0.6 ± 0.2	0.6 ± 0.3	0.6 ± 0.2
24:1 (n-9)	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.2
22:4 (n-5)	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
22:5 (n-3)	1.6 ± 0.6	1.7 ± 0.6	1.9 ± 0.1
T saturated	29.8 ± 3.0	29.7 ± 3.4	30.6 ± 5.0
T mono-unsaturated	21.3 ± 2.0	23.6 ± 2.3	19.4 ± 2.3
T poly-unsaturated	44.5 ± 5.3	42.1 ± 4.3	45.3 ± 7.4
Unsaturated p/s ratio	1.5 ± 0.3	1.5 ± 0.3	1.6 ± 0.5

ease, a relationship may exist between phagocytosing leukocytes, CP and FB. Under normal physiological conditions the acute phase proteins, especially FB, do not significantly influence the activation of PMNLs in the plasma [33]. But under pathological conditions when these acute phase proteins are non-specifically increased, FB may increase the biological activity of PMNLs as a function of its concentration [33]. Increased PMNLs activity resulted in plasminogen activation and release of plasmin which triggered additional fibrinogenolysis [35,36]. A FDP concentration of 200 mg/dl [33] increased SI emission. Fibrin in a similar concentration partially inhibited the SI emission. We found activated PMNLs stimulated FB and FDP activity and increased oxidation products in the plasma. Weijenberg et al. [66] showed that PMNLs may have a decisive role in arterial occlusion. They believe that adhesion of leukocytes (especially activated by PMNLs) to the coronary vessel wall, or their incorporation into thrombi, can have serious consequences as a result of the release of reactive oxygen species which can promote atherosclerosis and lysis of erythrocytes [62,64].

CP is a plasma protein that carries ≈ 95% of the total circulating copper in the plasma [67]. The presence of activated PMNLs may also be involved in whether intact CP increases the oxidation of LDL. Ehrenwald et al. [67] found highly purified human CP enhanced copper ion-mediated oxidation of LDL in vitro. They

found CP, degraded to a complex containing 115 and 19-kD fragments, inhibited cupric ion oxidation of LDL. The antioxidant capability of the degraded CP was similar to that of other acute phase proteins, including albumin [16]. These data indicated that the concentration of oxidation products rather than the concentration of cholesterol in the plasma identified stenosis in cardiac catheterized patients.

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Relationship between classic risk factors, plasma antioxidants and indicators of oxidant stress in angina pectoris (AP) in Tehran

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Abstract

Cardiovascular disease (CVD) in general seems to be the leading cause of death in the Eastern Mediterranean Region (EMR) including Iran. This may be due to classic risk factors such as high triglyceride (TG), high total cholesterol (TC), and low levels of high density lipoprotein cholesterol (HDL-C). The impact of antioxidants as potentially protective risk factors against early coronary heart disease (CHD) is unknown in Iran. Therefore, relationships between angina and plasma antioxidants and indicators of lipid peroxidation were investigated in a case-control study. In this study, 82 cases of previously undiagnosed angina pectoris (AP), identified by a modified WHO Rose chest pain questionnaire and verified by electrocardiography during treadmill exercise testing, were compared with 146 controls selected from the same population of over 4000 male civil servants aged 40–60 years. Subjects with AP declared significantly less physical activity and had higher serum TG [means (S.E.M.) 2.32 (0.18) versus 1.61 (0.07) mmol/l] but lower HDL-C [1.01 (0.04) versus 1.18 (0.03) mmol/l] than age-matched controls. Levels of total serum cholesterol, low-density lipoprotein cholesterol (LDL-C) and lipoprotein(a) [Lp(a)] were not significantly different between the two groups, while the ratio of LDL-C/HDL-C was significantly higher [4.51 (0.23) versus 3.54 (0.11)] for subjects with AP than for the controls. There was no significant difference in plasma levels of α -tocopherol, vitamin C, α - and β -carotene. However, retinol [1.90 (0.06) versus 2.09 (0.05)] and β -cryptoxanthin [0.398 (0.04) versus 0.467 (0.03)] were significantly lower in AP. Furthermore, angina cases exhibited a higher index of lipid peroxidation than controls (e.g. malondialdehyde, MDA; 0.376 (0.010) versus 0.337 (0.009) μ mol/l). On multiple logistic regression analysis, retinol with odds ratio (OR) of 0.644 [95% confidence interval (CI); 0.425–0.978], β -cryptoxanthin, with an OR of 0.675 (CI; 0.487–0.940), oxidation indices, MDA with OR of 1.612 (95% CI; 1.119–2.322) and LDL-C/HDL-C ratio with OR of 2.006 (95% CI; 1.416–2.849) showed the most significant independent associations with AP in this group of Iranians. In conclusion, the state of lipid peroxidation as well as the status of special antioxidants may be co-determinants of AP in Iran, in parallel with the influence of classical risk factors for cardiovascular disease. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Antioxidants; Lipid peroxidation; Malondialdehyde; Autoantibodies; Lipoproteins; Coronary heart disease

1. Introduction

Cardiovascular diseases (CVD) are the leading cause of death in the Eastern Mediterranean Region includ-

ing The Islamic Republic of Iran [1]. Despite the lack of accurate mortality data and modern medical care, there is enough evidence that CVD is increasing in Iran. The proportion of deaths due to CVD reached around 38% in 1989 (1983–1989) [2]. The national survey data have suggested that elevated plasma levels of classic risk factors such as a high total cholesterol (TC), triglyceride (TG) and low density lipoprotein cholesterol (LDL-C) might be important underlying causes of this

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increased mortality [3,4]. A recent community-based programme showed that 64% of a healthy normal population in Tehran were suffering from some kind of dyslipidaemia [5].

In industrialized countries, where classical risk factors for coronary heart disease (CHD) such as blood pressure, plasma cholesterol and smoking have been known for some time, corresponding public education and other interventions have led to a decline in the mortality rate in these countries in the last three decades [6]. However, in the present decade it has been suggested that other factors such as plasma antioxidant vitamins and peroxide concentrations play an important role in the etiology of CHD including angina pectoris (AP). Oxidative modification of low density lipoprotein (LDL) has been inferred to be important in the development of atherosclerosis, since modified LDL is readily taken up by macrophages, and as a consequence, leads to foam cell and plaque formation [7,8]. High levels of lipid peroxidation were also reported to be associated with angina pectoris [9,10] and progressing coronary artery disease [7,8,11].

The most abundant diet-derived antioxidants available in plasma to protect against reactive oxygen species are α -tocopherol (vitamin E) and ascorbic acid (vitamin C) [12]. α -Tocopherol is the major lipophilic antioxidant present in the outer lipid monolayer of LDL, which may protect the polyunsaturated fatty acids against oxidation by accepting electrons to form α -tocopheryl radicals [13,14], whereas carotenoids [14] and retinol (vitamin A) [15] are minor lipophilic antioxidants. Vitamin C, in the aqueous phase of the plasma, permits regeneration of α -tocopherol [16]. Cross cultural studies in Europe [17,18], Scotland and Finland [for review see Ref. [19]] have revealed an inverse relationship between the plasma vitamins C and E and CHD [17,18]. Other studies of large cohorts have shown that correction of an inadequately low dietary intake of vitamins E [20] and C [for review see Ref. [19]] reduces CHD expression.

Although the mortality rate of CHD seems to be increasing in urban areas of Iran, there has not been any systematic study on associations of risk factors with angina pectoris and CHD. A study of previously undiagnosed AP may provide most valuable information on an early stage of CHD with mild or transient clinical symptoms, which is not yet biased by recent changes of life style or any other treatment. In Edinburgh, AP of middle-aged males was associated with low plasma levels of vitamins A, C, E and carotene [21]. A small pilot study on young students in Tehran, found a higher level of plasma malondialdehyde (MDA) as an indicator of lipid peroxidation, but similar amounts of α -tocopherol and β -carotene and even higher levels of some other carotenoids (lycopene, canthaxanthin, lutein + zeaxanthin) as compared with a matched group

of Austrians [22]. Results of a household food security survey in Tehran [23] indicated that 96% of the population of Tehran consume vitamin C in excess of and 32% consume retinol below the recommended intake [23], of which only 18% is from meat and dairy products.

Generally, the dietary habits of Iranians are different from those prevailing in industrialized countries and their life style has been influenced to some extent by war in recent decades. A recent nationwide survey found that the main sources of carbohydrates are rice (at least once a day) and bread (66% of energy comes from carbohydrates); oil or hard margarine as the main sources of fat. Twenty two percent of energy was derived from fat in which saturated fatty acids exceed the recommended level: the intake of animal protein was low [24]. Leafy vegetables such as parsley, coriander, dill weed, onions, spinach, horse radish, mint, watercress and other herbs are traditionally eaten every day, but other vegetable are consumed to a lesser extent and all mostly in pickled form. However, tomato puree, lemon juice and puree from pomegranate and plums are also used for enhancing the taste, but often requiring prolonged cooking times for stews. Green salad, yoghurt or pickled vegetables are served with every meal.

The aim of this study was to identify subjects with AP among middle-aged males in Tehran and to measure both classic risk factors, as well as plasma antioxidant concentrations and indicators related to lipoprotein oxidation as potentially independent predictor risk factors for AP.

2. Material and methods

2.1. Experimental design

The design of the investigation was based on a case-control study. The selected population consisted of men aged 40–60 years who were not taking any medication for treatment of CHD, hyperlipidaemia, diabetes or hypertension. They were also not taking vitamin supplements and had not undertaken recent dietary changes that might affect their plasma antioxidant status. Male employees of eleven different Governmental Organizations in Tehran were screened for previously undiagnosed AP. More than 4000 questionnaires were returned. Three hundred reported coronary chest pain according to a modified Rose chest pain questionnaire [25] and were invited for exercise test with a 97% response rate. The ECG revealed abnormalities after exercise loading in 104 subjects, but only 82 subjects with horizontal or downslope ST depression of equal or more than 1 mm in two or more leads, were admitted to the study. These 82 verified AP cases were matched with 146 male controls of similar age drawn from the

remaining population (3700) who had a negative response to the Rose chest pain questionnaire (reconfirmed by telephone), no history of CHD and a negative electrocardiography exercise test. All subjects completed documentation with informed consent.

Blood pressure was measured in the right arm after resting sitting for 10 min, by mercury sphygmomanometer. Two measurements of blood pressure were recorded at 5 min intervals, by a trained person. Height and weight were measured, and questionnaire data was collected on food frequency, years of education, numbers of cigarettes smoked per day, exercise habits, walking, past history of reported high BP, diabetes and dyslipidaemia, in the subjects and their first degree relatives. Four of the AP cases underwent coronary artery bypass surgery immediately after angiography.

Exercise testing and all blood sampling was performed during the period September 1997 to mid-March 1998, before the beginning of spring when stored fruit supplies gradually decline. In particular, it was important to finish sample collection before the celebrations of the Iranian New Year which starts on 21 March and lasts for 2 weeks, when people eat more nuts, sweets and fruits. The frozen samples were transferred on dry ice to the University of Graz, Austria, in early May 1998. The biochemical analyses were completed by the end of June 1998.

2.2. Materials

All solvents and further specified chemicals were analytical grade, supplied by Merck (Darmstadt, Germany) or Prochem (Wesel, Germany) or Sigma (St. Louis, MO). The kit to measure autoantibodies to oxidized LDL (oLab) was provided by Dr Franz Tatzber, KEG, Vienna, Austria.

2.3. Methods

2.3.1. Storage of plasma

Blood samples (30 ml) were taken by venipuncture of the antecubital vein, with brief use of a tourniquet, in the morning after 12 h fasting. Serum was obtained for lipid profile. For parameters other than vitamin C, blood samples containing 1 mg/ml EDTA, pH 7.4, were centrifuged at $2000 \times g$ for 10 min. The aliquots of plasma were stored at -80°C for 3–8 months. A separate aliquot of 0.5 ml of plasma EDTA was mixed with 0.5 ml of 10% metaphosphoric acid, within 30 min of venipuncture and stored at -80°C for assay of vitamin C.

2.3.2. Determination of lipid profile in serum

Cholesterol was measured by the CHOD-PAP method. TG by the GPO-PAP method, high density

lipoprotein cholesterol (HDL-C) was determined after separation with phosphotungstic acid and magnesium chloride, all using established kit methods from Boehringer, Mannheim, Germany. LDL-C concentration was calculated [26]. The methods and results were validated against the UK-NEQAS scheme and Boehringer QC standards.

2.3.3. Determination of lipid soluble antioxidants

Antioxidants were determined as described previously [27]. Briefly, 0.4 ml of plasma with 0.4 ml of water were mixed with an equal volume (0.8 ml) of ice-cold ethanol (containing 0.5 mg/ml butylated hydroxy-toluene (BHT)). A total of 2.0 ml of *n*-hexane (stored over bidistilled water containing $20 \mu\text{M}$ EDTA) was added and after centrifugation (3000 rpm, 5 min), a volume of 1.2 ml of hexane phase was transferred into a brown crimp vial and dried in a speed-vac for 10 min, at room temperature. The residue was dissolved in 0.3 ml mixture of ethanol:ethylacetate (10:1 v/v, containing 1 mg/l BHT). A sample (20 μl) was injected into an HPLC system (Lichrosphere 100 RP18 column, 5 μm , 125×4 mm: Merck, Germany). Separation was performed in isocratic mode with a mixture of acetonitrile/methanol/ethanol/water (60:50:20:2 v/v), containing 0.01% ammonium acetate, flow rate 1.2 ml/min. The effluent was monitored with two detectors in series, a UV-VIS detector (L4250, Merck, Hitachi) set to 450 nm for detection of carotenoids and a fluorescence detector (Jasco 821 FP) set initially to 325/500 nm for detection of retinol and after 3.9 min to 292/335 nm for detection of tocopherols. The intra assay coefficient of variation was 4–7% for all antioxidants.

