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Fish oil and vitamin E change lipid profiles and anti-LDL-antibodies in two different ethnic groups of women transitioning through menopause

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Background: **studies have investigated the relationship between the transition through menopause and cardiovascular diseases. White population, generally, have lower levels of traditional coronary heart risk factors, particularly dyslipidemia, hypertension, obesity, and diabetes, and lower rates of coronary heart disease mortality, than black population. Furthermore many studies have shown the cardioprotective and anti-inflammatory effects of omega-3 polyunsaturated fatty acids (eicosapentaenoic acid and docosahexaenoic acid) of marine origin. The aim of this study was to investigate the effect of omega-3 supplementation, combined or not with vitamin E, on oxidative biomarkers and lipid profiles in nonwhite and white women with dyslipidemia transitioning through menopause.**

Methods: **a randomized, double-blind, placebo-controlled trial was conducted. Seventy-four eligible women were assigned to receive: fish oil, fish oil plus vitamin E and placebo for three months. At baseline, 45 and 90 days blood sample for biochemical variables and biomarkers of oxidative stress were taken. Socioeconomic and lifestyle variables were collected with standardized questionnaires.**

Results: **after 90 days the fish oil plus vitamin E treated group had a significant decrease in total cholesterol and LDL-C. Furthermore, there was a decrease in anti-LDL- autoantibodies after 45 days. Plasma TBARS concentrations were increased after 90 days in the group receiving only fish oil when compared to the placebo and fish oil-vitamin E groups. All of the effects observed were independent of ethnic group.**

Conclusion: **supplementation with fish oil and vitamin E reduced total cholesterol and LDL-C, but had opposite**

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EL ACEITE DE PESCADO Y LA VITAMINA E MODIFICAN EL PERFIL LIPÍDICO Y LOS ANTICUERPOS ANTI-LDL EN DOS GRUPOS ÉTNICOS DIFERENTES DE MUJERES EN TRANSICIÓN HACIA LA MENOPAUSIA

Resumen

Introducción: **diversos estudios han investigado la relación entre la transición a la menopausia y las enfermedades cardiovasculares. Generalmente, la población de etnia blanca posee bajos niveles de factores de riesgo coronarios, particularmente dislipidemia, hipertensión, obesidad, diabetes y bajas tasas de mortalidad por enfermedades del corazón en comparación con la población de etnia negra. Además, varios estudios demostraron efectos cardioprotectores y antiinflamatorios provenientes de ácidos grasos poliinsaturados omega-3 (ácido eicosapentaenoico y ácido docosahexaenoico) de origen marino. El objetivo del estudio fue investigar el efecto de la suplementación de omega-3 combinado o no con vitamina E en biomarcadores oxidativos y perfiles lipídicos en mujeres blancas y no blancas con dislipidemia en transición hacia la menopausia.**

Métodos: **fue realizado un estudio randomizado, duplo-ciego, placebo-controlado. Setenta y cuatro mujeres elegibles fueron escogidas para recibir: aceite de pescado, aceite de pescado con vitamina E y placebo durante tres meses. Fueron recogidas muestras de sangre en de referencia, 45 y 90 días para realizar exámenes bioquímicos y de biomarcadores para estrés oxidativo. Las variables socioeconómicas y de estilo de vida fueron recogidas por medio de cuestionarios estandarizados.**

Resultados: **después de 90 días, el grupo tratado con aceite de pescado con vitamina E tuvo una disminución significativa para colesterol total y LDL-C. Además, hubo una disminución de anticuerpos anti-LDL después de 45 días. La concentración de plasma TBARS aumentó después de 90 días en el grupo que recibió solamente aceite de pescado, comparado con los grupos placebo y aceite de pescado con vitamina E. Todos los efectos observados fueron independientes del grupo étnico.**

Conclusión: **la suplementación con aceite de pescado y vitamina E redujo el colesterol total y LDL-C, pero tuvo**

effects on oxidative stress compared to supplementation with fish oil alone.

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Key words: Oxidative stress. Fish oil. Menopause. Vitamin E. Race.

un efecto opuesto en el estrés oxidativo comparado con la suplementación solamente con aceite de pescado.

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Palabras clave: Estrés oxidativo. Aceite de pescado. Menopausia. Vitamina E. Raza.

Abbreviations

MBW: Minimum Brazilian Wage.
BMI: Body mass index.
SFA: saturated fatty acids.
PUFA: polyunsaturated fatty acids.
MUFA: monounsaturated fatty acids.
TBARS: thiobarbituric acid reactive substances.
HDL-C: high-density lipoprotein cholesterol.
LDL-C: low-density lipoprotein cholesterol.
ANCOVA: analysis of covariance.
CVD: cardiovascular disease.
NCHS: National Center for Health Statistics.
EPA: eicosapentaenoic acid.
DHA: docosahexaenoic acid.
FHP: Family Health Program.
ANOVA: analysis of variance.
PBS: phosphate-buffered saline.
SPSS: Statistical Package for the Social Sciences.
NHC: National Health Council.
MDA: malondialdehyde.
FAME: fatty acid methyl ester.
HPLC: high-performance liquid chromatograph.

Introduction

Previous studies have investigated the relationship between the transition through menopause and cardiovascular disease (CVD)[1-3]. The decline in estrogen increases cardiovascular risk as a result of changes in atherogenic lipid profiles in plasma. In humans, cardiovascular risk is clinically associated with an increase in low-density lipoprotein cholesterol (LDL-C) and a decrease in high-density lipoprotein cholesterol (HDL-C)[1-3]. According to the National Center for Health Statistics- NCHS[4], the prevalence of CVD is also influenced by ethnic characteristics. In this respect, nonwhite women are one third more likely to die from heart disease or stroke than white women[5]. The racial issue is somewhat forgotten in clinical trials on CVD, although some improvement has been observed over the last decades. Nevertheless, only half of all trials published in high-impact journals report data on ethnic groups of the populations studied[6].

