Aged garlic extract supplemented with B vitamins, folic acid and L-arginine retards the progression of subclinical atherosclerosis: A randomized clinical trial

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ABSTRACT

Objectives. Previous studies demonstrated that aged garlic extract reduces multiple cardiovascular risk factors. This study was designed to assess whether aged garlic extract therapy with supplements (AGE + S) favorably affects inflammatory and oxidation biomarkers, vascular function and progression of atherosclerosis as compared to placebo.

Methods. In this placebo-controlled, double-blind, randomized trial (conducted 2005–2007), 65 intermediate risk patients (age 60 ± 9 years, 79% male) were treated with a placebo capsule or a capsule containing aged garlic extract (250 mg) plus Vitamin B12 (100 μg), folic acid (300 μg), Vitamin B6 (12.5 mg) and L-arginine (100 mg) given daily for a 1 year. All patients underwent coronary artery calcium scanning (CAC), temperature rebound (TR) as an index of vascular reactivity using Digital Thermal Monitoring (DTM), and measurement of lipid profile, autoantibodies to malondialdehyde (MDA)-LDL, apoB-immune complexes, oxidized phospholipids (OxPL) on apolipoprotein B-100 (OxPL/apoB), lipoprotein (a) [Lp (a)], C-reactive protein (CRP), homocysteine were measured at baseline and 12 months. CAC progression was defined as an increase in CAC>15% per year and an increase in TR above baseline was considered a favorable response.

Results. At 1 year, CAC progression was significantly lower and TR significantly higher in the AGE + S compared to the placebo group after adjustment of cardiovascular risk factors ($p<0.05$). Total cholesterol, LDL-C, homocysteine, IgG and IgM autoantibodies to MDA-LDL and apoB-immune complexes were decreased, whereas HDL, OxPL/apoB, and Lp (a) were significantly increased in AGE + S to placebo.

Conclusion. AGE + S is associated with a favorable improvement in oxidative biomarkers, vascular function, and reduced progression of atherosclerosis.

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Background

Several clinical reports, including meta-analyses, have revealed cholesterol-lowering effects of garlic supplementation in humans (Neil et al., 1996; Warshafsky et al., 1993). Such reports have additionally impacted public awareness on the potential cardiovascular benefits of garlic. Garlic and garlic extracts have been postulated to impart cardiovascular benefits through multiple mechanisms. For example, recent studies of aged garlic extract (AGE) have shown it as a modulator of multiple cardiovascular risk factors, in addition to LDL lowering (Dillon et al., 2003), including lowering blood pressure, reducing platelet aggregation and adhesion, preventing LDL oxidation, smoking-caused oxidative damage, and also directly suppressing atherosclerosis (Steiner et al., 1996; Campbell et al., 2001; Okuhira et al., 2000; Rahman and Billington, 2000; Gonen et al., 2005; Lau, 2001). In addition, aged garlic extract has been demonstrated to improve vascular function (Okuhara, 1994), inhibit endothelial cell damage and transform smooth muscle cells (Ho et al., 2001), suggesting that AGE may have an effect of controlling arterial function through inhibiting the damage of nitric oxide synthesis (Morihara et al., 2002).

A variety of oxidative biomarkers have been developed and validated to reflect the presence of oxidation-specific epitopes on lipoproteins (Tsimikas et al., 2006). These biomarkers can be direct, measuring specific epitopes such as oxidized phospholipids (OxPL) or malondialdehyde (MDA) epitopes on apolipoprotein B-100 or lipoprotein (a), on indirect, measuring autoantibodies and immune complexes to oxidized LDL (Choi et al., 2008; Holvoet et al., 2001; Itabe and Ueda, 2007). These measures have been shown to reflect various manifestations of anatomical cardiovascular disease and to predict new cardiovascular events (Fraley and Tsimikas, 2006). More recently, active investigation is being pursued in assessing the role of such biomarkers following therapeutic interventions (Tsimikas, 2007a). In particular, the OxPL/apoB measure, which detects OxPL on individual apoB particles, but not total OxPL in plasma, has been

shown to increase in patients following therapeutic interventions including statin therapy (Choi et al., 2008; Ky et al., 2008; Rodenburg et al., 2006; Tsimikas et al., 2004a,b), as well as in patients (Choi et al., 2008; Rodenburg et al., 2006; Silaste et al., 2004) and animals following low fat diets (Tsimikas et al., 2007a,b). Thus, it may serve as a new biomarker of early benefit of therapeutic interventions.

The effect of garlic on coronary atherosclerosis progression in humans has not been widely studied (Budoff et al., 2006). In addition, the effect of garlic on oxidative biomarkers and their relationship to coronary calcium and vascular function is not known. This randomized, placebo-controlled study was designed to assess the effect of aged garlic extract plus homocysteine-lowering vitamins and ʟ-arginine on coronary calcium, vascular function and lipid and oxidative biomarkers.

