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Fluorometric assay of lipoperoxides and chromatographic analysis of α-tocopherol and fatty acids as biomarkers of risk from coronary atherosclerosis

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Abstract

There has been growing interest in the quantitative determination of biochemical predictors of atherogenesis. The aim of the present study was to investigate association of lipoperoxidation biomarkers known to be pro-atherogenic (thiobarbituric acid reactive substance activity, TBARS) or anti-atherogenic (α-tocopherol) with the fatty acid status, and relate it to the coronary artery disease (CAD) as assessed by coronary angiography in patients with stable angina pectoris. We found that serum lipoproteins and TBARS did not differ significantly. However there was significant correlation of TBARS with total vitamin E ($P = 0.02$) and vitamin E in very low-density lipoprotein (VLDL) ($P = 0.02$) and low-density lipoprotein (LDL) ($P = 0.01$), with LDL-linoleic acid ($P = 0.01$), and high-density lipoprotein-linoleic acid ($P = 0.02$). There was significant correlation of total vitamin E ($P = 0.01$) and VLDL-vitamin E ($P = 0.01$) with the degree of CAD. We conclude that TBARS and α-tocopherol could not be evaluated as biomarkers for the severity of CAD among the patients with stable angina pectoris.

Keywords: TBARS; α-Tocopherol; PUFA; Coronary atherosclerosis

1. Introduction

Oxidation of unsaturated lipids in the low-density lipoprotein (LDL) particle initiates a complex sequence of events that leads to the development of atherosclerotic plaque and resulting coronary artery disease (CAD) [1]. The typical LDL particle contains 2700 fatty acid molecules incorporated into several lipid classes; about half of these fatty acids are polyunsaturated fatty acids (PUFA) [2], which are very oxidation sensitive. The most prevalent antioxidant in LDL is vitamin E: each LDL contains five to nine vitamin E molecules along with smaller amounts of several other antioxidants (such as gamma-tocopherol and beta-carotene) that are present at less than

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one molecule in the average LDL particle. α-Tocopherol is considered to be the most biologically active form of vitamin E. It is an effective lipophilic antioxidant, which protects lipid membranes against peroxidation. α-Tocopherol is able to prevent cell damage by reaction with free radicals. There are several chromatographic methods for the determination of vitamin E in various matrix [3]. A comprehensive review on vitamin E analysis has already been done [4–9]. We used the high-performance liquid chromatography (HPLC) system coupled with diode array detector for the determination of α-tocopherol in this study; this procedure is described in another part of this paper.

If oxidation of LDL is a common theme in CAD, oxidized products from LDL might be a useful biomarker of risk from CAD. In a comparison of men with proven CAD and controls, the CAD patients had thiobarbituric acid reactive substance activity (TBARS) level higher in the LDL of the patients than the controls [10]. In the Kuopio atherosclerosis prevention study in Finland [11], TBARS values of LDL were among the strongest predictors of atherogenesis. Both higher peroxides and TBARS and relative lack of α-tocopherol in their LDL has been shown in unstable angina compared to controls [12].

There has been growing interest in the quantitative determination of biochemical predictors of atherogenesis. As we reported previously, the common methods for this determination include fluorescence spectrometry, HPLC, GC and spectrophotometry [13,14]. Currently available techniques are often based on the measurement of TBARS concentration, which is an indicator of the extent of lipid peroxidation. TBARS are formed during the oxidation of PUFAs. In spite of the above-mentioned studies several unresolved questions remain, namely the association of the degree of coronary atherosclerosis with the measures of oxidative stress and/or in vivo correlation of lipid peroxidation with the LDL PUFAs quantity. The data on the association between LDL oxidation and severity of clinical manifestations of CAD are contradictory. The findings depend on the stability or instability of angina pectoris [12,15–17].

In the present study an association of lipoperoxidation biomarkers known to be pro-atherogenic (TBARS activity) or anti-atherogenic (α-tocopherol) with the fatty acid status has been investigated, and related to the degree of CAD as assessed by coronary angiography. Thus the approach of our laboratory was to stratify the patients with coronary atherosclerosis based on the severity of arterial occlusion in order to raise the important question of targeting high-risk individuals that may need antioxidant protection.

