Original Paper

Annals of Nutrition & Metabolism

Ann Nutr Metab 2008;53:86–90
DOI: 10.1159/000164691

Received: January 10, 2008
Accepted after revision: July 10, 2008
Published online: October 21, 2008

Plasma Antioxidants and Asymptomatic Carotid Atherosclerotic Disease

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Key Words
Atherosclerosis · Carotid arteries · Antioxidants · Lycopene · Vitamin A · Vitamin E · β-Carotene

Abstract
Background: Atherosclerosis remains clinically mute for a long time and frequently manifests itself with an acute cardiovascular event. The possibility of detecting this disease in a subclinical phase and reducing or reversing its progression is an issue of relevance. Published studies on the association between antioxidant vitamins and carotenoids and carotid intima-media thickness (CIMT) have been inconclusive. ***Methods:*** We enrolled 220 consecutive, asymptomatic participants. After carotid ultrasound investigation, a medical history was taken, a physical examination was performed and venous blood samples were collected. Venous blood samples were analyzed for concentrations of antioxidant vitamins and carotenoids. ***Results:*** Low concentrations of vitamin A ($p < 0.01$), vitamin E ($p < 0.001$), lycopene ($p < 0.01$) and β-carotene ($p < 0.001$) were significantly associated with carotid atherosclerosis (CIMT ≥ 0.8 mm). In addition, marginally higher body mass index, plasma haemoglobin and high-density lipoprotein cholesterol were also associated with carotid atherosclerosis, while other laboratory parameters considered in this study (total cholesterol, low-density lipoprotein cholesterol, triglycerides and C-reactive protein) were not significantly associated with carotid atherosclerosis. ***Conclusions:*** Low plasma concentrations of antioxidant vitamins (A, E, β-carotene) and lycopene were associated with early carotid atherosclerotic lesions as measured by CIMT. Regular intake of foods rich in lycopene and antioxidant vitamins may slow the progression of atherosclerosis.

Introduction

Cardiovascular diseases, specifically coronary heart disease and cerebrovascular diseases, are the most common causes of morbidity and mortality worldwide [1]. Moreover, cerebrovascular disease is the most important cause of long-term disability in Western societies. Oxidative stress, particularly from the oxidation of low-density lipoprotein cholesterol in the artery wall, induces an inflammatory reaction [2] which stimulates recruitment of monocytes and their differentiation into macrophages. Macrophages accumulate lipids intracellularly, forming foam cells, which results in increased thickness of the arterial wall [3–5]. Antioxidants, which may inhibit lipid peroxidation, could play an important protective role [6, 7] against the formation of simple and complex athero-

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sclerotic lesions, which progressively protrude into the arterial lumen, causing stenosis or occlusion [4, 8]. In particular, increased carotid intima-media thickness (CIMT) represents an early phase of the atherosclerotic process [9–11] and is widely used as a marker of subclinical atherosclerosis which correlates with established coronary heart disease [12, 13].

Few studies have examined the relationship between plasma antioxidant concentrations and CIMT, and those that have report conflicting results. The aim of this study was to assess the relationship between asymptomatic elevated CIMT and plasma levels of antioxidants (vitamin A, vitamin E, β-carotene and lycopene).

Patients and Methods

Subjects

We enrolled 220 consecutive, asymptomatic participants (109 males, 111 females) who presented to our institute (Cardiology Unit of 'San Camillo de Lellis' Hospital, Manfredonia, Italy) between July and December 2007. The participants ranged in age from 45 to 65 years and underwent carotid ultrasound investigation of the extracranial carotid arteries.

Inclusion and Exclusion Criteria and Definitions of Clinical Parameters

Participants were identified as asymptomatic if they had not experienced a transient ischaemic attack, amaurosis fugax or stroke. Participants were excluded if they had symptomatic carotid artery disease that necessitated revascularization therapy, current infectious or inflammatory disease, recent operations or endovascular interventions, bilateral carotid occlusion, monolateral/bilateral stent implantation or monolateral/bilateral endoarterectomy.