2.3.4. Determination of vitamin C with HPLC

Vitamin C was measured according to the method of Bui et al. [28]. The frozen plasma-EDTA, in metaphosphoric acid, was thawed, mixed for 5 s and centrifuged on an Eppendorf centrifuge (Hamburg, Germany, Mod.5415C) at $10\,000 \times g$, for 4 min. A volume of 0.1 ml of supernatant was mixed with 0.4 ml of HPLC-eluent, centrifuged and injected by the autosampler into the HPLC System (Lichrosphere 100 RP18, 5 mm, 250×4 mm). The HPLC-eluent was prepared by adding 4.3 ml of 70% perchloric acid and 100 mg EDTA to 1 l of bidistilled water. Flow rate was 1 ml/min. The effluent was monitored with an electrochemical detector set to +0.6 V against an Ag/AgCl reference electrode filled with 3 M LiCl. Peak quantification was performed with at least two standard mixtures of ascorbic acid. The time between thawing and HPLC separation did not exceed 3 h.

2.3.5. Determination of MDA in plasma

MDA was determined using a slight modification of the HPLC method of Rabl et al. [29]. The plasma

samples were thawed immediately before the assay and 100 μ l was mixed with 100 μ l water, 300 μ l of 0.15 mol phosphoric acid/l, 10 μ l of BHT, 4% methanolic solution and 100 μ l 0.6% thiobarbituric acid (TBA) and incubated at 95°C for 60 min. The chromogen was extracted with 1.25 ml butanol-1 and fractionated by HPLC with fluorometric detection (excitation wavelength: 525 nm, emission wavelength 550 nm). MDA-TBA adduct was calibrated with tetramethoxypropane standard solution.

2.3.6. Measurement of autoantibodies to oLab

Determination of oLab (EliTec, Bisamberg, Austria) was performed according to the method of Tatzber and Esterbauer, 1995 [30]. Briefly, human LDL (50 μ g protein/ml) in PBS, (pH 7.4), was oxidized with CuCl_2 at a final concentration of 1.66 μ M for 24 h. Microtitration plates were coated using a concentration of LDL at 5 μ g protein/ml (250 μ l/well, 1.25 μ g protein) in phosphate carbonate buffer (pH 9.6) overnight. Unspecific binding sites were blocked with 1% bovine serum albumin (BSA) in PBS (pH 7.4) for 2 h at room temperature. The sera of the subjects were diluted 1:21 in PBS and incubated for 2 h at 37°C in the wells coated with oLDL. After washing, 100 μ l of antihuman IgG horse radish peroxidase conjugate was added to each well and incubated for 45 min at room temperature. Tetramethylbenzidine was used as a chromogenic substrate and the absorbance was read at 450 nm. The absorbance of samples collected before supplementation was taken as the 100% value for each subject, the measurements of oLab obtained after supplementation were calculated as a percent of this value.

2.3.7. Determination of lipoprotein (a) [Lp(a)] and apolipoprotein (a) [apo(a)]

Quantitation was performed by a sandwich assay on the DELFIA system (LKB-Pharmacia) [31]. In brief, a polyclonal rabbit antibody was purified by immunoaffinity by passing over a column loaded with plasminogen, and coated onto 96-well Costar plates. The purified antibody was free of any detectable cross reactivity against plasminogen, or other plasma constituents as tested by Western bolt analysis using Glu_1 -plasminogen. Non-specific bindings sites were blocked by exposure to 250 μ l of 0.5% (w/v) BSA for 30 min. Two hundred μ l aliquot of the samples were added to the wells and incubated for 2 h at 20°C. After three successive washing steps with 50 mM Tris-HCl, pH 7.7, the polyclonal antibody against apo(a), labelled with Europium (Eu) was added to the wells and incubated additionally for 2 h at 20°C. Fluorescence was determined in a DELFIA reader after 15 min. Plasma samples were diluted 1500–3000-fold.

2.4. Statistical analysis

Statistical comparisons between angina cases and the controls were carried out using unpaired *t*-tests. The non-parametric Wilcoxon test was used for comparing non-normally distributed data. For comparison of the percentage of smokers and of genetic history in first degree relatives, chi-squared tests were used. Multiple logistic regression with a forwards stepwise approach was used to determine the independent predictors of AP in the total sample. The following variables were not normally distributed and were log transformed before analysis: α , β -carotene, β -cryptoxanthin, γ -tocopherol, oLab, YadjMDA and triglycerides. Variables entered into the logistic regression included lipid profile, LDL-C and HDL-C (entered as LDL-C/HDL-C ratio, not individually, as this term can give more power in risk assessment [32]), and all the water soluble and lipid soluble antioxidants and indices of lipid peroxidation, whether they were statistically significant or not. Correlations between variables were calculated with Pearson (denoted *r*) or Spearman's rank correlations (denoted *r_s*), according to whether variables followed a normal distribution. There was a strong positive correlation between cholesterol ($r = 0.498$, $P = 0.0001$) and triglyceride ($r_s = 0.440$, $P = 0.0001$) and α -tocopherol, therefore α -tocopherol was adjusted for these two parameters according to the procedure of Jordan et al. [33], before entering into the logistic regression. There was a weak correlation between the other antioxidants (e.g. β -cryptoxanthin) and TC ($r_s = 0.279$, $P = 0.0001$) and TG ($r_s = 0.014$, $P = 0.841$), therefore they were entered without adjustments. Although, the correlation between MDA and TC ($r_s = 0.223$, $P = 0.002$) and TG ($r_s = 0.221$, $P = 0.002$), were not strong, MDA was adjusted for both TC and TG according to the procedure of Jordan et al. [33] in view of the high TG in AP cases. However, the relationship between MDA and angina was similar whether or not such an adjustment was made. The protective and the risk factors for angina pectoris were evaluated by logistic multiple regression with adjustment for other variables entering the statistical model. OR and their 95% confidence limits are presented as the relative odds associated with a standard deviation increase in that particular variable. A *P* value less than 0.05 was considered significant. All values shown in the table are mean (S.E.M.). All statistical analyses were performed by using the computer software SPSS Rel.6.1.2.

Note: The reason the number of analyses is not consistent is due either to insufficient plasma for all analyses for some subjects, or lack of time, as only 2 months were available to complete all the biochemical analyses. Regarding blood pressure and body mass index (BMI), their entry did not affect the outcome of the logistic regression, therefore they were omitted.

Also, the physical activity value was self-declared and unmeasurable, of interest but considered less reliable than the biochemical measurements and it was also omitted from the logistic analysis.

3. Results

3.1. Anthropometric measurements, smoking and BP

In the present case-control study the apparently healthy controls were compared with subjects with previously undiagnosed AP regarding sex, age (40–60), profession (civil servants of the Governmental staff) and a medium-low socioeconomic status with similar mean years of education and a similar BMI around 25. Also, they declared that they did not drink alcohol.

There was no significant difference in the BP between these two groups (Table 1) and levels were within the reference range according to European and National Cholesterol Educational Program guidelines [34,35]. The percentages of cigarette smokers were similar in the two groups, however, the percentages of those with a family history of CHD in first degree relatives, before

age of 60, were significantly higher (Table 1) in the angina group than in the controls.

3.2. Lipid profile

There were no significant differences in TC and LDL between the two groups (Table 2). TG (44%) and ratio of LDL-C /HDL-C were significantly higher (27%) in the cases than controls (Table 2) and HDL-C was significantly lower (15%) in the cases than controls. There was a significant inverse correlation between HDL and plasma TG ($r_s = -0.433$, $P = 0.0001$). It has been suggested that HDL-C < 1.0 mmol/l and/or fasting triglycerides > 2.0 mmol/l and TC > 5.0 mmol/l are markers of increased coronary risk [34,35]. Seventy five percent of either angina subjects or controls had TC > 5.0 mmol/l; 46% of the angina and 24% of controls had TG > 2.0 mmol/l; and 53% of angina cases and 26% of the controls had HDL-C < 1.0 mmol/l. Thus, these classical risk factors occurred at least twice as often in the angina cases as the controls, in combination with a lower declared physical activity and an increase in the family background of premature CHD ($P = 0.0055$ and $P = 0.0063$, respectively).

Table 1

Anthropometric data and blood pressure and smoking habits in controls and subjects with previously undiagnosed angina pectoris (AP), in Tehran^a

Parameter	n	Control	n	Angina	P
Age (years)	146	48.4 (0.38)	82	48.7 (0.54)	0.586
EDU	146	14.3 (0.27)	82	13.6 (0.37)	0.068*
BMI	146	24.6 (0.24)	82	25.1 (0.34)	0.207
BP (mmHg)					
Systolic	146	123.5 (1.1)	82	126.6 (1.3)	0.124*
Diastolic	146	77.10 (0.7)	82	78.7 (1.0)	0.269*
Activity (min)	146	270 (20)	82	181 (20)	0.0055*
Smoking % > 5/day	146	13.0%	82	15.9%	0.553**
History of CHD in the 1st relative < 60	146	17.1%	82	32.9%	0.0063**

^a Data are presented as mean (S.E.M.). Education by years EDU; weight (kg)/height (m)²; BMI; blood pressure, BP; walking + exercise/week. Activity (min); smoking more than 5 cigarettes/day considered as smoker. Data were compared with the unpaired *t*-test.

* Data were compared by non-parametric, Wilcoxon two sample test.

** Data were compared by χ^2 test.

Table 2

Serum levels of major lipids in subjects with previously undiagnosed angina pectoris and controls, in Tehran^a

Parameter (mmol/l)	n	Control	n	Angina	P
TC	146	5.78 (0.09)	82	6.13 (0.15)	0.053
LDL-C	146	3.87 (0.09)	82	4.09 (0.14)	0.215
HDL-C	146	1.18 (0.03)	82	1.01 (0.04)	<0.0001*
LDL-C/HDL-C	146	3.54 (0.11)	82	4.51 (0.23)	<0.0001
TG	146	1.61 (0.07)	82	2.32 (0.18)	<0.0001*
Lp(a) (mg/dl)	140	21.5 (1.7)	79	29.6 (3.5)	0.131*

^a Values are presented as mean (S.E.M.). Data were compared with the unpaired *t*-test. Total cholesterol, TC; high density lipoprotein cholesterol, HDL-C; low density lipoprotein cholesterol, LDL-C; triglyceride, TG; lipoprotein (a), Lp(a).

* Data were compared by non-parametric Wilcoxon two sample test.

Table 3
Comparison of plasma antioxidants and oxidation indices in subjects with previously undiagnosed angina pectoris and controls in Tehran^a

Antioxidants ($\mu\text{mol/l}$)	<i>n</i>	Control	<i>n</i>	Angina	<i>P</i>
Vitamin C	141	55.6 (1.9)	80	51.1 (2.2)	0.137
α -Tocopherol	138	24.0 (0.5)	77	26.3 (1.0)	0.053
α -Tocopherol/TC ($\mu\text{mol}/\text{mmol}$)	138	4.21 (0.08)	77	4.38 (0.16)	0.348
Yadj Vitamin E	138	23.3 (0.40)	77	23.5 (0.64)	0.820
γ -Tocopherol	138	2.67 (0.13)	77	2.75 (0.19)	0.816*
Retinol	138	2.09 (0.05)	77	1.90 (0.06)	0.025
β -Carotene	138	0.391 (0.03)	77	0.352 (0.03)	0.199*
α -Carotene	138	0.061 (0.007)	77	0.044 (0.004)	0.462*
Lycopene	138	0.719 (0.024)	77	0.654 (0.036)	0.118
β -Cryptoxanthin	138	0.467 (0.03)	77	0.398 (0.04)	0.041*
Canthaxanthin	138	0.266 (0.007)	77	0.222 (0.020)	0.802
Lutein + Zeaxanthin	138	0.659 (0.023)	77	0.618 (0.032)	0.296
Oxidation indices MDA ($\mu\text{mol/l}$)	111	0.337 (0.009)	77	0.376 (0.010)	0.0001*
YadjMDA	111	0.335 (0.009)	77	0.363 (0.011)	0.0021*
Olab (U/ml)	134	315 (24)	77	362 (33)	0.122*

^a Data are presented as mean (S.E.M.). Data were compared with the unpaired *t*-test. MDA, malondialdehyde; oLab, autoantibodies to oxidized LDL; YadjE and YadjMDA, α -tocopherol and MDA were adjusted to mmol/L cholesterol and mmol/L triglycerides according to Jordan et al. [33]; (Yadj = $Y - B_1(x_1 - x_{1,0}) - B_2(x_2 - x_{2,0})$, 5.71 mmol TC and 1.36 mmol TG (median control of this population), were taken as standard value for cholesterol and triglycerides, respectively.

* Data were compared by the non-parametric Wilcoxon two sample test.

3.3. Lp(a)

The mean level of Lp(a) was (37%) higher ($P = 0.131$: ns) in the cases than the controls (Table 2). Plasma level of Lp(a) above 30 mg/dl was found in 21% of controls and 33% of angina cases.