2. Experimental

2.1. Materials and methods

2.1.1. Reagents

Methanol and *n*-hexane for preparing standard solutions were obtained from Merck (Darmstadt, Germany). Ethanol denaturised with 5% methanol for deproteinization procedure was from Lachema (Brno, Czech Republic) and distilled methanol for HPLC were at p.a. purity from Penta (Praha, Czech Republic. Helium 4.6 and nitrogen 6.0 were from Linde (Praha, Czech Republic). Stock solution of α-tocopherol (500 μmol l^{-1}) was prepared in *n*-hexane. Working standard solution of α-tocopherol was prepared from stock solution by dilution with methanol to 20 μmol l^{-1} in volumetric flask and stored at 4 °C. A stability of stock and working solutions was 6 months.

2.1.2. Apparatus and software

The HPLC instrumentation was a Perkin Elmer (Norwalk, USA) composed of a Series LC 200 pump, Series LC 200 autosampler, LC Column Oven 101 and LC 235C Diode Array Detector attached to the Perkin Elmer Turbochrom Chromatography Workstation version 4.1. We used 150×4.6 mm reverse phase column of 5 μm Pecosphere C18 Perkin Elmer (Norwalk, USA). Centrifuge 400 R (Heraeus, Hanau, Germany) was used for centrifugation of blood samples.

2.2. Procedures

2.2.1. Sample preparation

Blood samples were drawn from the peripheral vein after 12 h overnight fasting. The samples were centrifuged immediately ($1300 \times g$, 10 min). After separation the serum was stored at -85 °C or the extracts were prepared immediately. 500 μl of serum was deproteinized by cool ethanol (500 μl, 5 min) denaturised with 5% methanol. In the liquid–liquid extraction (LLE) procedure, 2500 μl of *n*-hexane was added to 500 μl of serum and this mixture was extracted 5 min by Vortex. After centrifugation (10 min, $1600 \times g$) the aliquot (2000 μl) of clean extract was separated and evaporated under nitrogen. The residue was dissolved in 400 μl methanol and analyzed by reversed-phase HPLC.

2.2.2. HPLC procedure

HPLC separation was carried out using methanol as a mobile phase at a flow rate of 1.2 ml min^{-1} in ambient temperature. Injection volume of the sample was 100 μl and the eluent was monitored at 295 nm.

2.3. Subjects

Eighty hyperlipidemic patients (age 33–74 years) who underwent an elective coronary angiography for CAD, were divided into four groups based on the severity of CAD (group 1 = luminal narrowing $< 50\%$, $n = 21$; luminal narrowing of 50% or more is defined as a significant lesion, and the patients are referred to as having one (group 2, $n = 15$), two (group 3, $n = 27$) or three vessel disease (group 4, $n = 17$). Patients were counseled to follow the diet, which limits dietary cholesterol to < 300 mg day, saturated fats to $< 10\%$ of total calories, and total fats to $< 30\%$ of total calories. Furthermore, patients were treated with hypolipidemic drugs (statins, resins or fibrates). Blood samples were drawn before coronary angiography. All subjects gave informed consent to participate in the study and the local ethical committee, Charles University, Teaching Hospital, Hradec Králové, Czech Republic, accepted the protocol of the study.

2.4. Spectrofluorimetric method for TBARS determination

After collection the samples were centrifuged, 10 min, $1400 \times g$ (Hereaus, Germany), serum was separated and frozen in -25 °C before analyses. 20 μl of serum was analyzed after extraction with *n*-butanol treatment with thiobarbituric acid; the modified method of Yagi [18] was used. Thiobarbituric reactive substances (TBARS) were determinated spectrofluorometrically (excitation wavelength 528 nm, emission wavelength 558 nm) using LS—5-luminescence spectrometer (Perkin Elmer, Norwalk, USA).