Methods

The study is a placebo-controlled, double-blind, randomized trial (conducted 2005–2007) to determine whether commercially available aged garlic extract plus B vitamins, ʟ-arginine and folate (AGE + S) (Kyolic 108, Wakunaga Nutritional Supplement, CA, USA) can influence the rate of atherosclerotic plaque burden measured by coronary artery calcium, improve vascular function and favorably change biomarkers of oxidative stress.

The CONSORT (Consolidated Standards of Reporting Trials) statement is outlined in Fig. 1 (Moher et al., 2001). Aged Garlic Extract (AGE, Kyolic®), provided by Wakunaga of America (Mission Viejo, CA), was formulated by soaking sliced raw garlic in aqueous ethanol for up to 20 months at room temperature. The extract was then filtered and concentrated at low temperature. The AGE used in this trial contained 305 g/l of extracted solids. AGE contains primarily water-soluble organosulfur compounds such as S-allylcysteine and S-allylmercaptocysteine and small amounts of oil-soluble organosulfur compounds (Budoff et al., 2006). The finished product (Kyolic) used in this clinical study was commercially available and provided by Wakunaga of America Co., Ltd (Mission Viejo, CA). The Investigational Review Board of Los Angeles Biomedical Research Institute at Harbor-UCLA approved this research project. The trial was initiated prior to requirements for registering trials at clinicaltrials.gov.
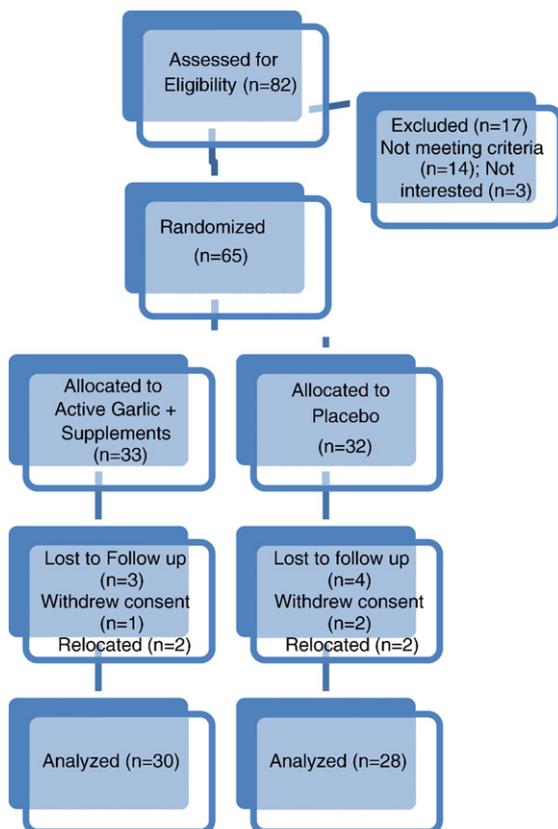
Fig. 1. Flow chart for patient enrollment and follow up.

Study population

We enrolled 65 asymptomatic patients (age 60 ± 9 years, 79% male) with CAC>30, intermediate to high risk for coronary artery disease, with a 10-year Framingham risk of developing coronary artery disease (Wilson et al., 1998) of 10–20%. Subjects with established cardiovascular disease, stroke, diabetic retinopathy, peripheral vascular disease, underlying infection, cancer, systemic inflammation status, immunosuppression, end-stage renal or liver disease, creatinine>1.4 mg/dl, triglycerides >400 mg/dl, known hypersensitivity to garlic therapy; weight >300 lb; drug or alcohol abuse, partial ileal bypass or known gastrointestinal disease limiting drug absorption; and patients currently using garlic supplements were excluded.

All the patients signed informed written consent after careful explanation and review of the protocol. Patients underwent CAC, Digital Thermal Monitoring (DTM) of vascular function assessment and oxidative biomarker measurement at baseline and after 12 months (04/01/2006 to 04/02/2007). They were randomized to garlic plus supplements or placebo in a double-blinded manner, using numbered containers, assigned to a computer generated randomization chart by the nurse coordinator. All participants, personnel administering interventions and physicians involved in the study were blinded to group assignment. A capsule of placebo or commercially available AGE (250 mg) plus vitamin B12 (100 μg), folic acid (300 μg), vitamin B6 (12.5 mg) and ʟ-arginine (100 mg) was given daily for the duration of 1 year. All participants were educated on a low-cholesterol diet and instructed to avoid any direct form of garlic and antioxidant supplementation.