3.4. Oxidation indices

Indices of lipid peroxidation such as MDA were significantly higher (12%) in the cases than controls ($P = 0.0001$, Table 3). Also, when MDA was standardized for lipid [yadjMDA, which incorporated cholesterol and triglycerides [33]] it was significantly higher in the cases than controls ($P = 0.0021$). Circulating autoantibodies against oLab were also 27% higher in the cases than controls, but at marginal significance only if outlying values were excluded ($P = 0.049$, Table 3). There was no correlation between MDA and oLab ($r_s = -0.051$, $P = 0.505$).

3.5. Vitamin C

Plasma vitamin C level was close to the optimal level recommended to protect against CHD [19,20,34,36], with no significant differences between the cases and controls (Table 3). Plasma vitamin C was positively correlated with the number of oranges consumed per day ($r = 0.271$, $P = 0.0001$). Vitamin C was significantly correlated with β -cryptoxanthin ($r_s = 0.372$, $P = 0.0001$).

3.6. Lipophilic antioxidants

Plasma retinol was significantly (9%) lower in the cases than in controls. β -Cryptoxanthin exhibited noticeable significant differences between the two groups. The control group had significantly higher β -cryptoxanthin (15%) than in the cases (Table 3). β -Cryptoxanthin was also weakly correlated with the number of oranges consumed/day ($r_s = 0.217$, $P = 0.002$). The plasma carotene (α - β -carotene) seemed to be similar to the levels recorded for Austrians [22] and other European males [21], yet β -carotene did not reach desirable recommended levels [36]. The absolute amount of α -tocopherol was 9.6% higher in the cases than controls, the difference was not statistically significant. When α -tocopherol was standardized for concurrent cholesterol-rich carriers, the α -tocopherol status within lipids (α -tocopherol/TC) as well as lipid standardized α -tocopherol (YadjE, which incorporates cholesterol and TG [33]) the values were clearly below the recommended desirable levels [36], but without any differences between cases and controls (Table 3). Although β -carotene did not exhibit any differences between the two groups, levels were lower than recommended for primary prevention for both controls and cases [36].

3.7. Multiple regression analyses

Considering all the variables assessed for AP and controls, plasma levels of retinol, β -cryptoxanthin, LDL-C/HDL-C and MDA were independently associ-

ated with AP in this Iranian population (Table 4). For one standard deviation (S.D.) increase in retinol and \log_e (β -cryptoxanthin), there was a 0.644 and 0.675-fold decrease in the odds of angina. However, for an increase of one S.D. in \log_e (YadjMDA) and LDL-C/HDL-C, there was 1.612 and 2.006-fold increase for risk of angina, respectively.

4. Discussion

According to the guidelines of the European Atherosclerosis Society [34] and the National Cholesterol Education Program [35], the AP cases had approximately twice the target levels of lipid-related cardiovascular risk, and the position of the control population was also not ideal.

Thus levels of HDL were below the guidelines (53% of AP and 26% of controls had HDL < 1.0 mmol/l), TGs were higher (46% of AP and 24% of controls had TG > 2.0) but levels of TC were not significantly different between two groups (75% of AP or controls had TC > 5.0 mmol/l). Some 75% of the population of Tehran suffer from some kind of dyslipidaemia [5]. The levels of HDL were lower, but TG and LDL-C/HDL-C ratio were significantly higher in the cases than controls (Table 2). It is well established that high HDL is considered to be antiatherogenic and associated with lower risk for CHD [37,38] and may mitigate the toxic effect of LDL [39]. On the other hand, an increase in total triglycerides and a decrease in HDL are reported to be associated with progression of coronary atherosclerosis [40]. As generally reported, and in the present study, TG level was not an independent predictor of AP in the logistic regression, but the ratio of LDL-C/HDL-C was an independent predictor (OR 2.006; CI 1.416–2.849). This was mainly due to low levels of HDL which might respond to increases in physical activity and changes of the dietary habits such as less reliance on hard saturated margarine [24].

No significant differences in vitamin C levels were observed between the cases and the controls. The con-

centration of plasma vitamin C was apparently sufficient since it was above the critical threshold of 50 $\mu\text{mol/l}$ suggested for antioxidant protection of LDL against CHD [36]. It was even 45% higher than levels reported for an AP group studied in Edinburgh [21]. Indeed, the consumption of vitamin C in Iran is rather high compared to other vitamins [23] and exceeds the intake recommended from a household survey [23]. Although it has previously been reported that α -tocopherol status (< 20–25 $\mu\text{mol/l}$ with α -tocopherol/TC ratios < 4.25) can play a key role in AP [21] and CHD [18–20], in this study there was no significant difference between levels for angina and controls. In this study, level of plasma α -tocopherol was 14.5% and the ratio of α -tocopherol/TC was 19.9% higher than those reported in the Edinburgh AP study in which vitamin E inadequacy was the most prominent risk factor [21]. Therefore, it was unlikely that any vitamin E inadequacy specifically contributed to AP in Tehran. Also, there was no significant difference in levels of α - and β -carotene between the two groups although both were below recommended levels for protection against CHD [36].

In contrast to vitamin C, E, and carotene, in the present study the levels of retinol and β -cryptoxanthin were significantly lower in the AP than controls (Table 3) and were predictors of AP with OR 0.644 ($P = 0.0392$) and 0.675 ($P = 0.0205$) for retinol and β -cryptoxanthin respectively (Table 4). In this study, the mean levels of retinol in AP (1.9 $\mu\text{mol/l}$) were 20% below that found for European population with AP [21] and 10% below the general recommended levels for protection against CHD [19]. According to a cross cultural study, this plasma level of retinol was associated with an increased mortality rate for CHD [17,18]. The low level of retinol in this population in Tehran may not only be due to inadequacy of preformed retinol in the diet [23] but could also reflect reduced retinol formation from carotenoids with potential pro-vitamin A activity such as α - and β -carotene and β -cryptoxanthin. As there was no significant difference for the carotenes between the AP and controls, plasma β -cryptoxanthin might be

Table 4
Odds ratio (relative risk) of antioxidant, lipid and oxidation indices evolving from Logistic multiple regression analysis with statistical significance^a

Variables	Regression coefficient (B)	S.E. of (B)	S.D. of controls	OR	95% CI	P value
Retinol	-0.745	0.361	0.59	0.644	0.425–0.978	0.0392
β -Cryptoxanthin	-0.485	0.209	0.81	0.675	0.487–0.940	0.0205
YadjMDA	1.910	0.745	0.25	1.612	1.119–2.322	0.0103
LDL-C/HDL-C	0.512	0.132	1.36	2.006	1.416–2.849	0.0001
Constant	0.553	1.239				

^a Odds ratios (OR) were calculated for an increase in each variable equivalent to one standard deviation (S.D.) (estimated from control group data) of the variable. Thus odds ratio was calculated as $\exp(B \times S.D.)$. The 95% confidence interval (CI) was calculated as $\exp[(B \pm 1.96 \times S.E. \text{ of } B) \times S.D.]$. Each OR given was adjusted for all other variables in data. β -cryptoxanthin and YadjMDA were log transformed before entering multiple logistic regression, because this produced a more normally distributed variable. LDL-C/HDL-C; low/high density lipoprotein cholesterol.

causally related to the lower level of plasma retinol. However, although low levels of β -cryptoxanthin were a weak but significant independent predictor of AP, this does not necessarily imply any exclusive causal relationship between AP and carotenoids such as β -cryptoxanthin. Since β -cryptoxanthin is a relatively specific marker of consumption of oranges and tangerines [41], the latter will provide other antioxidants with presumed CHD-protective properties such as bioflavonoids and polyphenols [19]. Vitamin A deficiency has been reported in Iran [23] and this study population may require a higher intake of performed vitamin A such as oily fish and certain dairy products and its precursors found in varieties of coloured vegetables.

In the present study, the low levels of retinol and β -cryptoxanthin in AP were associated at least with a significantly higher susceptibility towards lipid peroxidation in the AP cases than controls as indicated by an increase in plasma MDA (Table 3), as reported by previous studies [9–11]. As we measured MDA in the presence of BHT, a phenolic antioxidant, MDA precursors or even preformed MDA may at least in part have occurred *in vivo*. Increased plasma MDA was an independent predictor of AP (OR 1.612; $P = 0.0103$; Table 4). However, in a study of AP in Aberdeen [9], high levels of plasma lipid peroxidation products were accompanied by differences in plasma levels of vitamin E but not of vitamins C or A. In the present study, high levels of lipid peroxidation was associated with a lower plasma level of retinol and β -cryptoxanthin but not with lower levels of vitamins C and E. In other studies [19–21], lower levels of vitamins C, E and carotene were associated with angina and this may be related to the special dietary habits in different countries. A high level of lipid peroxidation in the patient in Tehran could well accelerate the process of atherosclerotic plaque formation [7,8], particularly in the presence of low retinol and accelerate AP. It has been shown that supplementation with vitamin E [42,43], β -carotene and retinol retards the process of oxidation [15,22,44]. The underlying cause of lipid peroxidation is not known, but it could partly be due to lack of antioxidants, or to oxidised fat accumulated during processing, storage and/or cooking. Overall this study supports the hypothesis that lipid peroxidation is involved in AP and CHD respectively and that cardiovascular health requires the concurrent adequacy of various antioxidants [9,11,19–21].

5. Conclusion

A high ratio of LDL/HDL and increased tendency towards lipid peroxidation, together with low levels of retinol and β -cryptoxanthin in conjunction with some genetic disposition of the patients and a low declared

physical activity, may contribute to the promotion of AP in Tehran. In full accordance with findings from other authors it is therefore advisable that the Iranian people should modify their life style by consuming a greater variety of fruits and more vegetables and, in particular, those rich in retinol precursors such as oranges, tangerines, carrots as well as sources of performed retinol such as milk and its products and oily fish. For individuals who have a very low level of retinol perhaps short term retinol supplementation may be advisable: long term supplementation can be associated with toxic effects. Furthermore, it is recommended that Iranians, and in particular this selected population, should replace saturated margarine [24] with a moderate intake of less saturated fats such as sunflower-oil and the more stable olive oil.

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Effects of Vitamin E on Chronic and Acute Endothelial Dysfunction in Smokers

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- OBJECTIVES** The aims of this study were to determine whether chronic or acute impairment of flow mediated vasodilation (FMD) in the brachial artery of smokers can be restored or preserved by the antioxidant vitamin E.
- BACKGROUND** Transient impairment of endothelial function after heavy cigarette smoking and chronic endothelial dysfunction in smokers result at least in part from increased oxidative stress.
- METHODS** We studied 22 healthy male smokers (mean \pm SD, 23 ± 9 cigarettes per day) randomly assigned to receive either 600 IU vitamin E per day ($n = 11$, age 28 ± 6 years) or placebo ($n = 11$, age 27 ± 6 years) for four weeks and 11 age-matched healthy male nonsmokers. Flow mediated vasodilation and endothelium-independent, nitroglycerin-induced dilation were assessed in the brachial artery using high resolution ultrasound (7.5 MHz) at baseline and after therapy. Subjects stopped smoking 2 h before the ultrasound examinations. At the end of the treatment period, a third scan was obtained 20 min after smoking a cigarette (0.6 mg nicotine, 7 mg tare) to estimate transient impairment of FMD.
- RESULTS** Flow mediated vasodilation at baseline was abnormal in the vitamin E (5.3 ± 3.8 , $p < 0.01$) and in the placebo group (6.4 ± 3.5 , $p < 0.05$) compared with nonsmoking controls (11.6 ± 4.7). Using a two-way repeated measures analysis of variance (ANOVA) to examine the effects of vitamin E on FMD, we found no effect for the grouping factor ($p = 0.5834$) in the ANOVA over time but a highly significant difference with respect to time ($p = 0.0065$). The interaction of the time factor and the grouping factor also proved to be significant ($p = 0.0318$). Flow mediated vasodilation values remained similar after treatment for four weeks in both groups but declined faster after smoking a cigarette in subjects taking placebo compared with those receiving vitamin E (p values from successive differences for the time/group factor: 0.0001/0.0017). The transient attenuation of FMD (calculated as the percent change in FMD) was related to the improvement of the antioxidant status, estimated as percent changes in thiobarbituric acid-reactive substances ($r = -0.67$, $p = 0.0024$). Nitroglycerin-induced dilation did not differ between study groups at baseline or after therapy.
- CONCLUSIONS** These results demonstrate that oral supplementation of vitamin E can attenuate transient impairment of endothelial function after heavy smoking due to an improvement of the oxidative status but cannot restore chronic endothelial dysfunction within four weeks in healthy male smokers. (J Am Coll Cardiol 2000;35:277-83) © 2000 by the American College of Cardiology

Endothelial dysfunction occurs at an early stage of atherosclerosis and has been observed in coronary and peripheral arteries of long-term smokers (1,2) as well as after heavy cigarette smoking (3,4). The precise mechanism of smoking-related endothelial dysfunction is not well understood and very likely multifactorial. However, recent clinical

and experimental observations strongly suggest a role of oxygen-derived free radicals (5-7). Cigarette smoke contains large amounts of free radicals, which may degrade nitric oxide released from the endothelium and also produce highly reactive intermediates, resulting in endothelial injury.

Epidemiologic studies have found an inverse association between the frequency of coronary artery disease (CAD) and dietary intake of antioxidant vitamins (8,9). However, a possible cause and effect relation between the intake of antioxidants and a reduction in the complications of CAD has thus far only been shown for vitamin E (10).