2.5. HPLC analysis of α-tocopherol and retinol

Vitamin E (α-tocopherol) and vitamin A (retinol) were analyzed by reversed-phase HPLC technique (LC 200, Perkin Elmer, Norwalk, USA). The mobile phase 100% methanol, flow rate 1.2 ml/min, column Pecosphere 5 C_{18} RP, 4.6×150 mm; 5 μm (Perkin Elmer, Norwalk, USA) were used. The vitamins were detected simultaneously with diode-array detector at 325 and 290 nm for vitamin A and vitamin E, respectively, after extraction with *n*-hexane (Perkin Elmer, Norwalk, USA) [19,20].

2.6. Density gradient ultracentrifugation of lipoproteins

Serum lipoprotein fractions were prepared by density gradient ultracentrifugation (Beckman TL 100, Palo Alto, CA). The lipoprotein fractions were distinguished as very low-density lipoprotein (VLDL) < 1.006 g ml^{-1}; intermediate density lipoprotein (IDL) < 1.019 g ml^{-1}; LDL < 1.063 g ml^{-1}; high-density lipoprotein (HDL) > 1063 g ml^{-1} [21].

2.7. Assay of cholesterol and triacylglycerols

Total concentration and/or lipoprotein fraction concentration of cholesterol [22] and triacylglycerols [23] were assessed enzymatically by conventional diagnostic kits (Lachema, Brno, Czech Republic) and spectrophotometric analysis (cho-

lesterol at 510 nm wavelength, triacylglycerols at 540 nm wavelength) (ULTROSPECT III, Pharmacia LKB Biotechnology, Uppsala, Sweden).

2.8. Gas chromatography (GC) analysis of fatty acids

Free fatty acids (FFA) in serum, including saturated fatty acids (SUFA), monounsaturated fatty acids (MUFA) and PUFA, and FA of lipoprotein fractions were measured using capillary GC. Plasma free fatty acids were extracted from 0.5 ml of plasma with 0.5 ml of 0.9% NaCl. 5 ml of extraction mixture [isopropanol–heptane–0.5 M H_2SO_4, 400:100:10 (v/v/v)] with 3 ml of internal standard (heptadecanoic acid) was added according to modified Dole method [24]. Lipids from lipoprotein fractions were extracted using extraction mixture [*n*-hexane–isopropanol, 3:2 (v/v)] with 0.1% butylated hydroxytoluene (BHT) following Bligh and Dyer [25]. The fatty acid methyl esters were then formed by heating for 30 min with BF_3 [boron trifluoride–methanol complex, in excess methanol (CA. 12 wt.% BF_3)]/methanol. Profiling of fatty acid-methyl esters (FAME) was performed by Hewlett–Packard gas chromatograph 5890 II (Palo Alto, USA) equipped with flame ionization detector and HP 3396 A integrator. FAME were separated on a SP-2330 Fused Silica Capillary Column 30 × 0.25 mm i.d., 0.20 μm film (Supelco, Inc., Bellafonte, USA). Injection port and FID temperatures were both 250 °C. Oven temperature was programmed to change from 120 to 230 °C at 4 °C min^{-1}. Helium was used as the carrier gas, splitting ratio was 1:100. Individual fatty acids were identified by relative retention times determined with known standards.

2.9. Statistical analysis

Results are expressed as the mean ± SEM for normally distributed data, and as the median and range for skewed data. Differences between groups were tested using the One-Way analysis of variance. Correlation between variables was assessed using the Pearson correlation test. Differences were considered significant at P less than 0.05. Analyses were performed with procedures available in SPSS (Sigma Stat 2.03, San Rafael, USA, 1997).

3. Results and discussion

The most developed theory for the etiology of atherosclerosis and CAD proposes that the oxidation of the LDL particle plays a key role [1]. As a measure of oxidative stress in the patients with CAD, blood levels of the following parameters are being evaluated: (i) blood lipids, (ii) TBARS, and (iii) vitamin E.

3.1. Serum lipoproteins

Serum lipoproteins, i.e. total cholesterol, VLDL cholesterol, intermediate-lipoprotein-density (IDL) cholesterol, low-density-lipoprotein (LDL) cholesterol and high-density-lipoprotein (HDL) cholesterol, nor the total triacylglycerols and triacylglycerols in VLDL, IDL, LDL and HDL did not differ significantly between groups 1, 2, 3 or 4 (Table 1). Moreover, the lipoprotein concentrations approached the target values, recommended by recent authorities [26]. These results documented well-established control of hyperlipidemia using hypolipidemic drugs.

