ORIGINAL RESEARCH

Aterofisiol® in carotid plaque evolution

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Background: In patients with carotid stenosis, the risk of plaque rupture is related to the composition of the atherosclerotic plaque rather than to its magnitude. In this regard, we evaluated the effects of a supplement, Aterofisiol,® containing omega-3 (EPA [eicosapen acid] DHA [docosahexaenoic acid]), vitamin K_2, vitamin B_6, vitamin B_{12}, oligomeric proanthocyanidins (OPC) and resveratrol on the composition of atherosclerotic plaque and on neurological symptoms in patients with carotid stenosis undergoing carotid endarterectomy.

Methods: The study was randomized, prospective, and double-blinded. Eligible patients were of both sexes, with carotid stenosis >70% who underwent endarterectomy. Enrolled patients were randomly allocated to receive either one tablet of acetylsalicylic acid 100 mg (Cardioaspirin®) + one tablet of Aterofisiol every 24 hours or one tablet of Cardioaspirin + one tablet of placebo every 24 hours. Each treatment was started 30 days before the surgery and was stopped 5 days before the surgery. The plaques were removed "en bloc" using standard surgical technique.

Results: During the study period, 214 patients (135 men and 79 women) were enrolled for intent-to-treat and randomized in two groups: Group A: 107 patients (68 men and 39 women) were treated with Cardioaspirin + Aterofisiol. Group B: 107 patients (67 men and 40 women) were treated with Cardioaspirin + placebo. At the end of the study, 202 patients participated fully (103 patients in Group A and 99 patients in Group B), making up the protocol evaluation population (94.4%). The mean lipid content of removed plaques was significantly lower ($P<0.05$) in Group A. We recorded a significantly lower incidence of neurological symptoms in Group A in comparison with Group B ($P<0.05$).

Conclusion: In the study, Aterofisiol showed to be effective in reducing the amounts of cholesterol and lipids in the plaques and in reducing adverse neurological events in the study group with respect to controls.

Keywords: Aterofisiol®, carotid stenosis, endarterectomy, carotid plaque, neurological symptoms, cholesterol

Introduction

The World Health Organization ranks cardiovascular diseases (CVD) as the leading cause of death worldwide, and showed that CVD constitute 48.6% of the ten main causes of death.[1] It has also been estimated that of the 83.6 million American adults with CVD, about 18% have atherosclerosis[2] and the presence of atherosclerotic plaque into the carotid artery is a risk factor for transient ischemic attack (TIA) and stroke.[3]

Usually, atherosclerotic plaque contains a coating of soft material formed by macrophages and lipids and a core of harder fibrous tissue; the size of the nucleus atheroma, the thinness of the cap, and the density of macrophages are the main determinants of the risk of plaque rupture.[4,5] This risk is related to the composition of the atherosclerotic plaque rather than to its magnitude; plaques with high concentration of lipids are more susceptible to rupture with subsequent formation of blood clots.[6,7] The plaque rupture often occurs on the surface during growth and is related to changes within the plaque as well as to an increase in the circumferential pressure outside the plaque.

To date, several therapeutic strategies are available to reduce both mortality and morbidity in patients with TIA or stroke. In particular, in asymptomatic patients with an atherosclerotic plaque <70% or in symptomatic patients with a plaque <50%, both statins and aspirin are prescribed. Statins are usually used to stabilize plaque, reducing its vulnerability and reducing the inflammation; aspirin is used to inhibit platelet aggregation, reducing the progression of the stenosis.[8,9]

Several authors[10–14] have reported that statins are able to improve clinical symptoms, in several vascular (such as aneurysms and stroke) and nonvascular diseases (such as diabetes, obesity, periodontitis) through the control of inflammation, particularly reducing the matrix metalloproteinase expression,[15] and recently, some drugs as well as nutraceuticals have been developed to control vascular symptoms through the control of the same mechanism involved in the inflammation.[16–21]

Because of the high incidence of atherosclerotic disease, the search for substances that can counter it is always on-going. Particular attention is being given to substances that reduce the lipid content of plaques and to agents capable of counteracting the local inflammation that seems to be a determining factor for this condition. In fact, some studies have suggested that a chronic systemic inflammation, even of low grade, may be involved in atherosclerosis and its main complications.[22–24]

In this regard, we evaluated the effects of a food supplement, Aterofisiol® containing omega-3 (EPA [eicosapentaenoic acid] DHA [docosahexaenoic acid]), vitamin K_2, vitamin B_6, vitamin B_{12}, oligomeric proanthocyanidins (OPC), and resveratrol, on the composition of atherosclerotic plaque and on neurological symptoms and cerebrovascular events in patients undergoing carotid endarterectomy.