In Brazil, the rate of cardiovascular mortality is 1.14 times higher among black women[7]. Despite this observation, studies have consistently demonstrated that these changes alone are not sufficient to explain the processes involved in CVD[8,9]. Within this context, many potential biomarkers have been proposed; for example, oxidized LDL and its autoantibodies (anti-oxidized LDL) have been detected in human plasma and atherosclerotic lesions under different environmental conditions[10].

There is extensive literature documenting the cardioprotective and anti-inflammatory effects of omega-3 polyunsaturated fatty acids (eicosapentaenoic acid, EPA, and docosahexaenoic acid, DHA) of marine origin[11-13]. Furthermore, studies on vitamin E, the most prevalent natural antioxidant vitamin, suggest that the supplemental use of this vitamin may lower the risk of coronary events[14-16]. To clarify these possibilities, the aim of the present study was to investigate the effect of omega-3 supplementation, combined or not with vitamin E, on oxidative biomarkers and lipid profiles in nonwhite and white women with dyslipidemia transitioning through menopause.

Materials and methods

Study population and design

A randomized, double-blind, placebo-controlled trial was conducted by the Family Health Program (FHP), Ministry of Health, Brazil. Women aged 40 to 70 years with low habitual fatty fish and seafood intake, who met at least two of the following criteria, were included in the study at baseline: total cholesterol > 200 mg/dL, LDL-C > 140 mg/dL, HDL-C < 50 mg/dL, and triglycerides > 150 mg/dL. Subjects were not eligible for study participation if they were of Asian ethnicity, had positive drug or alcohol test results, were receiving lipid-lowering medications or omega-3 supplementation, had a body mass index (BMI) ≥ 35 kg/m^2, had CVD or other comorbidities, bleeding disorders, kidney or liver disease and diabetes, or were allergic to fish or crustaceans.

Standardized questionnaires were used to collect information on menopause, age at menopause, ethnicity, educational level, cigarette smoking, number of pregnancies, history of oophorectomy, use of hormone therapy, hypertension, diabetes, and medications, as well as the volunteer's physical activity and habitual diet. Because of their participation in the FHP, all volunteers were undertaking walking activities that were classified as light. Furthermore, none of the participants presented any restrictions in their habitual diet. Women who met the initial prescreening criteria underwent a clinical screening examination consisting of height and weight measurements, BMI calculation, and laboratory tests for the determination of total cholesterol, HDL-C, LDL-C and triglycerides.

The subjects were allocated as follows: fish oil (fish group, 10 nonwhite and 12 white women) receiving daily fish oil [one capsule containing 1 g of n-3 polyunsaturated fatty acids (540 mg EPA and 360 mg DHA) and one capsule of placebo containing mineral oil]; fish oil plus vitamin E (fish+VitE group, 10 nonwhite and 9 white women) receiving daily fish oil [one capsule containing 1 g of n-3 polyunsaturated fatty acids (540 mg EPA and 360 mg DHA)] and one capsule of vitamin E (containing 400 mg vitamin E/ alpha-tocopherol) and placebo (placebo group, 9 nonwhite and 9 white women) receiving daily two capsules of placebo (containing mineral oil). The treatment was administered before the two main meals of the day for 3 months. The subject participation and flow diagram of the progress through the study are shown in Figure 1.

Adherence was evaluated by counting the remaining capsules and by evaluating changes in the concentrations of specific plasma fatty acids at the end of each treatment period.

Ethics

The clinical protocol of this study was approved by the Ethics Committee of the School of Public Health and the study was conducted in accordance with the ethical guideline of the National Health Council (NHC). All subjects gave their informed consent.

For treatment administration, gelatin-based softgel capsules of approximately 1 g were manufactured in accordance with good manufacturing practices. The capsules containing the test and placebo medications had the same shape and appearance.

Sample collection and biochemical analyses

Baseline data of all outcome measures were collected from the participants before the first treatment. These measurements were repeated after 90 days. Biochemical parameters were measured in blood samples at baseline and after 45 and 90 days.

Peripheral blood samples were collected after a 12-hour fast and were immediately centrifuged at 700 *g* for 15 minutes at 20°C. All samples were kept frozen (-80°C) until the time of analysis and all tests were performed in duplicate. Serum total cholesterol, HDL-C, and triglyceride levels were measured by validated routine laboratory methods based on immunoenzymatic and colorimetric tests, using an automated analyzer (Roche Hitachi 912 Chemistry Analyzer). LDL-C was obtained using the Friedewald equation.

Detection of anti-LDL- autoantibodies

Anti-LDL$^-$ monoclonal antibodies were characterized using the protocol described by Damasceno et al.[17] LDL$^-$ previously isolated by fast protein liquid chromatography was used for coating the plates. LDL$^-$ was diluted in 0.25 M carbonate-bicarbonate, pH 9.6, to reach a final concentration of 0.5 μg protein/well. The plates were incubated for 12 hours at 4°C. After this period, the plates were blocked with 50.0 g/L skim milk (Molico, Nestlé, Araçatuba, SP, Brazil) in phosphate-buffered saline (PBS) for 2 hours and washed four times with PBS in an automated plate washer. The plasma samples diluted 1:800 in PBS (v/v) were added and the plates were kept at room temperature for 2 hours. The plates were then washed four times with PBS. Next, human anti-IgG peroxidase (Sigma Chemical, St. Louis, MO, USA) was added at a proportions of 1:100 in PBS. The plates were incubated for 90 minutes and washed four times. The color reaction was developed by adding 3,3',5,5'-tetramethylbenzidine as chromogenic substrate. The plates were incubated for 30 minutes at room temperature protected from light. The reaction was blocked by the addition of 50 μL of 2 M H_2SO_4 and absorbance was monitored in a plate reader (Spectracount®, Canberra Company, Meriden, CT, USA) at 450 nm. For interpretation of the results, the mean absorbance minus the background was applied to the curve of the standard equation for human IgG (Sigma Chemical) (0.18-11.7 mg/mL). The results are expressed as equivalents of anti-IgG human anti-LDL$^-$.