Participants were classified as current smokers if they answered 'yes' to the question 'have you smoked cigarettes, cigars, or a pipe within the past 30 days?'. Body weight was measured using a balance scale. During the height and weight measurements, the subjects wore light clothing and no shoes. Body mass index was computed as the ratio of weight in kilograms to the square of height in meters. Arterial hypertension was diagnosed in subjects with blood pressure values >140/90 mm Hg measured repeatedly (at least twice) and was assumed to be present in patients taking antihypertensive drugs [14]. Diabetes mellitus was defined according to the clinical practice recommendations 2002 criteria of the Expert Committee of the American Diabetes Association [15]. Hyperlipidaemia was defined as an elevation of low-density lipoprotein cholesterol values above 130 mg/dl and was assumed to be present in all patients taking lipid-lowering therapies [16].

The diagnosis of peripheral artery disease was performed according to the American College of Cardiology/American Heart Association practice guidelines [17]. Stroke was defined as a neurological deficit evaluated after 24 h by a neurologist or internist according to the American Heart Association/American Stroke Association Council guidelines [18]. History of myocardial infarction was assessed according to the consensus document of the Joint European Society of Cardiology/American College of Cardiology Committee for the redefinition of myocardial infarction [19].

The study was performed in accordance with the Helsinki Declaration of 1975, as revised in 1983, and approved by the ethical committee of San Camillo de Lellis Hospital. All patients had to provide written informed consent.

Carotid Ultrasound Investigation

Carotid ultrasound investigations were performed by means of a colour-coded Acuson Sequoia C512 (Siemens Medical Solutions USA Inc.) carotid duplex machine with a 7.5-MHz linear transducer. The investigation included longitudinal and transverse examinations of the carotid arteries. Diameters of both the left and right carotid arteries were measured and calculated at the site of maximal stenosis in the extra-cranial common carotid arteries according to the European Carotid Surgery Trial method [20]. The CIMT measurements were performed 10 mm proximal to the carotid bulb or 20 mm proximal to the flow divider. CIMT was measured between the leading edge of the first echogenic line (lumen-intima interface) and the second echogenic line (upper layer of the adventitia) in the far (deeper) artery wall. All measurements were performed on frozen, enlarged images (×2) at the end of a heart cycle (end diastole), with the transducer in the mediolateral direction [21]. Measurements were performed in both common carotid arteries, and the larger of the two values was used in data analysis. Offline analysis of the CIMT was performed using video images based on the Atherosclerosis Risk in Communities study protocol [22]. Carotid atherosclerosis was defined as a CIMT between 0.8 and 1.2 mm, while carotid plaque was defined as focal echo structures encroaching into the vessel lumen where the CIMT was >1.2 mm. These cut-offs were chosen because they were used in previous randomized clinical trials [23, 24].

Clinical and Laboratory Data

After carotid ultrasound investigation, medical history (hypertension, diabetes mellitus, family history of atherosclerosis, myocardial infarction, hyperlipidaemia, angina pectoris, peripheral artery disease, history of prior cerebral accident) and data from a physical examination [age, gender, body mass index, blood pressure, cardiac rate, smoking habits (smoker, ex-smoker or non-smoker)] were collected.

Venous blood samples were obtained at the baseline visit. Blood analyses included haemoglobin, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides and C-reactive protein (measured at the Analysis Laboratory of San Camillo de Lellis Hospital) and plasma concentrations of vitamin A, vitamin E, lycopene and β-carotene (measured at the Clinical Biochemistry Laboratory, Chieti, Italy). All investigators and laboratory personnel were blinded to the subjects' status. Antecubital venous blood samples from all subjects were handled identically and blindly through all stages of the blood collection, storage, retrieval and analytic processes.