Materials and methods
Study design
The randomized, prospective, double-blind, and parallel groups study was performed in the Department of Clinical Medicine and Surgery, "Federico II" University of Naples, between April 2013 and November 2014.

This study was approved by the Institutional Review Board – Independent Ethics Committee (IRB-IEC) of the Interuniversity Center of Phlebolymphology-International Research and Educational Program in Clinical and Experimental Biotechnology, and before the beginning of the study, all participants were informed about the aim, procedures,

risks, and benefits of the study. Before the beginning of the study, all participants signed written informed consent.

Patients
Eligible patients were women and men older than 20 years with carotid stenosis >70% who had undergone endarterectomy. Patients with embolic cerebrovascular stroke, recent TIA history of drug allergies, psychiatric diseases, creatinine clearance <30 mL/min, or platelet count <100,000/mm³ were excluded. Simultaneous use of dextran, thrombolytic agents, anticoagulants, or heparin or heparinoids for topical use was not allowed during the study and was considered a reason for exclusion.

Experimental protocol
Clinical examination, laboratory findings (blood and urine evaluation), and Doppler ultrasonography were performed in all enrolled patients at the time of admission and during the follow-up. Enrolled patients were randomly allocated to receive either one tablet of acetylsalicylic acid 100 mg (Cardioaspirin®) + one tablet of Aterofisiol every 24 hours or one tablet of Cardioaspirin + one tablet of placebo every 24 hours.

For randomization, a computer program was used to generate a sequence of treatment allocations by block randomization using a random number generator. Investigators were blinded to the block size to avoid selection bias.

Aterofisiol and placebo were made available in identical packages. At the time of randomization, patients received a diary into which to write the tablets taken.

Each treatment was started 30 days before the surgery and was stopped 5 days before the surgery.

Plaques evaluation
204 plaques were removed "en bloc" using standard surgical technique. Particular attention was paid to minimize handling of the plaque during endarterectomy. All the plaques were lyophilized, placed into a saline solution, and stored at −70°C for biochemical analysis.

Lipids were extracted using a chloroform–methanol solution, and dry weight of lipids was determined after evaporation of the solution; lipids are expressed as milligrams of extracted lipid per milligram of plaque dry weight. Cholesterol from an aliquot of the lipid extract was quantified by the manual enzymatic method, and results are expressed as milligrams of cholesterol per milligram dry weight of the plaque. Subsequently, specimens were homogenized in acetic acid and then hydrolyzed using

HCl, HCl was removed by vacuum, samples were suspended, and collagen was determined using hydroxyproline colorimetric assay. Plaque calcium was measured by atomic absorption spectroscopy. Plaque lipid extract, after homogenization and hydrolysis, was diluted in lanthanum oxide; calcium absorbance was determined at 422 nm using a calcium/magnesium lamp. Results were expressed as mg Ca/mg dry weight of plaque.

Plaque morphology was assessed preoperatively with the Gray-Wheal classification method.[25]

Internal carotid artery (ICA) and common carotid artery (CCA) were also assessed by evaluating sonographic indexes such as peak systolic velocity (PSV), end diastolic velocity (EDV), and ICA/CCA PSV ratio.

Efficacy outcome

The efficacy outcome (Figure 1) was identified as a statistically significant difference ($P<0.05$) in lipid content of plaques between the two groups of treatment.

Drug adherence

At the end of the study, in both groups of patients, the adherence to the treatment was calculated using a formula based on the number of tablets consigned at the time of admission and on the number of tablets returned unused at the end of the study.

Security outcomes

During the follow-up performed 1, 7, and 30 days after the surgery, we evaluated the security outcomes, defined as minor (amaurosis fugax, reversible ischemic neurological deficit [RIND], TIA) or as major (stroke) neurological adverse drug reactions (ADRs) occurring in either the immediate (1st day) or the late (from 7 to 30 days) postoperative period. A secondary security end-point was the development of any
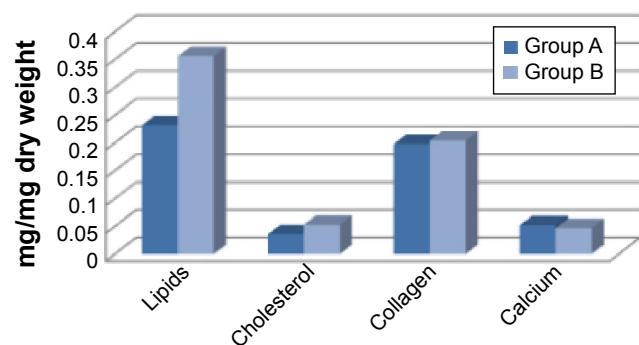

Figure 1 Efficacy outcome in lipid content of plaques between the two groups of treatment.
Notes: Group A patients were treated with Cardioaspirin® and Aterofisiol®. Group B patients were treated with Cardioaspirin® and a placebo.