Body mass index, hip circumference, blood pressure, fasting blood glucose, high sensitivity C-reactive protein (CRP), homocysteine, and lipid profile were obtained quarterly by standard techniques. All subjects were on background statin therapy as prescribed by their primary physician.

After randomization, participants returned at 3, 6, 9 and 12 months to assess their compliance with medication. 58 patients completed the study protocol. Drops outs were due to 3 patients relocating, and 4 patients withdrawing consent during the treatment period.

Demographics and laboratory measures

Information about age, gender, ethnicity, and medical history were obtained by questionnaires. Current smoking was defined as having smoked a cigarette in the last 30 days and quantitated as pack years. Alcohol use was defined as never, former, or current. Diabetes was defined as a fasting glucose >126 mg/dl or use of hypoglycemic medications. Use of antihypertensive and other medications were based on clinical staff entry of prescribed medications.

Resting blood pressure was measured two times in sitting position after a 5 min rest period using a Dinamap model Pro 100 automated oscillometric sphygmomanometer (Critikon, Tampa, Florida) and the average of the readings was recorded. Hypertension was defined as a systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, or use of medication prescribed for hypertension. Body mass index was calculated from the equation weight (kg)/height^2 (m^2).

Total and HDL cholesterol were measured from blood samples obtained after a 12-hour fast using the cholesterol oxidase cholesterol method and triglycerides measured from spectrophotometric reading after endogenous glycerol has reacted and before lipase is added to release the glycerol from the triglyceride. LDL cholesterol is calculated in plasma specimens having a triglyceride value <400 mg/dl using the formula of Friedewald et al. (1972).

CRP was measured using the BNII nephelometer (N High Sensitivity CRP; Dade Behring Inc., Deerfield, IL). Analytical intra-assay CVs ranged from 2.3–4.4% and inter-assay CVs ranged from 2.1–5.7%. S-allylcysteine (SAC) was measured in serum as previously described (Rosen et al., 2001) to determine compliance with garlic therapy.

Measures of coronary artery calcium

The electron beam tomography (EBT) studies were performed with an Imatron C-150XL Ultrafast computed tomographic scanner (Imatron, South San Francisco, California) in high-resolution volume mode using a 100-ms exposure time as previously described (28). Electrocardiographic triggering was used so that each image was obtained at the same point in diastole, corresponding to 40% of the RR interval. Proximal coronary artery was obtained with at least 30 consecutive non-enhanced images at 3-mm intervals. Total radiation exposure using this technique was <1 Rad per patient. Patients underwent similar repeat examination at the end of the study protocol. In the acquired images from non-enhancement, calcification was defined as a plaque of at least 3 contiguous

pixels (area 1.02 mm^2) with a density of >130 Hounsfield units. The amount of calcium was quantified using 2 different methods. First, the calcium score as described by Agatston et al. (1990) was calculated by multiplying the lesion area by a coefficient based on the peak density within that plaque. The total Agatston score was determined by summing the scores obtained for each lesion. The voxel size on EBT scans obtained with a 3-mm section thickness and a typical field of view of 30 cm^2 (pixel size = 0.586 mm) corresponded to $0.586 \times 0.586 \times 3 = 1.03$ mm^3. The original voxel was divided into the smaller voxels whose size is 0.201 mm^3 with the technique of isotropic interpolation for measuring a more precise volume. All voxels with a value greater than 130 Hounsfield units were defined as the calcified lesion. A total score in coronary artery by the Agatston and volumetric methods was determined by summing individual lesion scores from each of 4 anatomic sites (left main, left anterior descending, circumflex, and right coronary arteries).

A single experienced investigator (JK), blinded to clinical status of the participant and temporal relation of the scans, interpreted all studies on a commercially available software package (Neo Imagery Technologies, City of Industry, California). Patients were classified as CAC progressors if the CAC increased >15% over the one year study period, and as CAC non-progressors, if progression was <15%, as previously described (Raggi et al., 2004).

Digital Thermal Monitoring (DTM) of vascular function

After an overnight fast and abstinence from tobacco, alcohol, caffeine, and vasoactive medications, the left arm blood pressure was recorded in a sitting position 15 min before the DTM test (Omron HEM 705 CP semi-automated sphygmomanometer, Bannockburn, IL, USA). After resting in a supine position in a room with temperature 23° to 25 °C for 30 min, DTM of both hands was obtained during 3 min stabilization; 5 min cuff inflation to 50 mm Hg greater than systolic blood pressure, and 5 min deflation using an automated, operator-independent protocol (VENDYS-5000, Endothelix Inc., Houston, TX). DTM thermal probes, designed to minimize the area of skin probe contact and fingertip pressure, were attached to the pulp of the index finger, and thermal changes were traced continuously and digitalized automatically using VENDYS software (a computer based thermometry system with 0.006 °C thermal resolution and an automated compressor for measurement of blood pressure and controlled occlusion hyperemia).