Lipid peroxides

Lipid peroxidation was evaluated by the detection of derivative products from oxidation in plasma, substances that react with thiobarbituric acid-reactive substances (TBARS), mainly malondialdehyde (MDA)[18]. Briefly, plasma diluted in 0.02 M PBS, pH 7.4 (1:10, v/v), was mixed with 1 mL of freshly prepared reagent containing 0.046 M thiobarbituric acid, 0.92 M trichloroacetic acid, and 0.25 M HCl. After 30 minutes of incubation at 100°C, the samples were cooled on ice and centrifuged at 8000 *g* for 15 minutes at 4°C, and absorbance of the supernatant was read at 535 nm. Freshly prepared 1,1,3,3-tetramethoxypropane was used as the standard. The results are expressed as μmol TBARS/L.

Plasma alpha-tocopherol

Plasma alpha-tocopherol concentrations were measured in 200 μL of the samples[19]. After thawing, 200 μL ethanol was added and mixed (5 seconds). Next, 500 μL hexane was added to the samples, which were mixed for 2 minutes and centrifuged at 700 *g* for 5 minutes. A 250 μL aliquot of each supernatant was collected, dried under a nitrogen stream, and resuspended in 200 μL of the mobile phase (70% acetonitrile, 20% methanol, and 10% dichloromethane). Twenty microliter was injected into a high-performance liquid chromatograph (HPLC) (Shimadzu, System Controller SCL-10AVP) equipped

with a Rheodyne manual sample injector. A fluorescence detector (Merck Hitachi L7480) was used (λ_{exc} = 295 nm and λ_{emi} = 325 nm). The chromatograms were integrated using the Class VP software and alpha-tocopherol concentrations were calculated by constructing calibration curves. Separation was performed on a 5-μm HyperClone ODS C18 analytical column (Phenomenex, Torrance, CA, USA).

Plasma fatty acids

Lipid extraction and separation were carried out as described by Marmer & Maxwell[20]. Fatty acids were determined by saponification of aliquots of the lipid extract, in which lipids were methylated according to the method of Metcalfe et al.[21]. Fatty acid analysis was performed in a Shimadzu GC 2010 gas chromatograph equipped with an FID detector under the following conditions: split injection (1:30, injection volume 1 μL); capillary column SP 2560 (Supelco) (100 m, 0.25 mm, 0.2 μm film); injection port and detector temperatures of 250°C and 260°C, respectively; initial oven temperature of 140°C increased at 4°C/min to 240°C, which was maintained for 15 minutes; hydrogen as carrier gas at a flow rate of 1.5 mL/minute. The fatty acids were identified by comparing their retention times with those of pure standards (fatty acid methyl ester (FAME) 37, code 47885, Sigma Chemical Co.). Fatty acid composition is expressed as the percentage of total FAMEs.

Statistical analysis

The normality of the variables was evaluated using the Kolmogorov-Smirnov test. The delta values (final mean – initial mean) of the biochemical variables were compared between the placebo and intervention groups by analysis of variance (ANOVA), assuming the equality of variance determined by Levene's test.

Linear regression with a 95% confidence interval was used for analysis of covariance (ANCOVA), considering the biochemical variables (total cholesterol, triglycerides, HDL-C, LDL-C, alpha-tocopherol, TBARS, and anti-LDL$^-$ autoantibodies) as the dependent variables and the group (0 = placebo; 1 = fish oil or fish oil + vitamin E) as the independent variable of interest. Ethnicity (0 = white; 1 = nonwhite) was used as the control variable.

Statistical significance was defined as a *p* value of less than 0.05. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 20.0.

Results

Seventy-four eligible women were recruited from the FHP. Fifteen patients dropped out of the supplementation program. Eight white women dropped out after 30 days, five reporting discomfort during ingestion of the capsules, two giving up, and one starting antihypertensive treatment. Three white women dropped out after 60 days because of discomfort during ingestion of the capsules and three women after 65 days because they underwent surgery for correction of glaucoma. Thus, 59 women completed the trial (Figure 1).

The sample studied had a mean age of 51.6±7.8 years and low education level and household income. Most participants were non-smokers and non-drinkers. No differences in baseline biochemical, anthropometric, socioeconomic, lifestyle and dietary characteristics were observed between the placebo and intervention groups or between ethnic groups (Table I).

Plasma concentrations of TBARS were increased in the fish oil group after 90 days when compared to the placebo and fish+VitE groups ($p<0.001$ and 0.015, respectively) (Table II).

Multiple analyses confirmed the increase in TBARS after 90 days in the fish oil group (β=0.045, 95% CI=0.021, 0.069). A decrease in total cholesterol and LDL-C concentrations was observed in the fish oil-vitamin E group after 90 days (β=-30.993, 95% CI=-51.78, -10.201). Additionally, there was a reduction in anti-LDL$^-$ autoantibody concentrations after 45 days (β=-6.572, 95% CI=-11.882, -1.261). All of the effects observed were independent of ethnic group (Table III).