ADRs during the study; the development of ADRs was evaluated using both the Naranjo[26] scale and the Drug Interaction Probability Scale.[27]

Statistical analysis

All data are expressed as mean ± standard error medium. Student's t-test was performed in order to analyze the difference between each group with their control. Analysis of variance (ANOVA) was used to evaluate the differences between the groups. Differences identified by ANOVA were pinpointed by unpaired Student's t-test. The threshold of statistical significance was set at $P<0.05$. SPSS (SPSS Inc., Chicago, IL, USA) software was used for the statistical analyses. We defined this study as exploratory, and therefore we did not determine a power calculation. In light of this, these results could only be labeled as exploratory.

Results

Patients

During the study period, 214 patients (135 men and 79 women) were enrolled for intent-to-treat and randomized in two groups:

Group A: 107 patients (68 men and 39 women) were treated with Cardioaspirin + Aterofisiol

Group B: 107 patients (67 men and 40 women) were treated with Cardioaspirin + placebo.

At the end of the study, 202 patients participated fully (103 patients in Group A and 99 patients in Group B), making up the per protocol evaluation population (94.4%).

12 patients (5.6%) did not complete the study: 6 patients (2.8%; 2 patients in Group A – 0.9% – and 4 in Group B – 1.9%) were considered noncompleters due to deviation from protocol, and 6 patients (2.8%; 2 patients in Group A – 0.9% – and 4 in Group B – 1.9%) were lost to follow-up.

Mean age was about 58 years, a previous TIA was detected in about 12% of patients, and CVD in about 4.8% (Table 1).

We did not detect any difference in blood sugar, blood lipids, and blood pressure between the two groups at the time of inclusion (data not shown).

Ultrasound assessment of plaque morphology and velocimetry

According to the Gray-Wheal classification,[25] 10 patients of Group A (9.3%) and 6 patients of Group B (6.1%) ($P=0.3372$) had Type 1 plaques (predominantly echolucent with a thin echogenic cap); 11 patients of Group A (10.7%) and 12 patients

Table 1 Population characteristics of enrolled patients

Characteristics	Group A (Cardioaspirin® + Aterofisiol®) N=103	Group B (Cardioaspirin® + placebo) N=99	P-value
Age, years (mean±SD)	58.1±12.2	57.1±12.1	0.68
Sex, male	65 (63%)	62 (62.6%)	0.94
BMI, kg/m²	29.2±5.2	29.0±5.4	0.72
Pathologies			
Previous TIA	12 (11.6%)	12 (12.1%)	0.91
Previous stroke	7 (6.8%)	7 (7.07%)	0.93
Cardiovascular diseases	5 (4.8%)	5 (5.0%)	0.94
Diabetes mellitus	5 (4.8%)	7 (7.07%)	0.50
Thrombophilia	5 (4.8%)	6 (6.0%)	0.70
Medical therapies at the time of inclusion			
Antihypertensive drugs	77 (74.7%)	75 (75.7%)	0.86
Analgesic drugs	27 (26.2%)	26 (26.3%)	0.99
Antidiabetics	41 (39.8%)	40 (40.4%)	0.93
Anticoagulants	4 (3.9%)	3 (3.0%)	0.74
Statins	24 (23.3%)	23 (23.2%)	0.99
NSAIDs	3 (2.9%)	2 (2.0%)	0.68

Abbreviations: TIA, transient ischemic attack; NSAIDs, nonsteroidal anti-inflammatory drugs; SD, standard deviation.

of Group B (12.1%) (P=0.7471) had Type 2 plaques (intermediate echolucent lesions with small areas of echogenicity); 38 patients of Group A (36.9%) and 41 patients (41.4%) of Group B (P=0.5104) had Type 3 plaques (intermediate echogenic lesions with small areas of echolucency); 44 patients (42.7%) of Group A and 40 patients (40.4%) of Group B (P=0.7387) had Type 4 (uniformly echogenic lesions).