3.2. Serum TBARS and PUFAs

Methods of investigating LDL oxidation generally evaluate the oxidability of isolated LDL in the presence of a pro-oxidant during enough time to promote lipid peroxidation. Currently available techniques are based on the measurement of thiobarbituric acid-reactive substances (TBARS) concentration, which is an indicator of the extent of lipid peroxidation. In these methods the red fluorescent product is detected by spectrophotometry or fluorometry. The main product of fatty acid reaction with free radicals, malondialdehyde, reacts with thiobarbituric acid to give a product with fluorescence at 553 nm. Event though TBARS determination lacks specificity and there is great variability among all the reported techniques, it is still of value when studying purified

Table 1
Cholesterol and triacylglycerols in serum and lipoproteins

Variable (mmol l^{-1})	Group 1		Group 2		Group 3		Group 4	
	n	Mean (±SEM)	*n*	Mean (±SEM)	*n*	Mean (±SEM)	*n*	Mean (±SEM)
Total cholesterol	21	5.32(0.21)	15	5.57(0.28)	27	5.24(0.23)	17	5.44(0.15)
VLDL cholesterol	21	1.16(0.07)	15	1.32(0.11)	27	1.23(0.07)	17	1.41(0.07)
IDL cholesterol	21	0.88(0.04)	15	0.99(0.09)	27	0.86(0.06)	17	0.93(0.04)
LDL cholesterol	21	2.12(0.12)	15	2.11(0.18)	27	2.08(0.12)	17	2.06(0.08)
HDL cholesterol	21	0.98(0.04)	15	1.02(0.08)	27	0.96(0.05)	17	0.90(0.04)
Total triacylglycerols	21	1.53(0.10)	15	1.85(0.19)	27	1.60(0.19)	17	1.82(0.14)
VLDL triacylglycerols	21	1.02(0.09)	15	1.27(0.18)	27	1.08(0.15)	17	1.25(0.11)
IDL triacylglycerols	21	0.23(0.01)	15	0.27(0.03)	27	0.27(0.02)	17	0.31(0.04)
LDL triacylglycerols	21	0.14(0.02)	15	0.15(0.02)	27	0.17(0.03)	17	0.19(0.04)
HDL triacylglycerols	21	0.08(0.02)	15	0.05(0.01)	27	0.08(0.01)	17	0.10(0.03)

Serum lipoproteins, i.e. total cholesterol, VLDL cholesterol, IDL cholesterol, LDL cholesterol, high-density-lipoprotein (HDL) cholesterol and total triacylglycerols. Eighty hyperlipidemic patients (age 33–74 years) who underwent an elective coronary angiography for CAD, were divided into four groups based on the severity of CAD (group 1 = luminal narrowing $< 50\%$, $n = 21$; luminal narrowing of 50% or more is defined as a significant lesion, and the patients are referred to as having one (group 2, $n = 15$), two (group 3, $n = 27$) or three vessel disease (group 4, $n = 17$). Results in mmol l^{-1} were expressed as mean ± SEM. Significance was established if the P-value was less than 0.05; presented differences were not significant between groups.

systems like isolated lipoproteins. Thus, it is necessary to choose the most adequate technique for measuring TBARS [27]. In this study we used the method of Yagi [18] for the determination of TBARS—marker of lipid peroxidation and oxygen free radicals-induced damage.

Numerous theories have been proposed to explain the pathology of coronary atherosclerosis over the past decade and evidence has accumulated that among the theories, cellular and tissue damage by oxygen free radicals is gaining prominence, and oxidative stress is thought to play an important role in the pathogenesis of disorders associated with atherosclerosis [28–30]. Oxidative modification of LDL is important, and possibly obligatory, in the pathogenesis of atherosclerosis [31].

As an index of peroxidation of plasma lipids, we measured TBARS levels in the plasma. This approach has been criticized for its lack of specificity and reproducibility. There are many other parameters for determination of free radical damage (deoxyribonucleic acid degradations, superoxide dismutase, glutathione peroxidase and other enzymes, levels of carotenoids and others). However, evaluation of TBARS is one of the easiest and most frequently used tests for measuring lipid peroxidation, and TBARS is often assumed to be proportional to lipid peroxide-derived malonyldialdehyde, especially when all samples are assayed in one batch [15]. Therefore we have chosen the assay of TBARS that is routinely used in our laboratory also in our project.