Discussion

The present clinical trial evaluated the effect of separate or combined (with vitamin E) supplementation with fish oil in dyslipidemic women of two ethnic groups transitioning through menopause. We observed that: 1) supplementation with fish oil alone increased TBARS concentrations; 2) combined supplementation with fish oil and vitamin E reduced total cholesterol, LDL-C and anti-LDL$^-$ autoantibodies, and 3) the effects observed in the two intervention groups were independent of ethnic group.

The effects of fish oil supplementation on lipid profiles and oxidative stress have been described in the literature. Similar to the present results, Carrapeiro et al.[22] evaluated the effect of fish oil alone (2.4 g/day of EPA and DHA) and combined with statin administered in a crossover study of 43 women (mean age of 61 ± 8 years), most of them Caucasian (65%), for 6 weeks and observed an increase in plasma MDA (p=0.003) and superoxide dismutase activity (p=0.014) and a decrease in catalase expression (p=0.013). The authors suggested that the mechanism whereby omega-3 fatty acids increase oxidative stress is associated with changes in the expression and activity of antioxidant enzymes.

Although the present study demonstrated an increase in oxidative stress markers after supplementation with fish oil alone, the effect of omega-3 fatty acids on oxida-

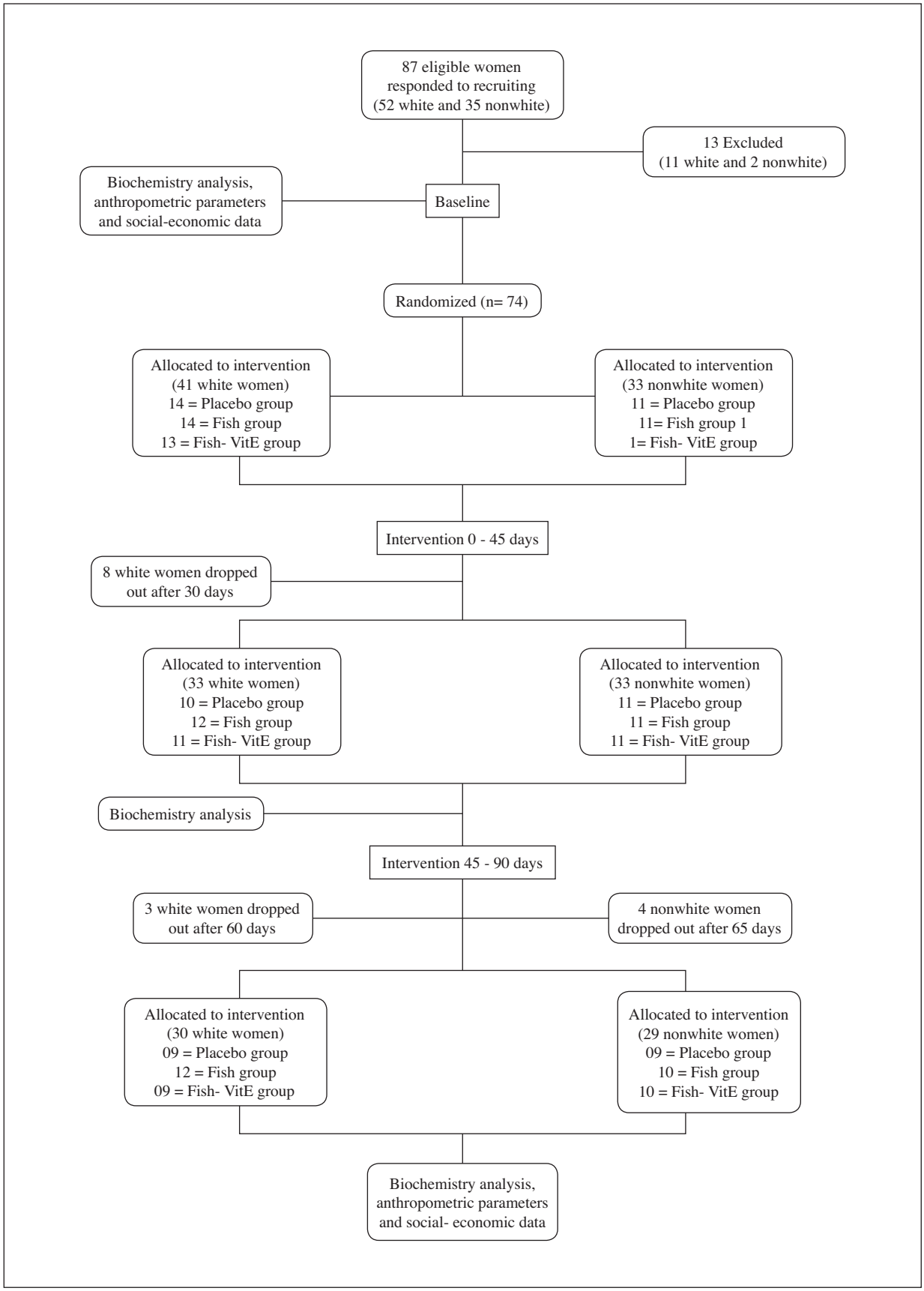

Fig. 1.—Flow of patients through the trial.