We did not detect any statistically significant difference in blood-flow rates (ICA – PSV, ICA – EDV, ICA/CCA PSV ratios) between the two groups (data not shown).

Efficacy outcomes

The mean lipid content of removed plaques was significantly lower (P<0.05) in Group A. In particular, the mean of lipids was 0.232±0.018 mg/mg dry weight in Group A and 0.356±0.022 mg/mg dry weight in Group B (P<0.05); the mean of cholesterol was 0.036±0.006 mg/mg dry weight in group A and 0.053±0.007 mg/mg dry weight in Group B (P<0.05) (Table 2). We did not record any statistically significant change in collagen or calcium content of plaques between the groups.

Security outcomes

We recorded a significantly lower incidence of neurological symptoms in Group A (n=8) compared with Group B (n=20) (P<0.05). In particular, during the study, we recorded the development of amaurosis fugax (4 patients in Group A and 9 in B), TIA (2 patients in Group A and 5 in B), RIND (1 patient in Group A and 4 in B), and stroke (1 patient in Group A and 2 in B). No cases of fatal stroke were recorded (Table 3). No other ADRs appeared during this study.

Discussion

Recently, it has been reported that inflammation plays a role in the destabilization of the plaque preceding the rupture, and therefore, statins as well as substances with anti-inflammatory actions could be used to improve the inflammation.[15–21] In fact, the generation of atherosclerotic plaques starts with a concentration-dependent transport of low-density lipoproteins (LDL) into the arterial wall, followed by modification of these lipids by oxidation and/or inflammation, invasion of macrophages and their transformation to foam cells, infiltration of smooth muscle cells, and production

Table 2 Efficacy outcome evaluation in Aterofisiol® group versus placebo group

Plaque components	Group A (Cardioaspirin® + Aterofisiol®) N=103	Group B (Cardioaspirin® + placebo) N=99	P-value
Lipids	0.232±0.018	0.356±0.022	0.032
Cholesterol	0.036±0.006	0.053±0.007	0.041
Collagen	0.198±0.012	0.204±0.010	0.68
Calcium	0.052±0.006	0.047±0.006	0.98

Notes: Unit of measure: mg/mg dry weight. All values are expressed as mean ± standard error medium.

Table 3 Security outcome evaluation in Aterofisiol® group versus placebo group

Cerebrovascular events	Group A (Cardioaspirin® + Aterofisiol®) N=103	Group B (Cardioaspirin® + placebo) N=99	P-value
Amaurosis fugax	4 (3.9%)	9 (9%)	0.038
TIA	2 (1.9%)	5 (5%)	0.025
RIND	1 (0.9%)	4 (4%)	0.048
Stroke*	1 (0.9%)	2 (2%)	0.125

Note: *Indicates non-fatal stroke.
Abbreviations: RIND, reversible ischemic neurological disease; TIA, transient ischemic attack.

of fibrin and extracellular matrix.[28] The anti-inflammatory role of resveratrol and omega-3 fatty acids, in combination with the effects of vitamin K_2, B_6, and B_{12}, seems to have a stabilizing effect on the atheromatous plaque opposing that mechanism. Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a nonflavonoid polyphenolic compound belonging to the stilbene group able. It has been reported in cultured endothelial cells that resveratrol shows anti-inflammatory effects through the inhibition of cyclo-oxygenase-2 (COX-2) and matrix metalloproteinase-9 (MMP-9).[29,30] Moreover, resveratrol has been shown to protect endothelial cells by inhibiting NADPH-oxidase and attenuating reactive oxygen species production, thereby showing antioxidant effects.[31,32] In contrast, several clinical studies have failed to report a clinical effect of resveratrol on plasma lipids,[33–37] but another in patients treated with statins and at high risk for CVDs documented that daily ingestion of 350 mg resveratrol-enriched grape extract containing 8 mg resveratrol for 6 months caused a 20% reduction of oxidized LDL cholesterol, but only a modest 4.5% reduction in LDL cholesterol,[38] suggesting that the antioxidant effect of resveratrol may be more important than its LDL cholesterol-lowering effect. Therefore, it may be postulated that resveratrol could increase the antiatherosclerotic effect of traditional lipid-lowering medications that work mainly by lowering the cholesterol and/or triglycerides levels.