Blood Sample Collection, Storage and Preparation

Blood samples were collected in polypropylene tubes containing EDTA 1 mM. Samples were stored in an ice box prior to centrifugation at 3,000 *g* for 10 min at 4°C. Then, 200-μl aliquots of

Table 1. Characteristics of the study population

	CIMT <0.8 mm (n = 95)	CIMT ≥0.8 mm (n = 125)	p value
Demographic data			
Age, years	45.2 ± 18.3	47.5 ± 17.2	0.34
Males/females	52/43	57/68	0.48
Body mass index	25.15 ± 3.22	26.12 ± 2.87	0.02
Blood pressure, mm Hg			
Systolic	124 ± 7	130 ± 4	<0.0001
Diastolic	84 ± 6	83 ± 5	0.18
Resting pulse, beats/min	64 ± 15	66 ± 13	0.29
Smoking status			
Current smokers	45 (47.36)	64 (51.2)	0.69
Ex-smokers	12 (12.63)	16 (12.8)	0.99
Non-smokers	38 (40)	45 (36)	0.64
Laboratory data			
Haemoglobin, g/dl	12.65 ± 0.81	12.88 ± 0.63	0.02
Total cholesterol, mg/dl	184.46 ± 14.90	191.00 ± 35.25	0.09
HDL-C, mg/dl	48.18 ± 4.64	49.62 ± 4.56	0.02
LDL-C, mg/dl	121.72 ± 10.39	124.25 ± 34.20	0.49
Triglycerides, mg/dl	127.20 ± 24.22	130.62 ± 35.27	0.42
CRP, mg/dl	4.29 ± 2.15	3.86 ± 2.23	0.15
Antioxidants			
Vitamin A, μmol/l	6.00 ± 0.73	2.71 ± 0.96	<0.0001
Vitamin E, μmol/l	86.69 ± 12.15	43.48 ± 8.49	<0.0001
Lycopene, μmol/l	0.92 ± 0.43	0.34 ± 0.19	<0.0001
β-Carotene, μmol/l	3.47 ± 0.72	0.809 ± 0.39	<0.0001

Values in parentheses represent percentages. HDL-C = High-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; CRP = C-reactive protein.

plasma were transferred into foil-wrapped polypropylene tubes. Plasma samples were either used for immediate extraction or stored in the dark at –80°C until analysis was performed. Sample preparation was performed as described by Lee et al. [25].

HPLC System and Conditions for Antioxidant Measurements

Vitamin A, vitamin E, lycopene and β-carotene concentrations were determined by HPLC. Two Waters 515 HPLC pumps (Whatman, Clifton, N.J., USA) equipped with a Waters auto-injector (model 717 plus auto-sampler) and a Waters 996 photodiode array detector were used as the HPLC system. Data acquisition and processing were performed using the chromatography software Empower-Pro (Waters). Analysis was performed by isocratic elution. The flow rate was 1.5 ml/min. The mobile phases used were as follows: (A) methanol/*n*-butanol/water (89.5/5/5.5 v/v/v) premixed and vacuum filtered through a 0.45-μm polypropylene membrane filter (Whatman) before use; (B) methanol/*n*-butanol/water (76/19.5/4.5 v/v/v) premixed and vacuum filtered before use as for A. The elution isocratic profile was 0–7 min 100% A, 7–15 min 100% B and 15–20 min 100% A for column re-equilibration. Auto-injections of 20 μl were performed at 5°C. The analytical column used was a replaceable Partisphere 5 C-18 cartridge with an inner diameter of 110 × 4.7 mm and particle size of 5 μm (Whatman, Waters Corporation, Milford, Mass., USA) protected by a guard cartridge (C-18, 5 μm) system and maintained at 45°C. The photodiode array wavelength ranged from 270 to 460 nm, and the chromatograms were extracted at 340 nm for vitamin A, 288 nm for vitamin E and vitamin E-acetate (used as internal standard) and 441 nm for lycopene and β-carotene. The run time was 20 min.

Statistical Analysis

Study participants were grouped into two categories according to CIMT, i.e. <0.8 and ≥0.8 mm. For each baseline characteristic, the mean value or corresponding percentage of study participants was calculated according to the CIMT category. The statistical significance of differences was examined using Student's t test (continuous variables) and the χ^2 test (categorical variables).

A two-sided p value <0.05 was considered statistically significant. Data were analyzed using SPSS statistical software (version 15.0 for Windows, SPSS Inc., Chicago, Ill., USA).

Results

Means (± SD) or percentages of patients for various demographic characteristics and established cardiovascular disease risk factors are summarized in table 1 according to CIMT category. Of the 220 participants who took part in the study, 125 subjects (56.8%) had carotid atherosclerosis, of whom 52 (41.6%) had disease localized to the common carotid arteries, 43 (34.4%) had disease localized to the internal carotid arteries and 30 (24%) had disease localized to the external carotid arteries.