Using the cuff occlusive reactive hyperemia procedure, temperature rebound (TR), calculated as [post cuff-deflation maximum temperature − baseline temperature] was measured an index of vascular reactivity. The coefficient of variation (CV) after a 1 day interval measured by Bland Altman model for the TR was 2.4% (Ahmadi et al., 2009).

Determination of oxidative biomarkers: OxPL/apoB, IgG and IgM IC/apoB, and IgG and IgM MDA-LDL autoantibody titers

Oxidized phospholipids on apolipoprotein B-100 particles (OxPL/apoB) were measured as described in detail previously (Tsimikas et al., 2005, Tsimikas et al., 2004a,b) by chemiluminescent enzyme-linked immunosorbent assay using the murine monoclonal antibody E06 which binds to the phosphorylcholine head group of oxidized but not native phospholipids. In brief, a 1:50 dilution of plasma was added to microtiter wells coated with the murine monoclonal antibody MB47 (5 μg/ml), which specifically binds apoB. Under these conditions, a saturating amount of apoB is added to each wall, and consequently, equal numbers of apoB particles were captured in each well for all samples. The content of OxPL/ apoB is then determined with biotinylated E06. The data are presented as relative light units per 100 ms; the OxPL/apoB measurement is independent of apoB (and LDL cholesterol) levels (Tsimikas et al., 2004a,b, 2006) and reflects OxPL on individual apoB particles, but not total OxPL in plasma.

Chemiluminescence enzyme-linked immunosorbent assays were also used to measure IgG and IgM autoantibodies to MDA-LDL and apoB-IC as described previously (Tsimikas et al., 2007a,b). Plasma lipoprotein (a) [Lp (a)] levels were measured by a chemiluminescent enzyme-linked immunosorbent assay with monoclonal antibody LPA4, as described previously (Tsimikas et al., 2004a,b).

Statistical analysis

All statistical analyses were performed using SPSS V 15.0 (SPSS Institute, Chicago, IL). Initial power analysis was based upon the results of a our previous placebo double-blind aged garlic extract feasibility study (Budoff et al., 2004). This study had over an 80% power to detect differences between the groups with group sizes of at least 20 participants per study. To accommodate for dropout, we enrolled 65 patients, with final retention of 58 participants. All continuous data are presented as a mean value ± SD, and all categorical data are reported as a percentage or absolute number. Student's t tests and Chi-square tests were used to assess differences between groups. Comparisons of all parameters between the active therapy and placebo were made with the Student's t test. Trends of all parameters during follow-up periods were examined using matched pair t tests. The association between the change of CAC, lipids, and endothelial function were made with correlation coefficients. The associations between changes in the two treatment groups over 1 year between groups (active therapy and placebo) for risk factors, including lipid profile, oxidation biomarkers, TR, and CAC were analyzed by logistic regression analyses. These analyses were adjusted for demographics, age, gender, and traditional cardiac risk factors. The results are reported as odds ratios (OR) for the logistic regression. Odds ratios were calculated for the highest vs. 2 lower tertiles of changes in temperature rebound (TR), LDL-C, HDL-C, Triglyceride, total cholesterol, homocysteine, CRP, Lipoprotein (a), OxPL/apoB, IgG and IgM autoantibodies to MDA-LDL and IC/apoB.

Results

Patient characteristics

The baseline clinical characteristics, lipid variables and oxidation biomarkers including OxPL/apoB, Lp (a), autoantibodies to MDA-LDL and immune complexes, as well as TR of vascular function and CAC were similar between AGE + S and placebo groups (Tables 1 and 2).

The effect of aged garlic extract and supplement therapy in CAC progression

After one year follow up, the volumetric CAC score increased from 347 ± 67 to 439 ± 77 in the placebo group compared to 291 ± 50 to 311 ± 48 in the AGE + S group, $p = 0.03$ for comparison of final scores between groups. After one year follow up, the AGE + S group had significantly less CAC progression compared to the placebo group (6.8% vs. 26.5%, $p = 0.005$, Table 2). In multivariable analysis adjusting for age, gender, hypertension, hypercholesterolemia, statin therapy, diabetes, family history of premature CHD and smoking status, CAC progression was significantly lower in the aged garlic extract group compared to the placebo group (OR: 0.35, 95% CI 0.1–0.85, $p = 0.03$).

The effect of aged garlic extract and supplement therapy in vascular function

The mean temperature rebound (TR) index at baseline was 0.57 and 0.56 on active therapy and placebo, respectively ($p = 0.9$) (Table 2). TR significantly increased in the AGE + S group compared with placebo (138% vs. 26.7%, $p = 0.001$).