Table I
Baseline characteristics and biochemical data of women according to intervention

Variables	*Placebo n=18*	*Fish n=22*	*Fish + VitE n=19*
Age (years)	54 (8)	52 (8)	50 (7)
Ethnicity (nonwhite)[#]	10 (53)	11 (46)	10 (50)
Educational level (0-8 years)[#]	15 (83)	17 (77)	14 (74)
Per capita monthly income (0-2 MBW)[#]	11 (61)	12 (55)	10 (53)
Smoking (yes)[#]	5 (28)	4 (18)	6 (32)
Alcohol intake (yes)[#]	3 (17)	4 (18)	2 (11)
Physical activity (sedentary)[#]	2 (11)	3 (14)	4 (21)
BMI (25.0-29.9 kg/m²)[#]	3 (17)	5 (23)	4 (21)
Total Cholesterol (mg/dL)	244 (38)	260 (47)	230 (31)
Triacylglycerols (mg/dL)	237 (118)	206 (157)	225 (135)
HDL-C (mg/dL)	47 (13)	54 (13)	54 (13)
LDL-C (mg/dL)	147 (34)	165 (34)	135 (33)
Alpha-tocopherol (μmol/L)	14.6 (4.1)	15.3 (4.6)	14.8 (4.1)
TBARS (μmol/L)	0.06 (0.04)	0.04 (0.04)	0.05 (0.03)
Autoantibodies anti-LDL- (mg/mL)	43.4 (6.9)	46.7 (10.6)	48.2 (4.1)
Fatty acids plasma			
SFA (%)	32.4 (4.3)	32.5 (11.6)	31.2 (4.3)
MUFA (%)	21.2 (5.0)	23.3 (9.3)	21.2 (4.1)
PUFA (%)	46.4 (8.5)	40.0 (17.8)	47.6 (7.2)
Ω 3 (%)	5.5 (6.2)	4.8 (5.7)	5.5 (3.2)
Ω 6 (%)	40.9 (6.1)	35.3 (16.5)	42.1 (6.5)

Data were reported as mean (standard deviation - SD) or [#]n (%). Minimum Brazilian Wage, (1 MBW approx. US$200); BMI: Body mass index; SFA: saturated fatty acids; PUFA: polyunsaturated fatty acids; MUFA: monounsaturated fatty acids; TBARS: thiobarbituric acid reactive substances; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol.

tive stress still produces conflicting results. The present clinical trial diverges from another crossover study involving 15 postmenopausal women with a mean age of 58 ± 6 years supplemented with fish oil (2.0 g EPA/day and 1.4 g DHA/day) for 5 weeks compared to sunflower and safflower oil. The authors concluded that there was no increase in oxidative stress estimated based on plasma F2-isoprostanes and MDA, although TBARS concentration was higher in the fish oil group compared to the other oils[23]. In another study from the same research group[24] evaluating only LDL oxidation, supplementation with fish oil alone did not increase total *ex vivo* LDL particle oxidation, especially when compared to sunflower oil. Other studies also found no changes in oxidative stress markers, lipid peroxidation or inflammation after short-term omega-3 supplementation[25,26].

Combined supplementation with fish oil and antioxidants has been described in the literature; however, there is still no consensus about the role of antioxidants in the reduction of oxidative stress induced by omega-3 fatty acids. In a randomized study in which 36 men were supplemented with fish oil alone (600 mg EPA and 400 mg DHA per day) and combined with antioxidants (30 mg vitamin E, 60 mg vitamin C and 6 mg beta-carotene), an increase in oxidative stress was observed which was not attenuated by the addition of antioxidants[27].

The effect of fish oil and alpha-tocopherol on the reduction of oxidative stress, measured as urinary TBARS and thiobarbituric-MDA adduct, has been demonstrated. TBARS decreased linearly with increasing alpha-tocopherol dose[28]. These findings agree with Meydani et al.[29] who observed an increase in MDA after 2 months of daily supplementation of women with fish oil (1,680 mg EPA, 720 mg DMA and 6 IU vitamin E), even when combined with vitamin E. The authors concluded that the vitamin E concentration of the capsules was not sufficient to provide adequate antioxidant protection, thus confirming the relationship with the dose administered.

To our knowledge, this is the first study describing a reduction in anti-LDL- autoantibodies after combined supplementation of fish oil and alpha-tocopherol. Experimental data have shown that vitamin E is the most

Table II
Clinical trial results of supplementation of fish oil and vitamin E by difference of means[1]

Variables	*Placebo Mean (SE)*	*Fish Mean (SE)*	*Fish + VitE Mean (SE)*
Total Cholesterol (mg/dL)			
45 days	-15.0 (8.2)	-26.7 (4.5)	-23.2 (5.9)
90 days	-2.4 (8.4)	-14.1 (6.1)	-17.2 (8.4)
Triacylglycerols (mg/dL)			
45 days	2.1 (19.0)	-31.1 (17.4)	-25.0 (13.1)
90 days	-1.0 (33.4)	-32.9 (18.6)	-15.7 (18.6)
HDL-C (mg/dL)			
45 days	2.4 (2.2)	2.8 (2.2)	0.5 (2.1)
90 days	3.0 (4.9)	2.2 (2.7)	-0.5 (2.5)
LDL-C (mg/dL)			
45 days	-7.6 (4.2)	-23.0 (4.7)	-14.8 (5.7)
90 days	12.2 (9.9)	-13.9 (5.6)	-9.8 (8.1)
Alpha-tocopherol (μmol/L)			
45 days	-0.2 (1.2)	1.3 (0.8)	1.6 (1.1)
90 days	4.8 (2.1)	4.2 (2.0)	2.4 (1.9)
TBARS (μmol/L)			
45 days	-0.02 (0.01)	0.02 (0.01)	0.01 (0.01)
90 days	-0.01 (0.01)[a]	0.05 (0.01)[b]	0.01 (0.01)[a]
Autoantibodies anti-LDL- (mg/mL)			
45 days	0.5 (1.3)	-4.3 (3.2)	-7.3 (2.0)
90 days	-11.4 (2.9)	-9.5 (3.0)	-14.7 (2.8)