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Abbreviations and Acronyms

ANOVA	= analysis of variance
CAD	= coronary artery disease
FMD	= flow-mediated dilation
LDL-C	= low-density lipoprotein cholesterol
NMD	= nitroglycerin-induced dilation
TBA	= thiobarbituric acid
TBARS	= thiobarbituric acid-reactive substances

With regard to the effects of antioxidant vitamins on endothelial function, controversial results have been obtained. Improvement of endothelial dysfunction in smokers was noticed in the forearm vasculature after intraarterial infusion of the watersoluble antioxidant vitamin C (11) and in the brachial artery after intravenous vitamin C infusion (12). The beneficial effect of vitamin C supplementation was associated with a decrease in thiobarbituric acid-reactive substances (TBARS) as an index of oxidative stress. In contrast, oral application of vitamin C for one month showed no beneficial effect on endothelial function in the forearm vessels of patients with hypercholesterolemia (13) but resulted in acute improvement of endothelium-dependent vasodilation in the brachial artery of patients with CAD when applied at a higher dose (14).

Recently, we demonstrated that the oral intake of vitamin E, one of the main lipid-soluble antioxidants in human plasma lipoproteins, in addition to lipid lowering therapy, restored vascular reactivity in the brachial artery of hypercholesterolemic men (15). In contrast, vitamin E alone failed to improve endothelial function in forearm circulation of patients with hypercholesterolemia (13). However, the effect of vitamin E supplementation on endothelial function in the brachial artery of smokers is unknown.

In this study, we hypothesized that vitamin E supplementation, through an antioxidant effect, may restore chronic or preserve acute impairment of endothelium-dependent vasodilation in the brachial artery.

METHODS

Subjects and treatment. In a randomized, double-blind, placebo-controlled trial, we studied 22 young, healthy male smokers (mean \pm SD, 23 ± 9 cigarettes per day) randomly assigned to receive either all-racemic alpha-tocopherol (Roche Austria, Vienna), the most active form of vitamin E, at a dosage of 600 IU ($n = 11$, age 28 ± 6 years) or placebo ($n = 11$, age 27 ± 6 years) for four weeks. Both substances were encapsulated without any visible difference and the ultrasound operator was blinded to the group assignment. The reliability of medication intake was controlled by taking pill counts, which revealed no essential irregularities. Two probands, one in each group, did not complete the study (withdrawal of consent). Thus, 22 smokers (11 probands in each group) could be statistically analyzed after conclusion

of the study. Both study groups were compared with an age-matched control group of 11 male nonsmokers to demonstrate that flow-mediated dilation (FMD) at baseline was abnormal among the smokers. No participant had a history of hypercholesterolemia, hypertension or diabetes mellitus. None of the participants took antioxidative drugs before this study. The investigation conformed with the principles outlined in the Declaration of Helsinki. Written, informed consent was obtained from all participants.

Assessment of vasodilation and blood flow. Endothelium-dependent FMD following reactive hyperemia and endothelium-independent nitroglycerin-induced dilation (NMD) were examined in the brachial artery according to the method described by Celermajer *et al.* (16). Using high resolution ultrasound (7.5 MHz linear array transducer), measurements of the right brachial artery were taken at rest after lying quietly for at least 10 min, after cuff deflation completing suprasystolic compression (250 mm Hg for 4.5 min) of the right upper arm and after sublingual application of 0.8 mg nitroglycerin. Scans of the brachial artery were taken proximal to the bifurcation of the radial and the ulnar artery above the antecubital fossa at end diastole, incident with the R wave on a continuously recorded electrogram by the same ultrasound operator. Diameter measurements were taken from one media-adventitia interface to the other for at least three times at baseline and every 30 s following reactive hyperemia and after administration of nitroglycerin. The maximum FMD and NMD diameters were calculated as the average of the three consecutive maximum diameter measurements following hyperemia and nitroglycerin, respectively. Vasodilation was then calculated as the percent change in diameter compared with baseline. The impairment of FMD after heavy smoking (delta FMD) was estimated as the percent change in FMD values compared with baseline.

In all subjects, blood flow was calculated at rest and within 15 s after cuff deflation by multiplying the velocity time integral of the flow signal by the heart rate and the vessel cross-sectional area ($3.14 \times D^2/4$). Reactive hyperemia was then calculated as percent change in blood flow during reactive hyperemia compared with baseline.

Ultrasound measurements were performed at baseline and after therapy for four weeks. Subjects stopped smoking 2 h before the examinations. At the end of the treatment period, a third scan was obtained 20 min after smoking a cigarette (0.6 mg nicotine, 7 mg tare) to estimate transient impairment of FMD.

The mean range of intraobserver difference for measurements of baseline diameter was $0.10 \pm 0.07\%$, that of %FMD $2.4 \pm 2.2\%$ and that of %NMD $2.7 \pm 1.9\%$ as reported elsewhere (17).

Determination of lipids and lipoproteins. Cholesterol and triglycerides were measured enzymatically using assay kits from Boehringer Mannheim (Mannheim, Germany). High-density lipoprotein cholesterol was measured from the

Table 1. Characteristics of the Study Groups

	Placebo (n = 11)	Vitamin E (n = 11)	Controls (n = 11)
Age (yr)	27 ± 6 (19-36)	28 ± 6 (22-34)	28 ± 5 (19-36)
Cigarettes per day	21 ± 4 (15-30)	24 ± 13 (5-40)	—
Pack-years	15.2 ± 11.6 (1.5-31.5)	11.8 ± 9.1 (1.5-38)	—
Triglyceride (mg/dL)	139 ± 94 (53-383)	147 ± 94 (70-323)	131 ± 64 (54-270)
Cholesterol (mg/dL)	191 ± 40 (111-242)	211 ± 24 (167-257)	188 ± 27 (161-243)
HDL-C (mg/dL)	39 ± 2 (26-50)	40 ± 3 (25-52)	48 ± 15 (26-75)
LDL-C (mg/dL)	124 ± 32 (62-173)	147 ± 28 (106-194)	121 ± 27 (88-163)
Baseline diameter (mm)	4.7 ± 0.9 (3.9-5.6)	4.9 ± 0.4 (4.5-5.8)	4.6 ± 0.7 (3.8-5.5)
Hyperemia (%)	334 ± 165 (175-674)	386 ± 147 (160-496)	359 ± 180 (154-577)

Controls = nonsmokers; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; placebo = smokers receiving placebo; vitamin E = smokers receiving vitamin E.

Values are mean ± SD (range). No comparisons were significantly different.

supernatant after precipitation with polyethylene glycol (Reagent A from Immuno A.G., Vienna, Austria). Low-density lipoprotein cholesterol (LDL-C) was calculated according to the Friedewald equation.

Alpha-tocopherol measurements in human plasma samples. The neutral lipid fraction (200 µl) including alpha-tocopherol, the most active form of vitamin E, was extracted in a basic extraction system consisting of 200 µl ethanol, 200 µl H₂O and 200 µl hexane. The upper organic layer (20 µl) containing alpha-tocopherol was analyzed by high-performance liquid chromatography using an ExSil 100 20 × 0.46 cm silica column (mobile phase: hexane/1% ethanol, 1 ml/min) and fluorescence detection (Hitachi, E: 295 nm/Em: 390 nm) (18). Quantitation was performed by peak area comparison with external alpha-tocopherol standards of known concentrations. The alpha-tocopherol serum level was then normalized to LDL-C because there is evidence in the literature that the vitamin E content in LDL-C and not in the whole plasma correlates with lipid peroxidation (19,20). The increase of alpha-tocopherol/LDL-C (delta alpha-tocopherol/LDL-C) under therapy was calculated as the percent change in alpha-tocopherol/LDL-C.

Determination of malondialdehyde with thiobarbituric acid. The measurements of TBARS are based on the reaction of malondialdehyde, a secondary breakdown product of lipid hydroperoxides, with thiobarbituric acid (TBA). The assay was performed as described previously (21). The plasma sample was mixed with two volumes of cold 10% trichloroacetic acid for protein precipitation. Following centrifugation, the supernatant was mixed with an equal volume of 0.67% TBA in a boiling water bath for 10 min. After cooling, the absorbency was measured at 532 nm, and concentration of malondialdehyde was calculated from epsilon = 153 000 M⁻¹ cm⁻¹ (21). Measurements were performed in duplicate. The decrease of TBARS (delta TBARS) under therapy was calculated as the percent change in plasma TBARS levels.

Statistical analysis. Results are expressed as mean ± standard deviation (SD). Differences between groups were analyzed using one factor analysis of variance (ANOVA) followed by Scheffe's test, when comparing all three study groups (including nonsmoking controls) and Student *t* test, when comparing the vitamin E with the placebo group only. To examine whether FMD can be restored or preserved by vitamin E, a two-way repeated measures ANOVA with the group factor vitamin E/placebo and the repeated factor time (three levels: baseline/after four weeks/after heavy cigarette smoking) was performed. Successive differences contrasts between neighboring points in time were calculated as supportive statistical analyses. Univariate analyses of the effects of delta alpha-tocopherol/LDL and delta TBARS on delta FMD as well as of cigarettes smoked per day on TBARS levels were performed with linear regression. Differences were considered significant at *p* < 0.05.

RESULTS

Clinical and biochemical parameters. No differences were found among all three study groups with regard to age, lipids and lipoproteins serum levels (Table 1). Alpha-tocopherol and TBARS plasma levels at baseline were similar in the placebo and the vitamin E group. Vitamin E supplementation for four weeks led to significant increases of alpha-tocopherol serum levels and alpha-tocopherol serum levels normalized to LDL-C as well as of delta alpha-tocopherol and delta alpha-tocopherol/LDL. The percent changes of TBARS under therapy were also different between study groups (Table 2).

Vasodilation and blood flow responses. Baseline diameter and increases in blood flow during reactive hyperemia were similar among the study groups at baseline (Table 1) and after treatment for four weeks, respectively. Thus, it can be assumed that the stimulus for endothelium dependent vasodilation was similar in all study groups and that it was not influenced by the study medication. Smoking a cigarette did not effect brachial artery diameter and blood flow.

Comparing the two study groups consisting of male

Table 2. Oxidation Parameters and Results of Ultrasound Measurements in the Brachial Artery

		Placebo (n = 11)	Vitamin E (n = 11)	p
FMD (%)	baseline	6.4 ± 3.8 (0.0-10.9)	5.3 ± 3.8 (0.2-13.9)	0.51
	after therapy	6.9 ± 3.5 (2.7-13.9)	6.9 ± 4.0 (0.8-12.4)	0.98
	after therapy and acute smoking	2.7 ± 2.8 (0.5-9.1)	5.8 ± 3.2 (0.4-11.3)	0.028
delta FMD (%)	after acute smoking	-0.77 ± 0.31 (-1.51--0.34)	-0.12 ± 0.37 (-0.83-0.44)	0.0002
TBARS (μmol/L)	baseline	0.10 ± 0.03 (0.06-0.15)	0.11 ± 0.04 (0.06-0.19)	0.66
	after therapy	0.13 ± 0.03 (0.08-0.17)	0.10 ± 0.03 (0.06-0.16)	0.056
delta TBARS (%)	after therapy	0.36 ± 0.42 (-0.13-1.08)	-0.06 ± 0.35 (-0.62-0.48)	0.033
alpha-tocopherol (μg/ml)	baseline	12.2 ± 3.8 (4.5-19.6)	14.5 ± 3.6 (9.6-20.7)	0.17
	after therapy	13.3 ± 3.8 (6.4-20.2)	23.5 ± 6.6 (13.0-32.6)	0.0005
delta alpha-tocopherol (%)	after therapy	0.07 ± 0.24 (-0.34-0.37)	0.62 ± 0.27 (0.18-1.07)	0.0002
alpha-tocopherol/LDL-C (μg/100 mg% LDL)	baseline	10.2 ± 3.4 (4.5-19.6)	10.7 ± 4.3 (9.6-20.7)	0.75
	after therapy	11.0 ± 2.9 (6.6-14.8)	16.7 ± 6.3 (7.6-28.4)	0.018
delta alpha-tocopherol/LDL-C (%)	after therapy	0.17 ± 0.40 (-0.44-0.96)	0.60 ± 0.42 (0.08-1.35)	0.035

FMD = flow-mediated dilation; LDL-C = low-density lipoprotein; TBARS = thiobarbituric acid-reactive substances. delta TBARS, delta alpha-tocopherol and delta FMD = percent changes after therapy and heavy smoking, respectively. Values are mean ± SD (range), p = for comparison of subjects receiving placebo and subjects receiving vitamin E (600 IU).

smokers with an age-matched control group of male non-smokers, FMD was impaired in both the vitamin E (5.3 ± 3.8 vs. 11.6 ± 4.7, p < 0.01) and the placebo groups (6.4 ± 3.5 vs. 11.6 ± 4.7, p < 0.05).

By performing a two-way repeated measures ANOVA, we found no effect for the grouping factor (group factor p value = 0.5834) in the ANOVA over time. With respect to time, there was a highly significant difference (time factor p value = 0.0065), and the interaction of the time factor and the grouping factor also proved to be significant (p = 0.0318), thus confirming the beneficial effect of vitamin E supplementation (Fig. 1).

Supporting statistical analyses revealed a stronger decline of FMD values after acute cigarette smoking in subjects taking placebo compared with those receiving vitamin E (p values from successive differences for the time/group factor: 0.0001/0.0017) (Fig. 2). However, FMD values did not change, neither after vitamin E nor after placebo intake for four weeks, when subjects stopped smoking at least 2 h before the ultrasound examination (p values from successive differences for the time/group factor: 0.3099/0.5756).

Nitroglycerin-induced vasodilation was similar in all study groups at baseline and after the treatment period.