It has been shown that oxidative stress contributes to restenosis and indicates that an elevated plasma level of TBARS may be a reliable predictor of restenosis. The plasma level of TBARS positively correlated with lumen loss of the coronary artery at the time of follow-up angiography [32]. Erythrocyte TBARS production was significantly higher in patients with coronary atherosclerosis than in the controls suggesting that erythrocytes from patients with coronary atherosclerosis are more susceptible to oxidation than those of controls [33].

If the oxidation hypothesis of atherosclerosis is right and oxidized LDL is an important factor in atherogenesis, which circulating levels may be higher in patients suffering from CAD [17]. Furthermore, one might expect an association between elevation of peroxidation of plasma lipids, oxidized LDL, TBARS and anatomical severity of disease (number of involved arteries, degree of

vessel narrowing, etc.). Thus the approach of our laboratory was to stratify the patients with coronary atherosclerosis based on the severity of arterial occlusion, measured by angiography (see Section 2.3) and to study correlation with the above mentioned oxidative stress markers. This would raise the important question of targeting high-risk individuals that may need antioxidant protection.

In our study, serum TBARS did not differ significantly between groups 1, 2, 3 or 4 (Table 2), and did not correlate with the degree of CAD. Thus TBARS was not an exact parameter of lipid peroxidation in stable angina pectoris. These results are in contrast with significantly higher TBARS in the group of patients with unstable angina, who were compared to stable angina or controls [12]. Elevated TBARS in unstable angina patients were reported also by others [15]. One of the main reasons for this contradiction may be the difference in the patient groups. Multiple brief myocardial ischemic episodes, as ischemia–reperfusion cycles are known to be associated with the production of free radicals, and, hence, the consumption of the chain-breaking antioxidants of plasma and elevation of plasma TBARS [16]. Therefore asymptomatic patients with stable angina in our study, and in the protocols of others did not reveal differences in oxidative parameters between CAD patients with stable angina pectoris and controls [15,17]. Another explanation would be reduced oxidative stress caused by the effect of well-established control of hyperlipidemia using hypolipidemic treatment in our patients.

PUFAs with a high degree of unsaturation of the n-6 and n-3 series could accelerate cell-mediated LDL peroxidation and thus aggravate the atherosclerotic process [34]. In our study, LDL-PUFAs did not statistically differ significantly between groups 1, 2, 3 or 4. However, there was significant correlation of TBARS with LDL-linoleic acid ($P = 0.01$) (Fig. 1), and HDL-linoleic acid ($P = 0.02$). As TBARS are proportional to lipid peroxide-derived malonyldialdehyde and are a measure of lipid peroxidation, the above findings relate to the oxidation hypothesis of coronary atherosclerosis.

3.3. Vitamin A and vitamin E in lipoproteins

Since lipid-soluble vitamins A and E are important antioxidants, we analyzed their serum content. The differences in vitamin A and vitamin E were not statistically different between groups 1, 2, 3 or 4 (Table 2). The hypothesis that links vitamin E and cardiovascular diseases posits that the oxidation of unsaturated lipids in the LDL particle, as well as the complex sequelae that flow

Table 2
TBARS, vitamin A and vitamin E in serum and lipoprotein fractions

Variable (μmol l^{-1})	Group 1		Group 2		Group 3		Group 4	
	n	Mean (±SEM)	*n*	Mean (±SEM)	*n*	Mean (±SEM)	*n*	Mean (±SEM)
TBARS	21	3.36(0.20)	15	3.31(0.22)	27	3.56(0.16)	16	3.03(0.16)
Total vitamin E	21	23.01(1.17)	15	23.97(1.35)	27	25.46(1.08)	17	27.20(1.81)
VLDL vitamin E	21	7.41(0.65)	15	8.48(0.89)	27	8.61(0.62)	17	10.76(1.30)
IDL vitamin E	21	3.90(0.26)	15	4.63(0.32)	27	4.61(0.33)	17	4.56(0.30)
LDL vitamin E	21	6.84(0.52)	15	7.63(0.47)	27	7.73(0.54)	17	7.36(0.39)
HDL vitamin E	21	4.60(0.30)	15	4.75(0.32)	27	5.51(0.51)	17	4.63(0.39)
Total vitamin A	21	2.29(0.19)	15	2.31(0.19)	27	2.26(0.12)	17	2.21(0.19)