Data were reported as mean (standard error - SE) of delta values (final mean – initial mean). [1]Used the ANOVA test to compare the study groups. Different letters stand for statistical difference. TBARS: thiobarbituric acid reactive substances; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol.

potent antioxidant in the prevention of coronary artery diseases and reduces atherosclerotic lesions induced by oxidized LDL, preventing their onset and progression[30]. Furthermore, Baynes & Dominiczak[31] related that the proliferation of endothelial and cardiac smooth muscle cells caused by the action of fatty acid peroxidation products can be blocked by antioxidants such as vitamins C and E. LDL modified by oxidation is an important atherogenic factor[32]. On the other hand, the formation of anti-oxidized LDL autoantibodies depends on individual susceptibility, including genetic and other factors such as the concentration of antioxidants and of unsaturated fatty acids in LDL[33]. The reduction in autoantibodies during combined supplementation may be explained by increased plasma immune complexes or by the individual immune response to the antigens[34].

Combined supplementation provided better lipid profile results, reducing total cholesterol and LDL-C. Intervention studies such as the Cambridge Heart Antioxidant Study (CHAOS)[35] and the Grouppo Italiano per ló Studio Della Sopravvivenza nell'Infarto Miocardito (GISSI) trial[36] indicate an effect of vitamin E on the incidence of coronary disease.

We evaluated two ethnic groups with different cardiovascular risks as reported in the literature[37,38]. However, no differences in baseline characteristics were observed in the population studied and no relationship could be attributed to ethnicity, with the effects observed being independent of race.

Although not statistically significant, the relationship between different ethnic groups and the effects on cardiovascular health require close attention since most data show differences between ethnic groups. However, there is still a lack of data, a fact limiting strategies designed to improve cardiovascular health, especially in the nonwhite population[39-41].

The strengths of the present study include its duration of 90 days, which is relatively long when compared to other studies. We also observed high compliance of the participants as demonstrated by capsule count and plasma EPA and DHA concentrations. This study also evaluated the influence of race on the findings, a fact

Table III
Clinical trial results of supplementation of fish oil and vitamin E by ANCOVA

Dependent variables	*Fish*		*Fish + VitE*	
	Crude β *(95% CI)*	*Adjusted for ethnicity* β *(95% CI)*	*Crude* β *(95% CI)*	*Adjusted for ethnicity* β *(95% CI)*
Total Cholesterol (mg/dL)				
45 days	-5.107 (-19.288;9.074)	-5.172 (-19.526;9.182)	-15.651 (-32.944;1.642)	-15.564 (-33.099;1.972)
90 days	-8.153 (-28.308;12.001)	-7.714 (-27.361;11.933)	**-22.960 (-44.184;-1.736)**	**-22.473 (-43.312;-1.633)**
Triacylglycerols (mg/dL)				
45 days	-38.031 (-89.508;13.447)	-38.798 (-91.133;13.538)	-28.482 (-74.727;17.762)	-28.735 (-75.538;18.068)
90 days	-37.307 (-110.489;35.875)	-38.232 (-112.661;36.196)	-14.886 (-92.585;62.814)	-15.104 (-93.935;63.727)
HDL-C (mg/dL)				
45 days	3.208 (-2.488;8.903)	3.465 (-2.175;9.104)	-0.530 (-6.847;5.788)	-0.695 (-6.982;5.592)
90 days	4.458 (-4.770;13.686)	4.702 (-4.590;13.994)	0.754 (-9.692;11.200)	0.847 (-9.742;11.437)
LDL-C (mg/dL)				
45 days	-12.367 (-25.366;0.633)	-12.449 (-25.446;0.548)	-11.889 (-25.679;1.901)	-10.731 (-24.542;3.080)
90 days	-17.097 (-36.855;2.661)	-16.807 (-36.317;2.703)	**-30.993 (-51.784;-10.201)**	**-29.396 (-50.552;-8.240)**
Alpha-tocopherol (μmol/L)				
45 days	1.795 (-0.748;4.338)	1.914 (-0.567;4.394)	1.780 (-1.592;5.151)	1.908 (-1.129;4.945)
90 days	-0.067 (-5.561;5.426)	0.014 (-5.543;5.571)	-2.366 (-8.191;3.458)	-2.182 (-7.646;3.281)
TBARS (μmol/L)				
45 days	0.019 (-0.001;0.039)	0.019 (0.000;0.039)	0.005 (-0.016;0.026)	0.005 (-0.016;.026)
90 days	**0.045 (0.021;0.069)**	**0.045 (0.020;0.069)**	0.006 (-0.015;0.027)	0.006 (-0.015;0.027)
Autoantibodies anti-LDL- (μg/mL)				
45 days	-2.251 (-8.903;4.400)	-2.085 (-8.753;4.582)	**-6.572 (-11.882;-1.261)**	**-6.531 (-12.005;-1.057)**
90 days	5.105 (-1.478;11.688)	5.248 (-3.756;9.247)	0.547 (-7.974;9.067)	-0.640 (-9.106;7.827)

Data were presented in Beta and confidence interval of 95% (CI 95%) obtained by analysis of covariance (ANCOVA). TBARS: thiobarbituric acid reactive substances; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol.

reported in few studies. Furthermore, highly specific and sensitive methods, such as gas chromatography and HPLC, were used for analysis of the outcomes described.