Relationship between oxidative stress, smoking habits and FMD. Univariate analyses over both study groups revealed significant inverse correlations between the transient impairment of FMD after acute smoking (delta FMD) and the improvement of the antioxidant status (delta TBARS, r = -0.67, p = 0.0024; delta alpha-tocopherol/LDL, r = 0.51, p = 0.016; Fig. 3). Alpha-tocopherol serum levels or delta alpha-tocopherol were not related to the impairment of FMD. The number of cigarettes smoked per day correlated with TBARS at baseline (r = 0.49, p = 0.025). No significant correlation was found between pack-years and TBARS levels. The close correlation between

delta FMD and delta TBARS (r = -0.71, p = 0.031) also remained following exclusion of the placebo group.

DISCUSSION

This study demonstrates a partially beneficial effect of vitamin E supplementation on endothelium-dependent vasodilation in the brachial artery of male smokers. Vitamin E supplementation for four weeks prevented the transient further impairment of endothelium-dependent vasodilation after acute smoking, while it showed no effect on chronic

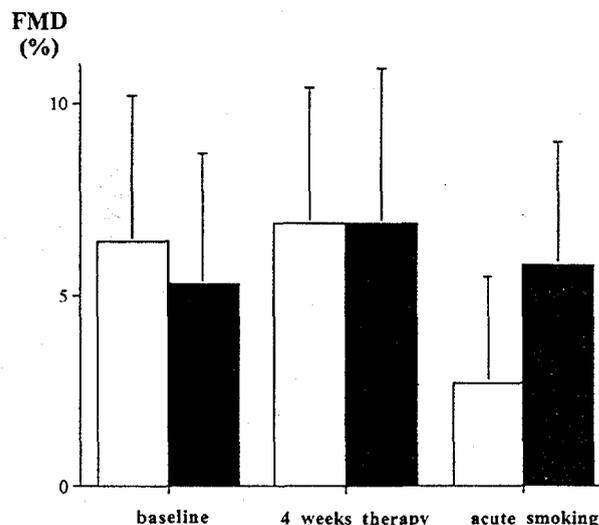


Figure 1. Bar graph showing FMD values of subjects receiving placebo and of subjects receiving vitamin E (600 IU/day) at baseline, after therapy for four weeks and after heavy smoking at the end of the treatment period. Time factor p value = 0.0065. Group factor p value = 0.5834. Interaction of the time factor and the grouping factor: p = 0.0318. FMD = flow-mediated vasodilation. Open square = placebo; closed square = Vitamin E.

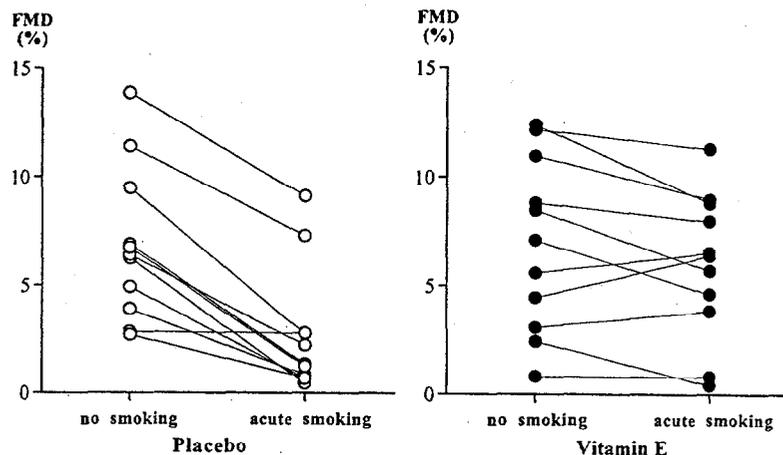


Figure 2. FMD values before and after heavy smoking at the end of the treatment period for subjects receiving placebo as well as for subjects receiving vitamin E (600 IU/day). P values for the time factor = 0.0001; p value for the group factor = 0.0017. FMD = flow-mediated vasodilation.

endothelial dysfunction in these subjects. In addition, the attenuation of transient endothelial dysfunction after acute smoking correlated with an improvement of the antioxidant status under vitamin E supplementation.

Effects of chronic smoking on endothelial dysfunction.

Endothelial dysfunction has been observed in both peripheral and coronary arteries of long-term smokers (1,2). The precise mechanism of this impairment by chronic smoking has not yet been elucidated but may be related to oxidative stress. In support of this, cigarette smoke has been shown to contain large amounts of free radicals such as superoxide anion and hydroxyl radicals (11,22), agents known to degrade endothelium derived relaxing factor and to impair

FMD (6). Superoxide anion, in turn, can rapidly react with nitric oxide to form peroxynitrite, a molecule with high cytotoxic potency (23,24). Moreover, it has been reported that cigarette smokers have higher rates of in vivo and in vitro lipid peroxidation (25). Thus, atherogenic effects of smoking may be mediated in part by free radical damage to lipids, in particular oxidative modification of LDL, which promotes foam cell formation (26,27), monocyte adhesion (28,29) and is cytotoxic to vascular cells (30).

Role of antioxidants in smokers.

Epidemiologic studies have shown that plasma levels of antioxidant nutrients, such as vitamin C and E, are significantly lower in smokers compared with nonsmokers (31). In the Health Professionals' follow-up study, a reduction in major coronary events among healthy male subjects taking 100 to 250 IU supplementation of vitamin E per day, with little further benefit at higher doses, was reported (8). In contrast, in a randomized therapeutic trial among Finnish smokers, no beneficial effect on cardiovascular events was observed for either vitamin E or beta-carotene intake (32). However, the dose of vitamin E applied in this study (50 mg per day) was below the protective range suggested in the Health Professionals' follow-up study. Therefore, a cause-and-effect relation between vitamin E intake and a reduction in cardiovascular events has thus far been suggested only for vitamin E supplementation in patients with angiographically proven coronary atherosclerosis (10).

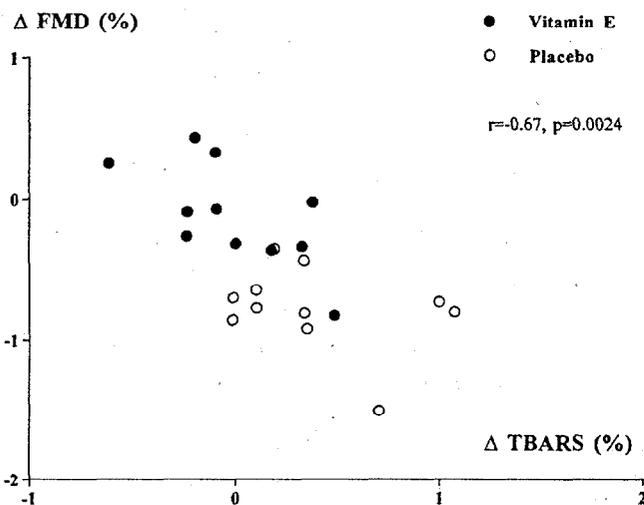


Figure 3. Transient impairment of FMD (delta FMD) after heavy smoking vs. improvement of the antioxidant status (delta TBARS) under vitamin E supplementation for four weeks. FMD = flow-mediated dilation; TBARS = thiobarbituric acid-reactive substances.

Effects of antioxidant vitamins on endothelial function in smokers.

Endothelium-dependent vasodilation in the forearm circulation of chronic smokers can be improved by acute intraarterial administration of vitamin C (11). Moreover, Motoyama *et al.* (12) demonstrated that an acute impairment of FMD in the brachial artery after heavy smoking is caused by an increase of oxidative stress within 10 min. This transient impairment of endothelial function

lasts for ≤ 90 min (4) and can be attenuated by intravenous infusion of vitamin C (12). In contrast, oral vitamin E supplementation did not improve endothelial function in the forearm circulation of normocholesterolemic chronic smokers (33). Our data suggest that the oral intake of vitamin E for four weeks is not able to reverse chronic endothelial dysfunction of smokers but does prevent the transient further impairment of endothelium-dependent vasodilation in the brachial artery after acute smoking. This acute effect is likely due to the antioxidant effect of vitamin E which is supported by its close correlation with

- 1) the decrease in TBARS, reflecting attenuation of oxidative stress under vitamin E supplementation and
- 2) the increase of alpha-tocopherol plasma levels normalized to LDL-C, suggesting reduced lipid peroxidation under therapy (19,20). Since TBARS are used to measure actual oxidative stress, it was not surprising that TBARS levels correlated with the total number of cigarettes smoked per day and not with the number of pack-years.

Interestingly, no improvement of chronic endothelial dysfunction was found, although the oxidative status was improved. These results are in accordance with previous observations in the forearm vasculature, in which vitamin E supplementation failed to improve endothelium-dependent vasodilation, although the susceptibility of LDL to oxidation could be reduced (34). However, the beneficial effect of vitamin E supplementation on the impairment of endothelial function after heavy smoking reported in this study is unlikely due to increased resistance of LDL-C to oxidation, as this process would last longer than the time elapsed between heavy smoking and the ultrasound examination (20). Thus, other mechanisms must be considered, such as the scavenging of superoxide or an improvement of the intracellular antioxidant status due to the accumulation of alpha-tocopherol in the vascular wall and in endothelial cells in particular (35).

A possible explanation for the discrepancy between the lack of effect on chronic endothelial dysfunction after vitamin E supplementation and its beneficial effect on transient endothelial dysfunction in our study may be the relatively low degree of oxidative stress while subjects refrained from smoking before the examination, compared with the burst of oxidative stress after heavy smoking. Recently, Heitzer *et al.* (33) revealed a beneficial effect of vitamin E supplementation on chronic endothelial dysfunction selectively in hypercholesterolemic smokers which was associated with a reduction of elevated autoantibody titers against oxidized LDL. Alternatively, chronic endothelial dysfunction caused by smoking may be due to other mechanisms not affected by the antioxidant or other properties of vitamin E. These mechanisms may include an increase in asymmetric dimethylarginine, an endogenous nitric oxide synthase inhibitor (36), as well as more long-lasting endothelial cell damage, due to cytotoxic intermediates (23,24).

Study limitations. A possible drawback of this study may be that we did not measure TBARS after heavy smoking. However, an acute increase of TBARS after heavy smoking as well as an attenuation of this increase by an antioxidant has been demonstrated previously (12). In this study, TBARS levels and alpha-tocopherol serum levels normalized to LDL-C were measured to estimate the attenuation of oxidative stress under vitamin E supplementation. The fact that TBARS levels were not significantly different in both study groups ($p = 0.06$) is most likely due to the high SD of this parameter (19). However, differences in delta TBARS, alpha-tocopherol/LDL-C and delta alpha-tocopherol/LDL-C strongly suggest an improvement in the antioxidant status of subjects taking vitamin E. Finally, we cannot exclude that the duration of vitamin E treatment may have been insufficient. However, long term vitamin E supplementation for four months also failed to restore endothelial function in long-term smokers with a lipid profile similar to our study subjects (33).

Conclusions. This study demonstrates that oral vitamin E supplementation can attenuate the transient impairment of endothelial function after heavy smoking due to an improvement of the antioxidant status, but cannot restore chronic endothelial dysfunction within four weeks. These data are consistent with the concept that vitamin E may in part be beneficial due to direct tissue effects that are different from the prevention of the formation of oxidized LDL-C.

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Possible role of high susceptibility of high-density lipoprotein to lipid peroxidative modification and oxidized high-density lipoprotein in genesis of coronary artery spasm

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Abstract

Recent study demonstrated high susceptibility of plasma LDL to lipid peroxidative modification in patients with variant angina. Oxidized stress state, especially oxidized LDL, may induce coronary artery spasm by its impairing effect of endothelium-dependent arterial relaxation, but precise mechanisms remain unclear. Study subjects included 93 patients who underwent coronary angiographic examination: 12 patients with coronary artery spasm provoked by ergonovine without organic stenosis (group I), 11 patients who did not demonstrate coronary artery spasm or organic stenosis (group II) and 70 patients with organic coronary artery stenosis (group III). Levels of plasma HDL-cholesterol and apoA-I in group I were similar to those in III but were significantly lower than those in II, although the other plasma lipid parameters were not different among the three groups. The levels of TBARS in plasma and HDL were significantly higher in group I than in II or III (2.94 ± 1.56 vs. 1.91 ± 0.35 or 2.23 ± 0.89 nmol MDA/ml and 1.23 ± 1.00 vs. 0.54 ± 0.37 or 0.70 ± 0.63 nmol MDA/mg protein; $P < 0.05$), although the levels of TBARS in LDL were not significantly different. In the monitoring curve of diene production during copper-induced lipid peroxidation of HDL, its propagation slope was steeper and levels of maximum diene absorbance was higher in group I as compared with that in II or III, but not found in those of LDL. These results suggested that high susceptibility of HDL to lipid peroxidative modification in group I may contribute to the genesis of coronary artery spasm, and oxidized HDL rather than oxidized LDL is more likely to be related to coronary artery spasm. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Peroxidative modification; Coronary artery spasm; LDL; HDL; Coronary artery disease

1. Introduction

Coronary artery spasm plays an important role in the pathogenesis of coronary heart disease (CHD) including variant angina pectoris, acute coronary syndrome and sudden cardiac death [1–3], although its precise mechanisms remain unclear.

Several studies have demonstrated that oxidized low-density lipoprotein (LDL) or lysophosphatidylcholine (Lys-PC) impairs the endothelium-dependent arterial

relaxation [4–7] and promotes the hyper-reactivity of coronary artery smooth muscle cells [8]. Recent studies demonstrated that plasma LDL obtained from patients with variant angina was highly susceptible to peroxidative modification [9] and they suggested that oxidized LDL may be related.