Serum TBARS, total vitamin E, and vitamin E in lipoprotein fractions: VLDL-vitamin E, IDL-vitamin E, LDL-vitamin E, HDL-vitamin E. Eighty hyperlipidemic patients (age 33–74 years) who underwent an elective coronary angiography for CAD, were divided into four groups based on the severity of CAD (group 1 = luminal narrowing $< 50\%$, $n = 21$; luminal narrowing of 50% or more is defined as a significant lesion, and the patients are referred to as having one (group 2, $n = 15$), two (group 3, $n = 27$) or three vessel disease (group 4, $n = 17$). Results were expressed as mean ± SEM. Significance was established if the P-value was less than 0.05; however, the differences were not statistically significant.

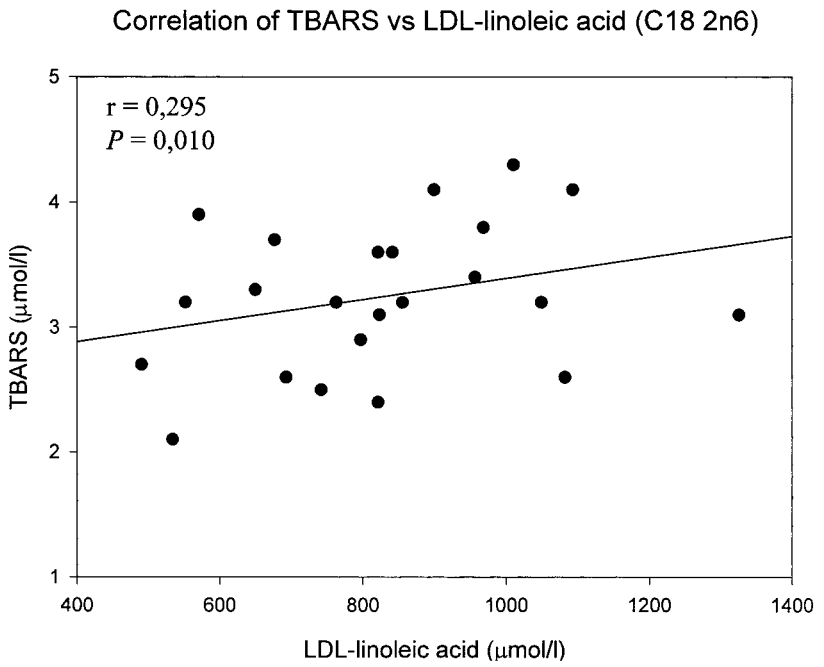

Fig. 1. Correlation of serum TBARS with LDL linoleic acid. Hyperlipidemic patients (age 33–74 years) who underwent an elective coronary angiography for CAD were examined. Blood samples were drowned before coronary angiography. Results are expressed as mean ± SEM. P-value of less than 0.05 was considered to be significant.

from this oxidation, play a crucial role in the pathogenesis of atherosclerosis. In our study, there was significant correlation of total vitamin E ($P = 0.01$) and VLDL-vitamin E ($P = 0.01$) with the degree of CAD (Table 3). In that respect it is noteworthy, that Dieber-Rotheneder et al. [35] demonstrated that the vitamin E content in LDL and not in whole plasma correlated with the susceptibility of LDL for oxidation. This probably relates to the fact that vitamin E distributes in plasma among all lipoproteins and the lipid free bottom fraction. Moreover, we found significant

Table 3
Correlation of coronary atherosclerosis, TBARS, vitamin A and vitamin E in lipoprotein fractions