Table 1
Baseline demographic and clinical characteristics of study subjects.

Variable	Garlic + S (N = 33)	Placebo (N = 32)	p value
Age in years — mean (S.D.)	60 (8)	61 (10)	0.40
Gender (% Male)	26 (79%)	25 (78%)	0.90
Hypertension (%)	18 (55%)	12 (38%)	0.20
Antihypertensive medication (%)	31 (94%)	32 (100%)	0.85
Hypercholesterolemia (%)	24 (73%)	26 (81%)	0.20
Statin therapy (%)	33 (100%)	32 (100%)	1.00
Diabetes mellitus (%)	1 (3%)	2 (6%)	0.70
Current smoker (%)	11 (33%)	12 (38%)	0.70
Family history of CVD (%)	21 (64%)	23 (72%)	0.50
Framingham risk score 10 year risk (S.D.)	13 (5)	15 (7)	0.70

Sample sizes in parenthesis, S.D. = standard deviation.
CVD = Cardiovascular disease; S = supplements.

Table 2
Comparison between cardiovascular risk factors among aged garlic extract (+ supplements) to placebo groups at baseline and 1 year.

Variable	Baseline			Follow up			Mean annual changes (%)		
	Garlic + S N = 33	Placebo N = 32	p	Garlic + S N = 30	Placebo N = 28	p	Garlic + S	Placebo	p
Coronary artery calcification	291 (50)	347 (67)	0.3	311 (48)	439 (77)	0.03	6.8	26.5	0.005
Total cholesterol, mg/dl	180.9 (41)	180.6 (31)	0.9	165 (29)	182 (30)	0.03	− 8.8	0.02	0.008
HDL-C, mg/dl	46.9 (9)	46.7 (12)	0.9	54.3 (7.8)	49.6 (8.9)	0.03	15.4	6.4	0.01
LDL-C, mg/dl	104 (34)	112 (28)	0.3	90.1 (29)	113 (27)	0.05	− 13.6	0.01	0.009
Triglyceride, mg/dl	109.3 (18)	105 (19)	0.8	89.9 (32)	94.2 (36)	0.36	− 17.8	− 10	0.08
Temperature rebound	0.57 (0.2)	0.56 (0.15)	0.9	1.36 (0.2)	0.71 (0.2)	0.04	138	26.7	0.001
Homocysteine, mg/dl	10.1 (2.4)	10.7 (2.2)	0.3	8.3 (2.7)	10.6 (2.6)	0.02	− 17.8	0.01	0.004
CRP, mg/L	0.31 (40)	0.22 (0.33)	0.4	0.21(0.42)	0.13 (0.37)	0.9	− 41	− 40.9	0.9
Lp (a), mg/dl	13.2 (10.7)	20.2 (19)	0.1	23.6 (9.8)	25.6 (14)	0.1	79	28	0.001
OxPL/apoB, RLU	4139 (913)	3231 (719)	0.4	6397 (522)	4419 (315)	0.008	54	36	0.001
IgG MDA-LDL, RLU	4054 (353)	3745 (271)	0.8	2544 (635)	3650 (410)	0.009	− 37	− 2.5	0.001
IgM MDA-LDL, RLU	2004 (206)	1800 (234)	0.3	1116 (2845)	2264 (117)	0.001	− 44	25	0.008
IgG IC/apoB, RLU	4840 (530)	4668 (392)	0.5	2179 (726)	4551 (663)	0.001	− 55	− 2.5	0.001
IgM IC/apoB, RLU	2553 (306)	2101 (258)	0.5	1845 (358)	1856 (389)	0.3	− 28	− 11.7	0.001

HDL = high density lipoprotein; LDL = low density lipoprotein; Lp (a) = lipoprotein (a); RLU = relative light units; S = supplements.
Mean (standard deviation).

The effect of aged garlic extract and garlic supplement therapy on lipid parameters, oxidative biomarkers, Lp (a), homocysteine and hsCRP

After one year follow up, total cholesterol (180.9 to 165 mg/dl, − 8.8%, $p = 0.008$) and LDL-C (104 to 90 mg/dl, − 13.6%, $p = 0.009$) decreased significantly, while HDL-C increased significantly (46.9 to 54.3 mg/dl, 15.4%, $p = 0.01$) in the AGE + S group. Lipid values did not significantly change in the placebo group over 1 year (Table 2).