High-density lipoprotein (HDL) has been considered as an anti-atherogenic lipoprotein [10–12]. In addition, it has been reported that HDL may have a direct effect on release of endothelial-derived relaxing factor or may have an indirect effect on it secondary to its effect on endothelial proliferation [13]. These findings suggested that HDL is also related to its effects on endothelial function and coronary artery vasoreactivity.

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Recently, there is growing evidence that oxidative modification of HDL could occur in vivo following by alteration of its conformation biologically and chemically [14–17]. Subsequently, oxidized HDL reduces its beneficial properties such as stimulation of cholesterol efflux from foam cells, and vasoregulatory functions described above. Moreover, it has also been reported that oxidized HDL could directly inhibit endothelium-dependent vasoreactivity [18]. These results suggested that oxidized lipoproteins, both LDL and HDL, may contribute to genesis of coronary artery spasm, while the possibility should also be considered that generation of oxidized lipoproteins could reflect an oxidative stress state predisposing to coronary artery spasm.

To investigate this hypothesis, we measured thiobarbituric acid-reactive substances (TBARS) and diene production during copper-induced lipid peroxidation of HDL and LDL in patients with or without coronary artery spasm.

2. 2. Material and methods

2.1. Subjects

Subjects in this study were selected from consecutive patients who underwent coronary angiographic examination to evaluate the coronary artery disease (CAD) and cardiac functions between January 1996 and January 1997. The following three groups were identified. Group I included 12 patients who had resting anginal attacks and documented positive ergonovine provocation test without any significant organic coronary artery stenosis. Group II included 11 patients with atypical chest pain who did not have organic coronary artery stenosis with negative ergonovine provocation test. Group III included 70 patients who did not have any resting anginal attack with angiographically documented CAD defined as 50% stenosis in at least one of major branch of coronary artery trees. Patients who were taking lipid-lowering or anti-oxidant medications such as probucol and antioxidative vitamins were excluded.

All patients who participated in this study gave their informed consent.

2.2. Coronary angiographic examination and ergonovine provocation test

Coronary angiographic examination was performed via right brachial artery or femoral artery by the standard method, after over-night fasting. Any calcium-channel blockers were stopped 3 days before the coronary angiographic examination. Blood pressure was monitored through the catheter and standard 12-lead electrocardiograms were recorded continuously

with a 6-channel recorder. Prior to ergonovine provocation test, a temporary pacing catheter was inserted into the right ventricle via the peripheral vein. After control coronary arteriograms were obtained, 0.2 mg ergometrine maleate was injected intravenously and repeat coronary arteriograms were obtained at 3, 6 min after injection. If significant changes occurred, which including anginal attacks or ischemic electrocardiographic changes with a significant vasoconstriction demonstrated angiographically, coronary arteriograms were obtained immediately and 2 mg of isosorbide dinitrate was injected into the coronary artery through the catheter.

Coronary artery constrictive changes following ergonovine provocation were assessed by quantitative coronary angiography (QCA) technique (QCA-Cardiovascular Measurement System: QCA-CMS, MEDIS medical imaging systems, Holland). The constrictive changes of coronary artery by ergonovine provocation were assessed by QCA technique and were expressed as percent change of segment diameter from diameter after nitroglycerin. We defined the coronary artery spasm to be present as $\geq 50\%$ change of diameter in at least one of the major coronary arteries.

2.3. Serum lipid samples and lipoprotein separation

Venous blood samples were obtained by venipuncture with Vacutainer tubes after a 12-h fasting period for the measurement of serum lipid profile. For separating the lipoprotein fractions, arterial blood samples were obtained via catheter sheath into tubes containing 0.04 N ethylenediaminetetraacetic acid (EDTA) disodium salt just before starting coronary angiographic examination. Plasma was immediately separated by centrifugation at 3000 rpm for 10-min. Then to obtain the LDL after removing very low-density lipoprotein using ultracentrifugation, samples taken from the bottom of the tube were adjusted in density to 1.063 mg/dl with adequate potassium bromide (KBr) solution and were then ultracentrifuged at 100 000 rpm for 4.5-h at 15°C (Beckman TL100.4 rotor) as previously described by Havel et al. [19]. After ultracentrifugation, LDL floating at the top of the tube was removed with pipette to another stock of tubes. Finally the HDL was obtained in same manner after adjusting the density to 1.21 mg/dl, and it was ultracentrifuged 100 000 rpm for 6-h at 15°C.

The stock samples were dialyzed to remove KBr and EDTA in the 2000 ml volume of phosphate buffer saline (PBS) with pH7.4 at 4°C in the dark place. After conclusion of dialysis, quantification of protein was performed by the Lowry method [20], and stock samples were stored at 4°C until the time to use for all oxidation studies.

2.4. Lipid peroxides level and peroxidative modification of lipoproteins

The lipid peroxides levels were determined by TBARS [21] in the plasma samples and in each lipoprotein fraction by using a lipidperoxide test kit (WAKO Junyaku, Osaka, Japan). This result was expressed as nmol malondialdehyde (MDA) equivalents. The susceptibility of both LDL and HDL to in vitro oxidation was assessed by the technique described by Esterbauer et al. [22]. Oxidative modification of lipoprotein containing 100 µg of protein was initiated by 5 µM CuSO₄ in final volume of 1 ml PBS at 37°C. The diene production during copper-induced lipid peroxidation was monitored by the change in 234 nm absorbance at 5-min intervals for 240 min with spectrophotometer (Beckman DU650). To assess the susceptibility to peroxidative modification, these results were expressed as lag time (min), propagation rate (min⁻¹) and maximum diene absorbance.

2.5. Statistical method

All results except lipoprotein(a) [Lp(a)] were expressed as mean ± S.D. Lp(a) was indicated by median and range. Intergroup comparisons concerning serum lipid levels except Lp(a), the generation of TBARS in plasma, LDL and HDL, and the diene production in LDL and HDL were made with Bonferroni multiple comparisons test after being analyzed by a one-way analysis of variance (ANOVA). As distribution plasma levels of Lp(a) was of highly skewed, the analysis of those levels was performed with Mann–Whitney's U analysis. To compare the prevalence of hypertension, smoking and diabetes mellitus with each group, χ^2 test was performed.

3. Results

3.1. Population characteristics and plasma lipids

Study subjects included 93 patients who underwent coronary angiographic examination; 12 patients with coronary artery spasm provoked by ergonovine without organic stenosis (group I), 11 patients who did not demonstrate coronary artery spasm or organic stenosis (group II) and 70 patients with organic coronary artery stenosis (group III). Table 1 shows clinical characteristics of each group. There was no significant difference in mean age among the three groups. The proportion of males was significantly higher in group III than in II ($P < 0.05$), although there was no difference between group III and I statistically. The prevalence of hypertension and diabetes mellitus were significantly higher in group III than in I ($P < 0.005$). The proportion of

current smokers was not significantly different among the three groups.

The levels of plasma HDL-cholesterol in group I was significantly lower than in II (42 ± 9 vs. 59 ± 19 mg/dl; $P < 0.05$) and was similar to that in group III. The levels of apoA-I in group I or II were also significantly lower than in III (116 ± 12 or 117 ± 25 vs. 134 ± 19 mg/dl; $P < 0.05$ each). Both the levels of apoB and Lp(a) were significantly higher in group III than in II (104 ± 19 vs. 90 ± 19 mg/dl and 29.5 (123.7) vs. 23.9 (17.2) as indicated median (range) mg/dl; $P < 0.05$ and < 0.005 , respectively). The levels of total cholesterol, triglycerides and LDL-cholesterol calculated by Friedewald formula were not significantly different among the three groups. This result may be explained by excluding the subjects with taking lipid lowering drugs. These results demonstrated that patients with CAD seemed to have conventional coronary risks, could be distinguished from the characteristics in patients with coronary artery spasm, which apart from low levels of plasma HDL-cholesterol and apoA-I.

3.2. Susceptibility to lipid peroxidative modification

Susceptibility to lipid peroxidative modification was determined by the levels of TBARS and diene production during copper-induced lipid peroxidation, shown in Table 2. Copper-induced lipid peroxidation was assessed by lag time, propagation rate and maximum diene absorbance as described previously. The levels of

Table 1
Clinical characteristics and lipid profile in study population^a

	Group I (n = 12)	Group II (n = 11)	Group III (n = 70)
Age (years)	60 ± 9	61 ± 7	62 ± 8
Sex (M/F)	8/4	7/4	61/9*
HT (%)	25 [§]	36	63
Smokers (%)	33	36	41
DM (%)	0 [§]	9	31
TC (mg/dl)	172 ± 31	187 ± 36	189 ± 30
TG (mg/dl)	141 ± 67	130 ± 45	141 ± 56
LDL-C (mg/dl)	104 ± 18	103 ± 45	141 ± 56
HDL-C (mg/dl)	42 ± 9*	59 ± 19	44 ± 13**
apoA-I (mg/dl)	116 ± 12*	134 ± 19	117 ± 25*
apoB (mg/dl)	97 ± 19	90 ± 19	104 ± 19*
LP(a) (mg/dl)	25.3 (85.2)	23.9 (17.2)	29.5 (123.7)**

^a HT, hypertension; DM, diabetes mellitus; TC, total cholesterol; TG, triglycerides; LDL-c, low-density lipoprotein-cholesterol; HDL-c, high-density lipoprotein-cholesterol; apo, apolipoprotein; Lp(a), lipoprotein(a) each value is expressed as mean ± S.D. except Lp(a). Lp(a) is expressed as median (range).

* $P < 0.05$ vs. group II.

** $P < 0.005$ vs. group II.

[§] $P < 0.005$ vs. group III.

Table 2
Comparison data of oxidative susceptibility among three groups^a

	Group I	Group II	Group III	P value
Plasma TBARS (nmol MDA/ml)	2.94 ± 1.56*	1.91 ± 0.35	2.23 ± 0.89	0.027
LDL TBARS (nmol MDA/mg LDL protein)	1.62 ± 0.74	1.32 ± 0.59	1.65 ± 0.95	NS
HDL TBARS (nmol MDA/mg HDL protein)	1.23 ± 1.00*	0.54 ± 0.37	0.70 ± 0.63	0.025
LDL lag time (min.)	46 ± 13	47 ± 8	46 ± 10	NS
Propagation rate (min. ⁻¹)	0.039 ± 0.008	0.041 ± 0.007	0.038 ± 0.008	NS
Max. diene absorbance	1.72 ± 0.39 [§]	1.57 ± 0.28	1.51 ± 0.33	NS
HDL lag time (min.)	20 ± 3	20 ± 7	20 ± 6	NS
Propagation rate (min. ⁻¹)	0.019 ± 0.005 [§]	0.017 ± 0.004	0.015 ± 0.005	0.039
Max. diene absorbance	0.66 ± 0.15 [†]	0.57 ± 0.008	0.52 ± 0.16	0.10

^a TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde; LDL, low-density lipoprotein; HDL, high-density lipoprotein each value is expressed as mean ± S.D. P value imply group differences analyzed by ANOVA. **P* < 0.05 vs. group II or group III.

[§] *P* < 0.05 vs. group III.

[†] *P* < 0.005 vs. group III.

TBARS in both plasma and HDL were significantly higher in group I than in II or III (2.94 ± 1.56 vs. 1.91 ± 0.35 or 2.23 ± 0.89 nmol MDA/ml; *P* < 0.05 and 1.23 ± 1.00 vs. 0.54 ± 0.37 or 0.70 ± 0.63 nmol MDA/mg protein; *P* < 0.05), although the levels of TBARS in LDL showed no significant differences. Levels of TBARS in plasma had positive correlation with those in LDL or HDL (data not shown). Based on the results from monitoring the curve of diene production during copper-induced lipid peroxidation (Table 2), lag time and propagation rate of LDL were similar in each group, except that levels of maximum diene absorbance in group I were higher as compared with those in III. In contrast, the monitoring curve of HDL (Fig. 1) demonstrated that the propagation slope was steeper and levels of maximum diene absorbance was significantly higher in group I as compared with the other two groups, although lag time was not different. These findings indicated high susceptibility of HDL to copper-induced lipid peroxidative modification in group I.

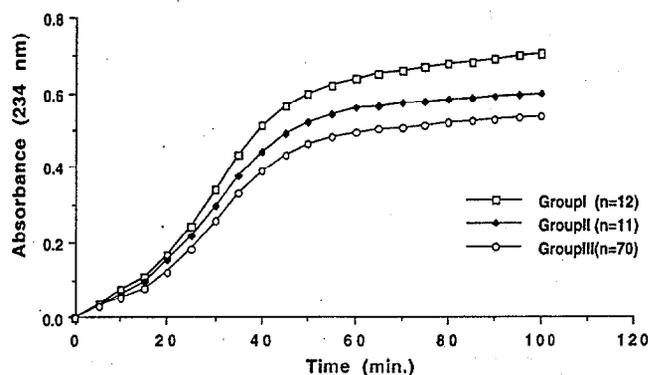


Fig. 1. The monitoring curve of copper-induced lipid peroxidation in HDL fractions. This monitoring curve shows the formation of conjugated dienes of HDL fractions. Mean values of those absorbances in each group were indicated. The oxidation was performed with 100 µg protein of HDL in PBS with 5 µM CuSO₄ at 37°C. The details are described in the methods.