Variable	Coronarography			TBARS		
μmol l	N	r	P	n	r	P
Vitamin A	78	−0.060	0.599	77	0.186	0.106
Vitamin E	78	0.248	0.012	77	0.264	0.020
VLDL vitamin E	78	0.287	0.011	77	0.256	0.025
IDL vitamin E	78	0.210	0.065	77	0.192	0.094
LDL vitamin E	78	0.136	0.234	77	0.297	0.009
HDL vitamin E	78	0.031	0.787	77	0.009	0.937
Coronarography	–	–	–	79	0.074	0.518

Correlation of the coronarography (Coronarography), based on the luminal narrowing, and/or TBARS with the serum vitamin A, total vitamin E and vitamin E in VLDL cholesterol, IDL cholesterol, LDL cholesterol and HDL cholesterol. The patients underwent an elective coronary angiography for CAD. Significance was established if the P-value was less than 0.05; n = number of patients, r = correlation coefficient.

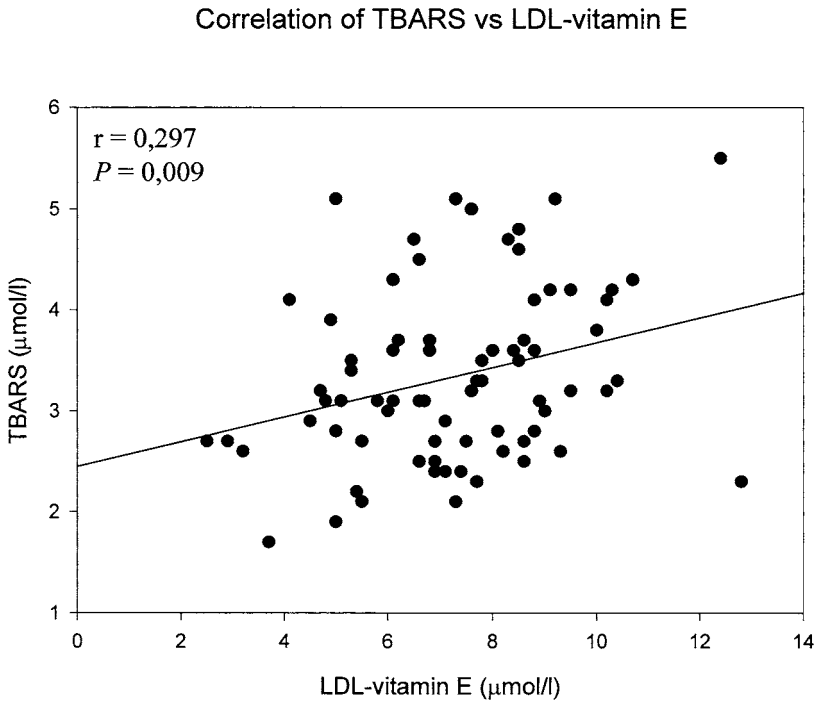

Fig. 2. Correlation of serum TBARS with LDL vitamin E. Hyperlipidemic patients (age 33–74 years) who underwent an elective coronary angiography for CAD were examined. Blood samples were drowned before coronary angiography. Results are expressed as mean ± SEM. P-value of less than 0.05 was considered to be significant.

correlation of TBARS with total vitamin E ($P = 0.02$) and vitamin E in VLDL ($P = 0.02$) (Table 3) and LDL ($P = 0.01$) (Fig. 2). Frei et al. also found that the susceptibility of LDL for oxidation correlates with the vitamin E content in LDL [36]. Others calculated the amount of LDL α-tocopherol and described it significantly lower in CAD patients as compared to controls [12].

In conclusion, the results of this study did not show difference in lipid peroxidation status between asymptomatic patients with stable angina pectoris. Both TBARS and α-tocopherol could not be evaluated as biomarkers for the severity of CAD in patients with stable angina pectoris. Proatherogenic lipoperoxidation, as revealed by TBARS, was not associated with the severity of CAD, which may be explained also by the effect of well-established control of hyperlipidemia using hypolipidemic treatment. Oxidation hypothesis of atherosclerosis has been supported by significant correlation of TBARS with LDL-linoleic acid and HDL-linoleic acid, and negative impact of CAD was compensated by antioxidation defense, namely total α-tocopherol and its VLDL- and LDL fraction.

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