The present study has some limitations such as the small number of participants. Nevertheless, we found a significant difference in some outcomes. Another limitation is the fact that the diet or physical activity of the participants during the intervention was not evaluated. However, we studied a low-income population which had low intake of fish and other sources of omega-3 fatty acids already at the beginning of the intervention. We therefore believe that no change occurred in the eating pattern during the intervention.

In view of their important role in oxidative stress and cardiovascular risk, the absence of biomarkers of inflammation would be a possible limitation of the study. However, previous studies could not obtain consistent results regarding the effect of omega-3 supplementation on plasma biomarkers of the acute-phase response[42,43], showing no dose-response effect[44].

Conclusion

Combined supplementation with fish oil and vitamin E reduced total cholesterol and LDL-C, but had opposite effects on oxidative stress compared to supplementation with fish oil alone. The results of this study support the hypothesis that α-tocopherol can protect LDL-C against oxidation and that supplementation reduces the levels of anti-oxidized LDL autoantibodies in nonwhite and white women with dyslipidemia transitioning through menopause.

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References

1. Wellons M, Ouyang P, Schreiner PJ, Herrington DM, Vaidya D. Early menopause predicts future coronary heart disease and stroke: the Multi-Ethnic Study of Atherosclerosis (MESA). *Menopause (New York, NY)* 2012;19(10):1081.
2. Tom SE, Cooper R, Wallace RB, Guralnik JM. Type and timing of menopause and later life mortality among women in the Iowa established populations for the Epidemiological study of the elderly (EPESE) cohort. *Journal of Women's Health* 2012;21(1):10-6.
3. Ebong IA, Watson KE, Goff Jr DC, Bluemke DA, Srikanthan P, Horwich T, et al. Age at menopause and incident heart failure: the Multi-Ethnic Study of Atherosclerosis. *Menopause* 2014;21(6):585-91.
4. Fryar CD, Hirsch R, Eberhardt M, Yoon S, Wright J. Hypertension, high serum total cholesterol, and diabetes: racial and ethnic prevalence differences in US adults, 1999-2006. *NCHS data brief* 2010(36):1-8.
5. Lloyd-Jones D, Adams R, Carnethon M, De Simone G, Ferguson TB, Flegal K, et al. Heart disease and stroke statistics—2009 update a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 2009;119(3):e21-e181.
6. Zhang T, Tsang W, Wijeysundera HC, Ko DT. Reporting and representation of ethnic minorities in cardiovascular trials: a systematic review. *American heart journal* 2013;166(1):52-7.
7. Brasil - Ministério da Saúde - DATASUS [homepage on the Internet]. Informações de Saúde (TABNET) - Taxa de mortalidade específica por doenças do aparelho circulatório [acess 12 dec 2014]. Available from: http://www2.datasus.gov.br/DATASUS/index.php
8. Bello N, Mosca L. Epidemiology of coronary heart disease in women. *Progress in cardiovascular diseases* 2004;46(4):287-95.
9. Silva IT, Mello A, Damasceno N. Antioxidant and inflammatory aspects of lipoprotein-associated phospholipase A2 (Lp-PLA2): a review. *Lipids Health Dis* 2011;10(170):1-10.
10. Grundtman C, Wick G. The autoimmune concept of atherosclerosis. *Current opinion in lipidology* 2011;22(5):327
11. Russo GL. Dietary n− 6 and n− 3 polyunsaturated fatty acids: from biochemistry to clinical implications in cardiovascular prevention. *Biochemical pharmacology* 2009;77(6):937-46.
12. de Oliveira Carvalho APS, Uehara SK, Netto JFN, Rosa G. Hypocaloric diet associated with the consumption of jam enriched with microencapsulated fish oil decreases insulin resistance. *Nutricion hospitalaria* 2014;29(5):1103-8.
13. Calder PC, Yaqoob P. Marine omega-3 fatty acids and coronary heart disease. *Current opinion in cardiology* 2012;27(4):412-9.
14. Tinkel J, Hassanain H, Khouri SJ. Cardiovascular antioxidant therapy: a review of supplements, pharmacotherapies, and mechanisms. *Cardiology in review* 2012;20(2):77-83.
15. Saremi A, Arora R. Vitamin E and cardiovascular disease. *American journal of therapeutics* 2010;17(3):e56-e65.
16. Riccioni G, D Orazio N, Salvatore C, Franceschelli S, Pesce M, Speranza L. Carotenoids and vitamins C and E in the prevention of cardiovascular disease. *International Journal for Vitamin and Nutrition Research* 2012;82(1):15-26.
17. Damasceno NR, Sevanian A, Apolinário E, Oliveira JM, Fernandes I, Abdalla DS. Detection of electronegative low density lipoprotein (LDL−) in plasma and atherosclerotic lesions by monoclonal antibody-based immunoassays. *Clinical biochemistry* 2006;39(1):28-38.
18. Esterbauer H and Cheeseman KH. Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal. In: Lester Packer ANG, editor. Methods in Enzymology: *Academic Press* 1990. p. 407-421.
19. Arnaud J., Fortis I., Blachier S., Kia D., Favier A. Simultaneous determination of retinol, a-tocopherol and B-carotene in serum by isocratic high-performance liquid chromatograph. *J Chromatogr B Biomed Appl* 1991;572:103-116.
20. Marmer WN, Maxwell RJ. Dry column method for the quantitative extraction and simultaneous class separation of lipids from muscle tissue. *Lipids* 1981;16(5):365-71.
21. Metcalfe L, Schmitz AA, Pelka J. Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. *Analytical chemistry* 1966;38(3):514-5.
22. Carrepeiro MM, Rogero MM, Bertolami MC, Botelho PB, Castro N, Castro IA. Effect of n-3 fatty acids and statins on oxidative stress in statin-treated hypercholestorelemic and normocholesterolemic women. *Atherosclerosis* 2011;217(1):171-8.