Polyunsaturated fatty acids omega-3 long-chain (n–3 LCPUFA), such as EPA and DHA, play an important role in the regulation of blood pressure, kidney function, blood clotting, and inflammatory and immunological reactions. In fact, the European Food Safety Authority (EFSA) established that to obtain the effects described for the reduction of blood pressure and triglyceride levels, there must be an intake of EPA and DHA of between 2 and 4 g/d, while for the maintenance of a normal cardiac function an intake of 250 mg is sufficient. Furthermore, as regards the dietary reference values, the group of experts concluded that the intake of 250 mg/d is adequate for the maintenance of overall cardiovascular health in adults and healthy children. Moreover,

an additional daily intake of 5 g of omega-3 long-chain does not raise safety concerns for the general population.[39,40]

Hyperhomocysteinemia increases the risk of atherosclerosis as well as venous disease.[41] It may result from folate deficiency or a genetic metabolic defect. The pathophysiologic mechanism is unknown, but may involve direct endothelial injury, stimulation of monocyte and T-cell recruitment, LDL uptake by macrophages, and smooth muscle cell proliferation. Epidemiological studies suggest that mild to moderate elevations in plasma total homocysteine (tHcy) concentrations are associated with an increased risk of atherosclerotic vascular diseases.[42–45] Elevated tHcy was independently associated with plaque morphology and increased plaque area, subclinical markers of stroke risk.[46] Lowering tHcy ought to slow the progression of atherosclerosis and prevent atherothrombotic events. Folate (folic acid), vitamins B_6, and B_{12} have been used to treat hyperhomocysteinemia.[47]

Menaquinone-7 (MK-7) is part of a family of vitamin K that are essential cofactors for the enzyme γ-glutamyl carboxylase, which is involved in the activation of γ-carboxy glutamate (Gla) proteins in the body. Gla proteins are important for normal blood coagulation and normality of bones and arteries. Menaquinones or vitamin K_2 (VK2) are formed usually by bacteria. Humans may obtain VK2 from fermented dietary sources, such as curd cheese and natto, a Japanese food.[48] The potential beneficial effects of menaquinones is being uncovered, and some studies suggest that VK2 reduces vascular calcification and the advancement of atherosclerosis in hypercholesteremic rabbits.[49] VK2 improves lipid profile by increasing high-density lipoprotein levels and decreasing total cholesterol levels and by playing an important role in the process of vascular calcification and plaque formation in atheroma, as shown in the Rotterdam study.[50] Vascular tissue and calcified plaques contain MGP, a vitamin K–dependent protein known to prevent excessive calcium deposition in bone.[51] Lack of vascular MGP resulted in excessive aortic and coronary calcification in knockout mice.[52] Calcified plaques are more prone to rupture, which will elicit a thrombotic response, thereby increasing the risk

of a coronary event. Another vitamin K–dependent protein found in the vessel wall is protein S. Together with activated protein C, this anticoagulant plays an important role in preventing clot formation at the inner surface of the vessel wall.[53,54] In recognition of the effect of VK2 on reducing the risk of coronary heart disease, the International Life Sciences Institute (ILSI Europe) recently recommended taking VK2, in addition toVK1.[50]

The combination of these substances may have a beneficial effect on atherosclerosis by reducing the lipid content of the plaque, the reduction of homocysteinemia, the anti-inflammatory effect, and favoring the stability of the wall.

As atherosclerosis is a multifactorial disease with endothelial dysfunction and the alteration of several molecular systems, it justifies the identification and modulation of various therapeutic targets as several studies have done in past and recent years.[19–21,55–58]

In this context, our study evaluated the effects of a food supplement, Aterofisiol based on omega-3 (EPA and DHA), vitamin K_2, vitamin B_6, vitamin B_{12}, OPC, and resveratrol, on atherosclerotic plaque composition and on neurological assessment in patients undergoing carotid endarterectomy.

The study found that carotid plaques removed from patients in Group B (treated with Cardioaspirin and placebo) contain higher amounts of cholesterol and lipids than the plaques removed from patients in group A (treated with Cardioaspirin and Aterofisiol), and the difference was statistically significant. Moreover, patients in group A developed a lower incidence of neurological accidents. The patients in our study are a representative sample of patients seen in clinical practice, with a clear predominance of men, with diabetics and smokers.

The study population was quite homogeneous with respect to patient demographics, clinical characteristics, and plaque composition, and this allows us to state that the evidence of better outcomes in patients treated with Aterofisiol were not biased by any factors related to patients or plaques variables.

Conclusion

In this study, we documented the efficacy of a new substance on atherosclerotic plaque and its constitution and on neurological assessment in patients with atherosclerosis and carotid stenosis.

Acknowledgment

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