After adjustment for cardiovascular risk factors, IgG and IgM autoantibodies to MDA-LDL and IC/apoB levels decreased significantly in the AGE + S group compared to placebo (Fig. 2 and Table 3). In contrast, OxPL/apoB and Lp (a) increased significantly more in the aged garlic extract and supplement group compared to the placebo group (Fig. 3 and Table 3). There was no association between aged garlic therapy use and CRP (Table 3).
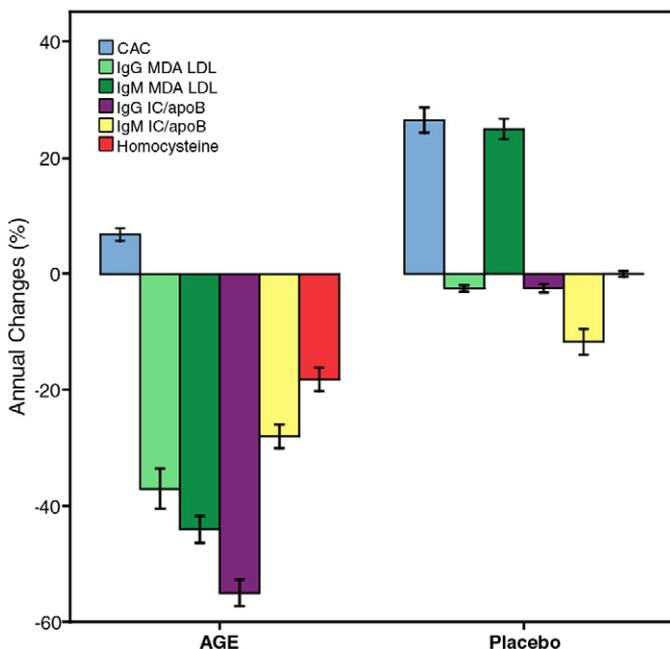
Fig. 2. Percent changes in CAC, biomarkers of oxidative stress and homocysteine (mean ± SD) from baseline to one year in the aged garlic extract and placebo groups.

Compliance with garlic therapy

SAC serum measurement is currently the only reliable human compliance marker used for studies involving garlic consumption since it is detectable and quantitatively increases in the blood after oral intake of garlic capsules (Rosen et al., 2001; Steiner and Lin, 1998). The average serum SAC level was significantly higher in patients randomized to aged garlic therapy as compared to those randomized to placebo, (36 parts per billion (ppb) vs. 15 ppb, $p < 0.05$).

Discussion

The current study demonstrates that aged garlic extract supplemented with vitamins B6 and B12, folic acid and L-arginine was associated with not only decreased CAC progression but also with concomitant improvement in vascular function following 1 year of treatment of asymptomatic intermediate risk patients. In addition, evidence of favorable effects on oxidative biomarkers were noted with increased levels of OxPL/apoB and Lp (a), which have been previously noted to increase in response to statins, low fat diets and athero-

Table 3
Odds ratios of progression of coronary artery calcification, vascular function and changes in lipid and oxidative biomarkers in AGE + S vs. placebo after 1 year of treatment.

Outcome	Aged garlic extract OR (95% CI)	p value
CAC progression	0.35 (0.1–0.85)	0.03
TR increase	2.21(1.12–4.9)	0.04
LDL-C	0.87 (0.71–0.90)	0.04
HDL-C	1.09 (1.03–1.13)	0.04
Triglyceride	0.97 (0.33–3.8)	0.60
Total cholesterol	0.92(0.81–0.98)	0.04
Homocysteine	0.87 (0.46–0.96)	0.03
C-reactive protein	0.92(0.35–3.5)	0.40
Lipoprotein (a)	1.78 (1.1–2.2)	0.02
OxPL/apoB	1.30 (1.1–1.8)	0.01
IgG MDA-LDL	0.74 (0.16–0.87)	0.02
IgM MDA-LDL	0.80 (0.1–0.9)	0.02
IgG IC/apoB	0.82 (0.05–0.9)	0.03
IgM IC/apoB	0.78 (0.1–0.9)	0.02