4. Discussion

The present study demonstrated lower levels of plasma HDL and high susceptibility of HDL to lipid peroxidative modification in patients with coronary artery spasm as compared with those in patients without coronary artery spasm. This result suggested that oxidized HDL may be one of the contributing factors to generate coronary artery spasm *in vivo* rather than oxidized LDL, at least, in our study population.

HDL is considered as an anti-atherogenic lipoprotein for its reverse cholesterol transport activity [10–12]. Recently other protective functions of HDL, which include an anti-thrombotic effects [23], prostacyclin stabilizing activity [24,25], and release of endothelial-derived relaxing factor to preserve normal endothelial cell function [13,26–28], has been postulated. Clinically, it has already been reported that the low levels of apoA-I, a major apoprotein in HDL, observed in patients with variant angina discriminated from normal control subjects or patients with CAD [29,30]. The others reported that there was positive correlation between the levels of plasma HDL-cholesterol and normal acetylcholine-induced coronary vasodilatory responses [13], and elevated levels of plasma HDL-cholesterol ameliorated abnormal vasoconstriction in both angiographically smooth and stenotic coronary artery segments [28]. In our study, the results of reduced levels of plasma HDL-cholesterol and apoA-I in patients with coronary artery spasm were, in part, consistent with previous studies. Therefore, it may be important to maintain a certain level of plasma HDL-C for its protective functions.

HDL has also been demonstrated to play a possible role in preventing the generation of oxidized LDL [31–34] and reversing the inhibitory effect of oxidized LDL on endothelium-dependent vasoregulation [35]. Oxidized LDL or increased content of Lys-PC in oxi-

dized LDL has been demonstrated to play an important role in impairment of endothelium-dependent arterial relaxation [4–7,18] and potentiate agonist-induced hyper-responsiveness of vasoconstriction by direct action on vascular smooth muscle [8]. Therefore, reduced levels of HDL might facilitate the generation of oxidized LDL and effect to impair normal vasoregulation cooperating with oxidized LDL. In addition, recent clinical study demonstrated that plasma LDL obtained from patients with variant angina was highly susceptible to peroxidative modification [9]. In our study, it showed significant higher levels of maximum diene absorbance and tendency toward higher levels of TBARS in LDL obtained from patients with coronary artery spasm as compared with patients who had high levels of plasma HDL-C such as group II, although it seemed to be less pronounced in association with coronary artery spasm rather than with those in HDL.

On the other hand, HDL obtained from patients with coronary artery spasm showed significantly elevated levels of TBARS, and significantly higher levels of propagation rates and maximum diene absorbance as compared with those in patients without coronary artery spasm. These results suggested that oxidized HDL, rather than oxidized LDL, may be related to coronary artery spasm, at least in our study population. There is growing evidence that oxidative modification of HDL also could occur *in vivo*, and alter biologically or chemically in its conformation, occasionally more rapidly than those in LDL [36,37]. Subsequently oxidized HDL reduces its beneficial properties such as stimulation of cholesterol efflux from foam cells [14–17], anti-oxidative activity [31–34]. In addition, it has been reported that oxidized HDL could inhibit the endothelium-dependent vasoreactivity [18] and moreover oxidized HDL converts itself into a cytotoxic particle such as oxidized LDL [38,39]. These reports suggested the possibility that oxidized HDL itself, aside from an ability of HDL, may be related to dysfunction of endothelium and/or hyper-reactivity of smooth muscle in coronary artery spasm. It should be also considered as another possibility that generation of both oxidized LDL and HDL could be simply reflecting oxidative stress predisposing to coronary artery spasm. Some studies have demonstrated that the antioxidant effect of probucol improved endothelium-dependent vasoconstrictive responses to acetylcholine, contributing to spasm in both animal experimental model and human [40,41]. More recent study has demonstrated that supplement of antioxidant vitamin E prevented the occurrence of attacks in patients with variant angina [42]. These findings indicate clinical expectation that intervention with antioxidants could prevent coronary artery spasm in patients and improve their anginal symptom.

In conclusion, high susceptibility of HDL to lipid peroxidative modification in patients with coronary artery spasm may contribute to genesis of coronary artery spasm, and oxidized HDL rather than oxidized LDL is more likely to be related to coronary artery spasm. Further studies are needed to elucidate whether prevention of peroxidative modification of HDL and/or correcting the levels of plasma HDL-cholesterol and apoA-I could improve anginal attacks in patients with coronary artery spasm.

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Anti-oxidant status and lipid peroxidation in patients with essential hypertension

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Background Lipid peroxidation and derived oxidized products are being intensively investigated, because of their potential to cause injury and their pathogenetic role in several clinically significant diseases. The view that an excess of lipid peroxidation products is present and relevant in the pathogenesis of human essential hypertension or in hypertension-induced damage has still not received definitive support.

Objective To evaluate both the extent of lipoperoxidation in essential hypertensive patients and the status of enzymatic and non-enzymatic antioxidants that potentially are able to modulate it.

Methods We selected 105 newly diagnosed essential hypertensives among those referred to our hypertension outpatient clinic and compared them with 100 normotensive controls matched for age. Plasma malondialdehyde was measured by high-performance liquid chromatography after reaction with thiobarbituric acid, as an end product of lipid peroxidation; serum selenium (Se), plasma copper (Cu) and zinc (Zn), vitamins A and E, erythrocyte superoxide dismutase and glutathione peroxidase levels were evaluated as indices of oxidant balance. Differences between the groups were tested by Student's *t* test and χ^2 test.

Results Compared with controls, essential hypertension patients had higher malondialdehyde and glutathione peroxidase activities ($P < 0.05$ for both) and Zn

concentrations ($P < 0.001$) and lower superoxide dismutase activities ($P < 0.005$), vitamin A ($P < 0.05$) and E ($P < 0.001$) levels and Cu concentrations ($P < 0.005$). We found no difference between Se levels of essential hypertensive and control subjects.

Conclusions Essential hypertension is associated with greater than normal lipoperoxidation and an imbalance in anti-oxidant status, suggesting that oxidative stress is important in the pathogenesis of essential hypertension or in arterial damage related to essential hypertension. *J Hypertens* 16:1267-1271 © 1998 Lippincott Williams & Wilkins.

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Keywords: essential hypertension, lipid peroxidation, anti-oxidants, malondialdehyde, superoxide dismutase, glutathione peroxidase, vitamins, trace elements, thiobarbituric acid

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Introduction

Lipid peroxidation and derived oxidized products are being intensively investigated, because of their potential to cause injury and their pathogenetic role in several clinically significant diseases [1]. Recent experimental evidence implicates oxygen radical reactions as playing a role also in the development of high blood pressure in some animal models [2]. However, the view that an excess of lipid peroxidation products can be present in humans and is of relevance in the pathogenesis of essential hypertension has still not received definitive support.

Lipid peroxidation is a reaction whereby molecular oxygen is incorporated into poly-unsaturated fatty acids

(PUFA) to yield lipid peroxides. Two general models of lipid peroxidation may be defined: enzymatic oxygenation of PUFA, which is catalysed by the eicosanoid-synthesizing enzymes cyclooxygenase and lipoxygenase, and non-enzymatic oxygenation of PUFA. The latter process, named 'PUFA autoxidation', is stimulated by reactive oxygen-containing species and transition metals and limited by anti-oxidants such as vitamins and free-radical-scavenger enzymes [3]. A variety of methods has been employed to detect and quantify lipid peroxidation. The most widely used is the so-called thiobarbituric acid (TBA) test, based on the reactivity of TBA towards malondialdehyde, a side product of enzymatic oxygenation of PUFA and an end product of oxidative degradation of

PUFA [2]. The present study was designed to evaluate both the extent of lipoperoxidation in a group of essential hypertension patients and the status of enzymatic and non-enzymatic anti-oxidants that potentially are able to modulate it.

Methods

Patients

Two groups of 105 essential hypertension patients and 100 normotensive controls matched for age and sex were selected from the population of the same restricted geographic area (Veneto region, northern Italy) between 1994 and 1997. For the control group, we used data from a cross-sectional study of a sample of 100 healthy subjects, selected as described previously [4]. Briefly, an initial group of 500 normal subjects was obtained by using tables of random numbers; a further selection was performed by the three practitioners operating in the area, who were familiar with the details of the clinical histories and lifestyles of the subjects. All subjects known to be suffering from chronic disease or acute intercurrent illness, as well as pregnant women and subjects taking any drug, including contraceptive pills were excluded from our study. Mean blood pressure levels of the control population were $131 \pm 13/81 \pm 6$ mmHg.

Essential hypertensive subjects were recruited from among the 1000 patients referred to our hypertension outpatient clinic between 1992 and 1996. As described previously [5], office blood pressure was evaluated by the same physician with the patient sitting after 5 min of rest, according to directives of the British Hypertension Society [6]: subjects who had diastolic blood pressures > 95 mmHg during three visits within 3 months, without any treatment, were recruited. These patients then underwent 24 h ambulatory blood pressure monitoring (ABPM), performed with a SpaceLabs 90207 device (SpaceLabs Inc., Redmond, Washington, USA). In accord with the indications of the first international consensus document on ABPM [7], we considered patients with daytime blood pressures $> 135/85$ mmHg hypertensive. For this group 24 h blood pressure levels were $142.5 \pm 12/90.5 \pm 7$ mmHg, daytime blood pressure levels were $145.9 \pm 13/92.7 \pm 7$ mmHg and night-time blood pressure levels were $132.6 \pm 14/83.7 \pm 8$ mmHg. Cuffs of appropriate width and length were used to avoid overestimation and underestimation of blood pressure values; ABPM data sets with more than 20% technical errors were discarded. Patients with secondary hypertension and essential hypertension patients with any other concomitant disease were excluded from our study.

None of the subjects were institutionalized, eating a special diet, or taking trace elements or vitamin supplements. A 7-day food record was randomly obtained from

a representative sample of subjects (50 essential hypertensive and 30 normotensive controls), to exclude the possibility of there being any significant differences in diet between essential hypertension patients and control subjects.

Biochemical analyses

Erythrocyte superoxide dismutase (SOD), and glutathione peroxidase (GSHPx) activities, plasma levels of vitamins A and E, serum level of selenium (Se) and plasma levels of zinc (Zn) and copper (Cu) were determined as described previously [8]. Lipoperoxidation was quantified by the assay of plasma malondialdehyde using a slight modification of a method published by Carbonneau *et al.* [9]. Briefly, after treatment beforehand of 250 ml plasma with 0.44 mol/l H_3PO_4 , protein-free extract was obtained by perchloric acid precipitation and allowed to react with TBA in H_3PO_4 for 45 min at $100^\circ C$. Then high-performance liquid chromatography (HPLC) separation was performed on Superspher 100 RP-18 column (150 mm \times 4.6 mm; Hewlett-Packard, Palo Alto, California, USA) with an eluting solution of methanol-phosphate buffer, 50 mmol/l, pH 6.8 (40:60, vol:vol), at a rate of flow of 1.5 ml/min. Malondialdehyde-TBA adduct was then quantified by ultraviolet-visible spectrophotometry (at $\lambda_{max} = 532$ nm; series 1050 spectrophotometer; Hewlett-Packard).

Statistical analysis

Statistical analysis was carried out with the aid of an Apple Macintosh SB/30 computer (Apple Co., Cupertino, California, USA), using the Systat 5.2.1 program (Systat Co., Evanston, Illinois, USA). Differences between the groups were tested by χ^2 or Student's t test. $P < 0.05$ was considered statistically significant.

Results

In Table 1 we summarize the main clinical and biochemical features of normotensive subjects and essential hypertension patients. The groups did not differ regarding age, sex, body mass index, smoking habits and levels of low-density lipoprotein (LDL) cholesterol, high-density lipoprotein cholesterol and triglycerides. Conversely, plasma malondialdehyde level, GSHPx activity and plasma Zn concentration were greater, whereas levels of vitamins A and E, SOD activity and plasma Cu concentration were lower in essential hypertension patients than they were in controls. Serum Se concentrations in members of these two groups were similar. Dietary analysis revealed no significant difference between essential hypertension patients and normotensive subjects in dietary intake of major nutrients (proteins, lipids, carbohydrates, saturated fatty acids, mono-unsaturated fatty acids and PUFA) and consumption of alcohol (Table 2).

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Table 1 Clinical and biochemical parameters of essential hypertensive and normotensive subjects

	Normotensives (n = 100)	Hypertensives (n = 105)	Significance ^a
Age (years)	45.9 ± 16	47.7 ± 11	NS
Sex (male/female)	49/51	63/42	NS
Body mass index (kg/m ²)	24.6 ± 3.2	26.2 ± 3.6	NS
Smokers (yes/no)	21/79	24/81	NS
HDLC (mmol/l)	1.51 ± 0.3	1.31 ± 0.3	NS
LDLC (mmol/l)	3.68 ± 1.1	3.95 ± 0.9	NS
Triglycerides (mmol/l)	1.51 ± 0.7	1.48 ± 0.5	NS
Plasma malondialdehyde (mmol/l)	0.65 ± 0.1	0.73 ± 0.2	P < 0.05
Erythrocyte SOD (IU/g haemoglobin)	8339 ± 2338	7194 ± 2057	P < 0.005
Erythrocyte GSHPx (IU/g haemoglobin)	6.58 ± 1.5	7.45 ± 2	P < 0.005
Plasma zinc (mmol/l)	14.84 ± 1.9	17.47 ± 4.6	P < 0.001
Plasma copper (mmol/l)	16.9 ± 2.3	15.7 ± 3.4	P < 0.01
Serum selenium (mmol/l)	1.16 ± 0.18	1.13 ± 0.25	NS
Vitamin A (mmol/l)	3.2 ± 0.9	2.9 ± 0.7	P < 0.05
Vitamin E (mmol/l)	41.2 ± 10	32.5 ± 10	P < 0.001

Values are expressed as means ± SD. ^aStudent's t test. HDLC, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholesterol; SOD, superoxide dismutase; GSHPx, glutathione peroxidase.