23. Higdon JV, Liu J, Du S-H, Morrow JD, Ames BN, Wander RC. Supplementation of postmenopausal women with fish oil rich in eicosapentaenoic acid and docosahexaenoic acid is not associated with greater in vivo lipid peroxidation compared with oils rich in oleate and linoleate as assessed by plasma malondialdehyde and F2-isoprostanes. *The American journal of clinical nutrition* 2000;72(3):714-22.
24. Higdon J, Du S, Lee Y, Wu T, Wander R. Supplementation of postmenopausal women with fish oil does not increase overall oxidation of LDL ex vivo compared to dietary oils rich in oleate and linoleate. *Journal of lipid research* 2001;42(3):407-18.
25. Wu W, Lu S, Wang T, Jou H, Wang T. Effects of docosahexaenoic acid supplementation on blood lipids, estrogen metabolism, and in vivo oxidative stress in postmenopausal vegetarian women. *European journal of clinical nutrition* 2006;60(3):386-92.
26. Ottestad I, Vogt G, Retterstøl K, Myhrstad MC, Haugen J-E, Nilsson A, et al. Oxidised fish oil does not influence established markers of oxidative stress in healthy human subjects: a randomised controlled trial. *British Journal of Nutrition* 2012;108(02):315-26.
27. Filaire E, Massart A, Rouveix M, Portier H, Rosado F, Durand D. Effects of 6 weeks of n-3 fatty acids and antioxidant mixture on lipid peroxidation at rest and postexercise. *European journal of applied physiology* 2011;111(8):1829-39.
28. Wander R, Du S, Ketchum S, Rowe K. alpha-Tocopherol influences in vivo indices of lipid peroxidation in postmenopausal women given fish oil. *The Journal of nutrition* 1996;126(3):643.
29. Meydani M, Natiello F, Goldin B, Free N, Woods M, Schaefer E, et al. Effect of long-term fish oil supplementation on vitamin E status and lipid peroxidation in women. *The Journal of nutrition* 1991;121(4):484-91.
30. O'Keefe Jr J, Lavie Jr C, McCallister B, editors. Insights into the pathogenesis and prevention of coronary artery disease. Mayo Clinic proceedings, 1995.
31. Baynes J & Dominiczak, M. Bioquímica Médica. São Paulo: *Manole* 2007 (2ed); 436p.
32. Toshima S-i, Hasegawa A, Kurabayashi M, Itabe H, Takano T, Sugano J, et al. Circulating oxidized low density lipoprotein levels a biochemical risk marker for coronary heart disease. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2000;20(10):2243-7.
33. Tinahones FJ, Gómez-Zumaquero JM, Garrido-Sánchez L, García-Fuentes E, Rojo-Martínez G, Esteva I, et al. Influence of age and sex on levels of anti-oxidized LDL antibodies and anti-LDL immune complexes in the general population. *Journal of lipid research* 2005;46(3):452-7.
34. Rossi GP, Cesari M, De Toni R, Zanchetta M, Maiolino G, Pedon L, et al. Antibodies to oxidized low-density lipoproteins and angiographically assessed coronary artery disease in white patients. *Circulation* 2003;108(20):2467-72.
35. Stephens NG, Parsons A, Brown M, Schofield P, Kelly F, Cheeseman K, et al. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *The Lancet* 1996;347(9004):781-6.
36. Franzoni MG, Santoro E, De Vita D, et al, on behalf of the GISSI Investigators. Ten-year follow-up of the first megatrial testing thrombolytic therapy in patients with acute myocardial infarction: results of the Gruppo Italiano per lo Studio della Sopravivenza nell'Infarto-1 study. *Circulation* 1998;98:2659–2665.
37. Bibbins-Domingo K, Pletcher MJ, Lin F, et al. Racial differences in incident heart failure among young adults. *N Engl J Med* 2009;360:1179-90.
38. Safford MM, Brown TM, Muntner PM, et al. Association of race and sex with risk of incident acute coronary heart disease events. *JAMA* 2012;308(17):1768-74.
39. Albert MA. Inflammatory biomarkers, race/ethnicity and cardiovascular disease. *Nutrition reviews* 2007;65(suppl 3):S234-S8.
40. Razzouk L, Muntner P. Ethnic, gender, and age-related differences in patients with the metabolic syndrome. *Current hypertension reports* 2009;11(2):127-32.
41. Stuart-Shor EM, Berra KA, Kamau MW, Kumanyika SK. Behavioral strategies for cardiovascular risk reduction in diverse and underserved racial/ethnic groups. *Circulation* 2012;125(1):171-84.
42. Balk EM, Lichtenstein AH, Chung M, Kupelnick B, Chew P, Lau J. Effects of omega-3 fatty acids on serum markers of cardiovascular disease risk: a systematic review. *Atherosclerosis* 2006;189(1):19-30.
43. Myhrstad MC, Retterstøl K, Telle-Hansen VH, Ottestad I, Halvorsen B, Holven KB, et al. Effect of marine n-3 fatty acids on circulating inflammatory markers in healthy subjects and subjects with cardiovascular risk factors. *Inflammation research* 2011;60(4):309-19.
44. Flock MR, Skulas-Ray AC, Harris WS, Gaugler TL, Fleming JA, Kris-Etherton PM. Effects of supplemental long-chain omega-3 fatty acids and erythrocyte membrane fatty acid content on circulating inflammatory markers in a randomized controlled trial of healthy adults. *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA)* 2014;91(4):161-8.