Logistic regression model adjusted for age, gender, DM, Hypertension, Hypercholesterolemia, Family History of CHD, Smoking status, Statin therapy.
Abbreviations: OR = Odds ratio, CI = Confidence intervals, LDL = low density lipoprotein, HDL = high density lipoprotein, CAC = coronary artery calcium.
Odds ratio of CAC progression (increase in CAC ≥ 15%/year) vs. CAC non-progression.
Odds ratio of highest vs. 2 lower tertiles of temperature rebound (TR), LDL-C, HDL-C, Triglyceride, Total Cholesterol, Homocysteine, C-reactive protein, Lipoprotein (a), OxPL/apoB, IgG and IgM autoantibodies to MDA-LDL and IC/apoB.
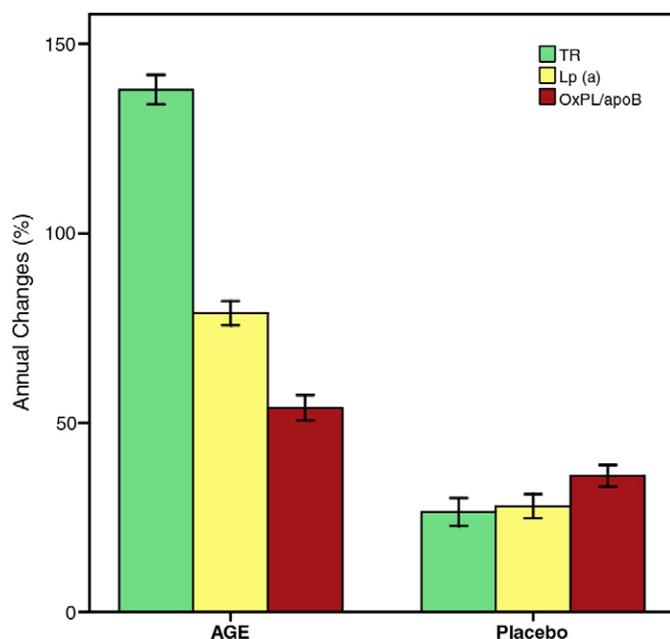
Fig. 3. Percent changes in vascular function measured by temperature rebound, OxPL/apoB and Lp (a) (mean ± SD) from baseline to one year in the aged garlic extract and placebo groups.

sclerotic plaque regression. Paralleling this were decreased levels of indirect measures of oxidative stress as measured by autoantibodies to MDA-LDL and apoB-immune complexes.

We previously studied the impact of aged garlic extract alone without the supplements evaluated in this study on the atherosclerotic plaque burden measured by changes in CAC (Budoff et al., 2004). After a one year follow up, changes in CAC were less in aged garlic extract alone as compared to placebo group ($7.5 \pm 9.4\%$ vs. $22.2 \pm 18.5\%$ and 45.2 ± 57.2 vs. 129.0 ± 102 volumetric score, respectively ($p<0.05$)). Our current study reconfirms our previous findings and provides evidence of a larger decrease in the progression of CAC with the combination of aged garlic therapy plus vitamins and L-arginine.

CAC is an excellent marker of overall coronary atherosclerotic burden (Budoff et al., 2006; Simons et al., 1992) correlating with both the total plaque burden and the presence of obstructive disease. In addition, there are numerous studies confirming the strong predictive power of CAC for clinical events in asymptomatic patients (Budoff, 2006; Detrano et al., 2008; Budoff et al., 2007). Mechanisms which may explain a significant decrease in the progression of CAC in AGE plus supplement group include a variety of potential anti-atherosclerotic effects, as shown recently by studies which have shown aged garlic therapy as a modulator of cardiovascular risk factors, (Steiner and Lin, 1998) including blood pressure (Dhawan and Jain, 2004), platelet aggregation and adhesion, total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL), LDL oxidation, smoking-caused oxidative damages, and microcirculation.

A study investigated molecular mechanism of aged garlic extract reported that it directly suppressed atherosclerosis (Steiner and Lin, 1998). In addition, inhibiting damage of the endothelial cells and transforming smooth muscle cells by aged garlic extract suggest that this compound may have an effect of controlling vascular function through inhibiting the damage of nitric oxide (NO) synthesis (Morihara et al., 2002). Our study demonstrated improvement in vascular function under the influence of garlic extract along with B vitamins, folic acid and L-arginine, with an improvement in fingertip temperature rebound. Similarly, Williams et al. (2005), in a randomized, placebo-controlled trial on 15 men with angiographically proven CAD, flow mediated vasodilation (FMD) through reactive hyperemia test increased significantly in aged garlic extract (44%) as compared to the placebo ($p = 0.04$).

We have also demonstrated that increased homocysteine levels are associated with increased plaque progression (Rasouli et al., 2005). Our previous study of aged garlic extract alone demonstrated significant reduction in homocysteine (Budoff et al., 2004). The current study reconfirms our previous work and demonstrated more reduction in homocysteine with the combination of aged garlic extract and the supplements. Ide et al. (2006) studied the effects of homocysteine on CD36 expression and foam cell formation in human monocytes/macrophages (THP-1) using flow cytometry and also evaluated the impact of aged garlic therapy on this process. They demonstrated that CD36 expression increased with incubation of THP-1 cells with homocysteine, also AGE therapy inhibits CD36 expression and OxLDL uptake in macrophages and suggested that AGE could modulate the formation of early atherosclerosis.