Table 2 Dietary parameters

	Normotensives (n = 30)	Hypertensives (n = 50)
Total energy intake (J/day)	9643 ± 2616	9572 ± 4296
Proteins (g/day)	96 ± 29	83 ± 23
Lipids (g/day)	76 ± 19	71 ± 37
Carbohydrates (g/day)	302 ± 125	326 ± 190
SFA (g/day)	25.5 ± 8.1	26.9 ± 14
MUFA (g/day)	35.9 ± 16	32.9 ± 18.4
PUFA (g/day)	9.7 ± 2.4	8.2 ± 3
Alcohol (g/day)	229 ± 200	145.5 ± 120

Values are expressed as means ± SD. SFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; PUFA, poly-unsaturated fatty acids. All comparisons are not significant by Student's t test.

Discussion

The result of the present study is that essential hypertension patients have an impairment of anti-oxidant status or a greater than normal response to oxidative stress, or both. This was proved both by the complex changes in levels of several enzymatic and non-enzymatic anti-oxidants and by the significant increase in amount of HPLC-separated malondialdehyde, one of the most reliable markers currently available for evaluating lipid peroxidation [3].

Levels of TBA-reactive substances have been demonstrated to be greater than normal in animal models of experimentally induced hypertension [10] and a higher than normal plasma level of malondialdehyde together with simultaneously lower than normal levels of erythrocyte SOD and vitamin E had already been observed for a small sample of essential hypertension patients [11]. Authors of no other studies, however, have evaluated all these variables with such a relatively large sample of untreated hypertensive subjects and corresponding age-matched and sex-matched controls.

Since a high amount of oxidative damage has been found to be involved in many diseases as well as in the process of ageing [1], particular care was adopted in order to include only healthy subjects in the control group and subjects suffering only from essential hypertension in the patient group. The study was conducted under unrestricted living conditions, but subjects taking preparations containing vitamins or other nutrients that could have influenced the parameters tested were not admitted. Dietary intake can also affect the oxidant-anti-oxidant balance, but analysis of dietary records revealed no difference in consumption of major nutrients and alcohol between the groups. Moreover, all people had normal nutritional indices (body mass index and levels of haemoglobin, haematocrit and serum albumin; data not shown), ruling out the possibility that our results may represent just the effects of different degrees of nutrition. Smoking was also taken into account, since the reactants present in cigarette smoke are potential sources of oxidative degradation both of membrane lipids and of vitamins [1]; however, the absolute numbers of smokers in the two groups were similar. Thus, after a careful evaluation of the majority of factors potentially able to influence the parameters examined, we could reasonably exclude the possibility that dietary, demographic or lifestyle habits could account for the observed differences between essential hypertension patients and controls.

One of the most relevant results of our study was the greater than normal plasma level of malondialdehyde observed in essential hypertension patients. This is probably the most widely discussed parameter of oxidative stress on biological samples. Malondialdehyde is a volatile side product of cyclooxygenase-catalysed and lipoxygenase-catalysed oxygenation of PUFA and the end product of non-enzymatic oxidative degradation of PUFA. The labile nature of pure malondialdehyde has, in practice, made direct measurement of its level diffi-

cult; however, malondialdehyde can readily be condensed with TBA to yield a fluorescent red adduct that is easily detectable. This method is of particular interest because of its relative procedural simplicity and sensitivity, but it is intrinsically non-specific, because a variety of non-lipid sources can react with TBA, leading to an overestimation of malondialdehyde level [3]. In this study we adopted all the precautions necessary to maximize the reliability of the test in terms of sensitivity and specificity [9]. Reaction with TBA was carried out after blood proteins and other substances that would react with TBA had been eliminated by precipitation with perchloric acid treatment, since TBA product reflects the actual lipid peroxidation when the analysed samples contain exclusively lipids [3]. Subsequent HPLC analysis allowed us to separate the malondialdehyde-TBA adduct from the other chemically distinct pigments generated during the test [3,9].

That active lipoperoxidation occurs in essential hypertension patients is also suggested by the greater than normal activity of the anti-oxidant enzyme GSHPx. This cannot be attributed to a greater than normal availability of its catalytic cofactor Se, since we observed no difference in levels of this trace element between essential hypertensives and controls. Rather, it might be due to induction of production of GSHPx by the high concentration of lipoperoxides, the natural substrates for this enzyme. Such a mechanism had been demonstrated to occur *in vitro* for erythrocyte GSHPx several years ago [12]. Interestingly, the oxidant-induced increase in erythrocyte GSHPx activity paralleled the release of malondialdehyde into the supernatant [13].

An apparent impairment of vitamin E and vitamin A status was a further condition characterizing our essential hypertension patients. Vitamin E is the main lipid-soluble anti-oxidant in human plasma and lipoproteins and acts as a chain-breaking anti-oxidant able to scavenge lipid peroxy radicals, which would otherwise propagate the chain reaction of lipid peroxidation. Vitamin A is able to quench singlet oxygen effectively and is the most abundant lipid-soluble anti-oxidant in LDL after vitamin E. Authors of observational studies have often found that low levels of vitamins associated with high incidence of coronary heart disease [14,15], due possibly to their ability to prevent atherogenic process. Results of previous experimental studies showed that both vitamins E and A may also influence vascular function by limiting the oxidation of LDL, which in turn inactivate endothelium-derived relaxing factor, the most powerful local vasodilating agent [16]. Furthermore, vitamin E is known to prevent proliferation of smooth muscle vascular cells and to preserve the normal endothelium-dependent vessel relaxation via the inhibition of protein kinase C [17].

Zn and Cu are known to influence lipoperoxidation by modulating activity of SOD and directly by acting as pro-

oxidants or anti-oxidants, since both have been shown both to promote [1,3] and to inhibit [18,19] formation of malondialdehyde. We previously found a higher than normal concentration of Zn in essential hypertensives, suggesting that they have greater than normal activities of some Zn-dependent enzymes, such as angiotensin converting enzyme and fatty acid desaturases [5]. A low concentration of Cu is consistent with the lower than normal activity of SOD found in our essential hypertension patients. It is of interest to note that similar deviations from normality have been reported to occur in some animal models of hypertension, in which SOD has been suggested to play a hypotensive role [18]. As a matter of fact, intravenous injection of a fusion protein consisting of human SOD and a C-terminal peptide that is able to localize itself within the endothelium was recently shown to produce a fall of blood pressure in spontaneously hypertensive rats but not in controls [2]. In addition, noradrenaline-induced hypertension in rats was reversed by administration of SOD [2]. These observations have emphasized the role of SOD, suggesting that oxidative stress on the arterial wall is important in the pathogenesis of hypertension [20]. Inferred mechanisms could be the direct destruction of nitric oxide by oxygen free radicals or the inhibition of production of prostacyclin (prostaglandin I₂) by lipid peroxides [11].

In conclusion, statistical analyses revealed significant differences between essential hypertension patients and controls with respect to almost all the parameters analysed in our study and each of these complex changes was consistent with the view that essential hypertension is associated with an impairment of anti-oxidant status or a greater than normal response to oxidative stress, or both. We therefore concluded that there is no serious threat to the validity of this hypothesis. It remains to be determined whether these events are simply consequences of tissue damage or are strictly involved in the primary pathogenetic mechanism of disease. Further appropriate studies to clarify this issue and to evaluate the possible favourable effects of anti-oxidant therapeutic strategies are necessary.

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Oxidized LDL and Atherogenesis^a

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ABSTRACT: A brief review of recent findings regarding the role of oxidized low-density lipoproteins (Ox-LDL) in atherogenesis. Lipid peroxidation and oxidative damage to LDL make arteries susceptible to chronic inflammation, which is known to cause alterations in arterial gene expression and promote lesion development. Treatment protocols implementing antioxidants for preventing cardiovascular diseases are suggested.

INTRODUCTION

Atherosclerosis and cardiovascular diseases remain the major cause of mortality in the United States and Europe.¹ High plasma total cholesterol and low-density lipoprotein (LDL) values show significant positive relationship to the development of atherosclerosis and cardiovascular diseases.² Recent evidence suggests that oxidation (Ox) of LDL plays an important role in the pathogenesis of atherosclerosis.^{2,3} The purpose of this article is to review recent findings regarding the role of oxidized LDL (Ox-LDL) in atherogenesis.

LDL OXIDATION

Peroxidation can damage LDL particles. Several reactive radical species can initiate lipid peroxidation. These include reactions involving lipoxygenases, superoxide anion, hydroxyl radical, peroxynitrate, heme proteins, ceruloplasmin, and myeloperoxidase. It has been demonstrated that 15-lipoxygenase, superoxide anion, peroxynitrate, and myeloperoxidase can oxidize LDL.⁴ Peroxynitrate may be formed in the arterial wall from the reaction of superoxide anion with nitric oxide.⁵ It is also possible that oxidized membrane lipids are transferred to LDL.

In addition to lipids, LDL oxidation also involves the modification of apoprotein B (ApoB). In Ox-LDL, ApoB is fragmented and contains covalently bound malondialdehyde and 4-hydroxynonenal conjugates.² These reactions change LDL properties so that it is metabolized through macrophage scavenger receptors.⁶

LDL antioxidant levels do not fully explain individual differences in the susceptibility of LDL to oxidative stress.⁷ It is likely that other factors, such as fatty acid

TABLE 1. Properties of minimally oxidized LDL and fully oxidized LDL

	Minimally Oxidized LDL	Oxidized LDL
Cellular lipid accumulation	No	Yes
Reactive aldehyde conjugates in ApoB	No	Yes
Metabolism through scavenger receptor	No	Yes
Metabolism through LDL receptor	Yes	No
Stimulates proinflammatory cytokines	Yes	No
Inhibits endothelial-derived relaxing factor	No	Yes
Immunogenic	No	Yes
Cytotoxic	No	Yes
Chemotactic for plasma monocytes	No	Yes

composition and LDL particle size, may also affect LDL oxidation. It has been shown that small, dense LDL particles are more susceptible to oxidation than larger LDL subfractions.⁷ On the other hand, LDL particles enriched with monounsaturated fatty acids are less prone to oxidation than particles enriched with polyunsaturated fatty acids.⁷

ATHEROGENIC PROPERTIES OF OXIDIZED LDL

Ox-LDL can cause lipid accumulation in macrophages and foam cell formation.² Ox-LDL is also cytotoxic to many cell types and chemotactic for monocyte macrophages. In addition, Ox-LDL can inactivate endothelial cell-derived relaxing factor.²

It is becoming increasingly evident that the biological properties of minimally oxidized LDL (MM-LDL) are different from those of Ox-LDL.⁸ TABLE 1 summarizes some of the properties of MM-LDL and Ox-LDL. MM-LDL seems to have several specific effects on gene expression.⁸ It can stimulate monocyte chemotactic factor-1 and macrophage colony stimulating factor-1 expression and activate prothrombotic properties in the vascular wall.⁸ However, MM-LDL can not cause lipid accumulation in arterial cells since it is not metabolized through scavenger receptors.² Ox-LDL appears to be immunogenic, causing autoantibody formation in humans and in experimental animals. According to preliminary results, presence of autoantibodies may predict the progression of atherosclerosis in human populations.^{4,9}

EVIDENCE FOR THE PRESENCE OF OXIDIZED LDL IN ATHEROSCLEROTIC LESIONS

It has been clearly shown that Ox-LDL is present in atherosclerotic lesions: (1) LDL isolated from human atherosclerotic lesions, but not from normal arteries, resembles Ox-LDL³; (2) epitopes characteristic of Ox-LDL can be demonstrated in atherosclerotic lesions by immunocytochemistry¹⁰; (3) atherosclerotic lesions con-

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TABLE 2. Evidence for the presence of oxidized LDL in atherosclerotic lesions

	References
LDL isolated from atherosclerotic lesions resembles Ox-LDL	3
Epitopes characteristic of Ox-LDL are present in atherosclerotic lesions	10
Atherosclerotic lesions contain immunoglobulins that recognize Ox-LDL	11
Serum contains autoantibodies against Ox-LDL	9, 10
Antioxidant treatment reduces atherogenesis in experimental animals	12

tain immunoglobulins that recognize Ox-LDL¹¹; (4) serum contains autoantibodies to Ox-LDL⁹; and (5) antioxidant treatment reduces the rate of atherosclerotic lesion development in experimental animals (TABLE 2). Antioxidants shown to be effective in animal models include probucol,¹² α -tocopherol,¹³ butylated hydroxytoluene,¹⁴ and diphenylphenylenediamine.¹⁵ It remains to be determined whether small quantities of Ox-LDL are present in plasma.⁴

CONCLUSIONS

Research has provided strong evidence that LDL oxidation plays an important role in the pathogenesis of atherosclerosis and cardiovascular diseases. There seems to be no doubt that lipid peroxidation and oxidative damage to LDL resemble chronic inflammation, which causes various alterations in arterial gene expression and promotes lesion development. Based on current knowledge about the role of Ox-LDL in atherogenesis, randomized placebo-controlled intervention trials using antioxidants are warranted to test the hypothesis that increased antioxidant protection could be useful in the prevention of cardiovascular diseases.

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