A significant association was identified between low concentrations of vitamin A ($p < 0.01$), vitamin E ($p < 0.001$), lycopene ($p < 0.01$) and β-carotene ($p < 0.001$) and evidence of carotid atherosclerosis as measured by CIMT. Marginally higher body mass index, plasma haemoglobin and high-density lipoprotein cholesterol were also associated with carotid atherosclerosis, while other laboratory parameters considered in the study (total cholesterol, low-density lipoprotein cholesterol, triglycerides and C-reactive protein) were not significantly associated with carotid atherosclerosis.

Discussion

The primary finding of our study is that low plasma levels of antioxidant vitamins and oxygenated carotenoids (vitamin A, vitamin E, β-carotene and lycopene)

are associated with carotid atherosclerosis. Other epidemiological studies have evaluated the relationship between antioxidant vitamins and evidence of subclinical atherosclerosis; however, the results have been inconclusive.

For example, in a cross-sectional study of 392 men and women (age 45–65 years), D'Odorico et al. [26] found no association between vitamin A plasma concentrations and the presence of carotid/femoral atherosclerosis. Similarly, Iannuzzi et al. [27] examined 310 women for early carotid atherosclerosis and found no association between intake or plasma concentrations of vitamin A and the presence of carotid plaques. In prospective cohort studies, Dwyer et al. [28] and McQuillan et al. [29] found no association between CIMT atherosclerosis or progression and vitamin A plasma concentrations. In a nested case-control study, Iribarren et al. [30] also failed to find an association between plasma vitamin A (retinol) concentration and CIMT. In contrast, a case-control study conducted by Polidori et al. [31] found a significant association between lower plasma vitamin A (retinol) concentrations and carotid and iliofemoral atherosclerosis.

The results of published studies regarding vitamin E and carotid atherosclerosis are also inconclusive. Iannuzzi et al. [27], Polidori et al. [31] and McQuillan et al. [29] found an inverse association between CIMT and plasma vitamin E concentration, whereas D'Odorico et al. [26], Dwyer et al. [28] and Giannetti et al. [32] found no association between vitamin E plasma concentration and the presence of carotid/femoral atherosclerosis. Even for the well-studied relationship between β-carotene and carotid atherosclerosis, the results of published studies are unclear and conflicting. Many studies have found that the consumption of β-carotene is inversely related to the risk of coronary heart disease [33, 34] and that the risk of carotid and femoral atherosclerosis decreased with increasing plasma β-carotene concentrations [26], suggesting a protective role for β-carotene in early atherogenesis. However, several well-conducted studies have found no association between β-carotene plasma concentrations and CIMT or peripheral vascular disease [29, 30, 32]. In contrast, the results of published studies of the association between lycopene and carotid atherosclerosis are generally concordant and show a significant inverse relationship between serum concentrations of lycopene and CIMT, supporting the hypothesis that plasma lycopene may decrease the risk of atherosclerosis and play an important role in the early stage of atherogenesis [29, 32, 35].

One important limitation of this study is the cross-sectional nature of our findings. Because antioxidant values, lipid profiles, anthropometrics and questionnaires were collected concurrently with the ultrasound assessment of carotid arteries, temporality cannot be inferred from these findings. Further studies which examine this important relationship are warranted.

Conclusions

In summary, we found a strong inverse relationship between antioxidant plasma levels and the presence of carotid atherosclerosis. Atherosclerosis remains clinically mute for a long time and frequently manifests itself with an acute cardiovascular event; therefore, the possibility of detecting the disease in a subclinical phase and reducing or reversing its progression is an issue of relevance. The evaluation of CIMT in asymptomatic patients with CUS allows us to identify atherosclerotic disease in its early phases, to evaluate disease progression and to monitor the effects of interventions. In particular, regular intake of rich foods rich in lycopene and other antioxidant vitamins may slow the progression of atherosclerotic processes and modify the early stages of atherosclerosis, with a consequent reduction in cardiovascular events.

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