In addition to garlic and homocysteine-lowering vitamins, active therapy included arginine supplementation. Arginine has been shown to increase NO bioavailability, which has been shown to reduce vascular dysfunction which underlies vascular stiffness, independent of other age-related vascular pathologies such as atherosclerosis. The activation/upregulation of arginase appears to be an important contributor to age-related vascular dysfunction by a mechanism that involves substrate (L-arginine) limitation for NOS3 and therefore NO synthesis (NOS) (Briley-Saebo et al., 2008). The activation/upregulation of arginase impairs NO production, also enhances production of reactive oxygen species by NOS. While arginase abundance is increased in vascular aging models, it appears that post-translational modification by S-nitrosylation of the enzyme enhances its activity as well (Tsimikas, 2008). Furthermore, arginase activation contributes to aging-related vascular changes by mechanisms that are not directly related to changes in NO signaling, including polyamine-dependent vascular smooth muscle proliferation and collagen synthesis.

The potent effect of aged garlic extract, B vitamins and L-arginine on biomarkers of oxidative stress is a novel observation. In this study a significant reduction in autoantibodies to MDA-LDL and apoB-immune complexes were noted in the AGE + S group compared to placebo. Previous studies have shown that OxLDL autoantibodies correlate with both the progression and regression of atherosclerosis in LDLR$^{-/-}$ mice in response to low fat diets and antioxidant vitamins (Tsimikas et al., 2006). In addition, a recent study in apoE$^{-/-}$ mice with magnetic resonance using micelles loaded with oxidation-specific antibodies demonstrated OxLDL in the vessel wall (Briley-Saebo et al., 2008). Furthermore, a recent study in humans has shown that autoantibodies to MDA-LDL and apoB-immune complexes are correlated with the extent of angiographically determined coronary artery disease (Choi et al., 2008). Reduction in OxLDL biomarkers with concomitant reduced progression of CAC and improved vascular function in response to aged garlic extract plus B vitamins and L-arginine, is consistent with the previous studies and suggests that part of the benefit may be mediated through reduction of oxidative stress and oxidative pathways.

Consistent with prior data on a variety of statins and low fat diets, aged garlic extract plus supplement also induced an increase in OxPL/apoB and Lp (a) levels (18). These data suggest that OxPL and Lp (a) are physically associated and that Lp (a) has a strong affinity for OxPL (Bergmark et al., 2008). Previous studies postulated that Lp (a) may function at a primordial level to bind and perhaps mediate the removal of oxidized fatty acids from OxPL/apoB, particularly as it is enriched in lipoprotein-associated phospholipase A2, an enzyme with the ability to hydrolyze oxidized fatty acids from OxPL/apoB (Choi et al., 2008). Furthermore, previous studies have shown a 50–100% increase of plasma OxPL/apoB levels in rabbits and monkeys, along with concomitant evidence of loss of OxPL from the vessel wall, suggesting that as OxPL/apoB rises, OxPL epitopes disappear in the vessel wall in response to therapeutic interventions (Miyazaki et al., 2005; Nakaishi et al., 2000).

Finally, previous studies have shown that vascular dysfunction measured by DTM correlates well with the severity and extent of coronary artery disease measured by CAC and CT angiography,

Framingham risk score, insulin resistance, metabolic syndrome and diabetes (Ahmadi et al., 2007, 2008a,b). Similar to statin therapies (Choi et al., 2008; Davignon and Ganz, 2004), current study provides evidence that aged garlic therapy plus supplement results in significant increases in OxPL/apoB and lipoprotein (a) levels, improves vascular function and decreases in apoB-immune complexes and OxLDL autoantibodies. In addition suggests that favorable effect of aged garlic therapy plus supplement with increases in OxPL/apoB, Lp (a), and vascular function may be associated with the reduction in the rate of CAC progression.

Limitations

The present study has several limitations. In our study CAC progression was defined as absolute changes in CAC as reported by Raggi et al. (2004) as a minimum annualized percent change of CAC score ≥15%. Analyses of the progression of CAC are statistically challenging because of a high degree of dependence on the baseline CAC score as well as inter-scan variability. However, Raggi et al. demonstrated a 17-fold increase in CVD events among progressors. Furthermore, this study was not designed to assess which components of the AGE + S capsule were most responsible for the observed benefits. A much larger study randomizing patients to individual components of the therapy would be needed. Both the placebo and active therapy groups were advised to adhere to a healthy lifestyle behaviors, but this was not formally assessed as to adherence or independent effects on the endpoints. A randomized trial of diet and lifestyle would be needed to better assess these potential interventions. Further studies addressing these relationships to clinical outcomes await results of large therapeutic studies in progress.

Conclusion

This study demonstrates that commercially available aged garlic extract with B vitamins, folate and L-arginine: 1) retards progression of CAC; 2) improves vascular function measured by DTM; 3) favorably influences markers of oxidative stress. Further longitudinal studies are warranted to evaluate whether these dietary supplements can decrease long term adverse cardiovascular events, particularly in high-risk patients.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ypmed.2009.06.018.

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