Increased Serum Titers of Autoantibodies Against Oxidized LDL Cholesterol in Young Healthy Adults: An Evidence of a Protective Effect of These Antibodies?

Ilias K. Karabinos[1], Spyridon Koulouris[1], Aggeliki Melpidou[2], Georgios Makris[1], Athanasios Kranidis[1], Nikolaos Papageorgakis[2], Ilias Moshovitis[1], Christos Kalofoutis[3], Zerar Zoulien[2], Nikolaos Exadaktylos[1], Anastasios Kalofoutis[3]

[1]1st Cardiology Dept. and [2]Biochemical Dept. of "Evangelismos Hospital" Athens, Greece, [3]Department of Biological Chemistry, Medical School University of Athens, Greece

Key words:
Autoantibodies, oxidized LDL, atherosclerosis, oxidative stress.

Introduction: Oxidative modification of LDL represents a crucial step in the initiation and development of atheromatic plaque. Autoantibody titers against oxidized LDL (aOxLDL) has been extensively used in many studies as an index of the degree of oxidation of LDL. Accumulating clinical and experimental data suggest that autoantibodies against oxidized LDL may exert a protective antiatheromatic role. The aim of our study was to assess aOx-LDL in young and middle aged healthy adults and search for any correlation with age and lipid profile.

Methods: We studied 243 healthy individuals, aged 17 to 78 years. Study population was composed of two groups: the group of young healthy adults (group A) aged 23.1±4.7 years (N=96) and the group of middle aged adults without clinical evidence of severe atherosclerosis (group B) aged 55.6±10.02 years (N=147). All study population was blood sampled so as to assess aOxLDL and standard parameters of lipid profile. aOx-LDL (mU/ml) were evaluated using an ELISA. Adults in group B had a transthoracic echocardiogram and a stress test to exclude at least severe coronary atherosclerosis.

Results: We found that group A had higher aOx-LDL titers compared to group B (592±490 vs 456±366 respectively, p=0.02). Group B had higher serum levels of cholesterol, triglycerides, LDL, HDL (paradoxically) and apoA , apoB compared to group A. We identified a statistical significant correlation only between aOx-LDL and both LDL (r=0.28, p=0.01) and apoA (r=-0.23, p=0,04) in group A. Multivariate linear regression analysis revealed that serum levels of LDL predicts aOx-LDL serum titers, independently from age, sex, smoking, hypercholesterolemia, obesity, family history of coronary artery disease, and lipid parameters.

Conclusions: Higher aOx-LDL serum titers in young healthy adults - who actually have little atherosclerosis- compared to middle aged individuals are suggestive of a protective antiatheromatic role for aOx-LDL. The independent positive correlation between aOx-LDL and LDL only in the group of young healthy adults suggests that aOx-LDL may exert this protective role during young age only, and its effectiveness may be diminished as the years pass.

Manuscript received:
November 14, 2002;
Accepted:
September 25, 2003.

Address:
Ilias K. Karabinos

17 Solomou St., 111 47, Galatsi, Athens, Greece
e-mail:
ilias.karabinos@mailcity.com

It is widely accepted nowadays that oxidative modification of low density lipoprotein (LDL) represents a significant parameter in the atheromatous plaque formation. Oxidative modification of LDL takes place in two stages. In the first stage (stage of mild oxidation), lipids of LDL, mainly various pho-spholipids such as phosphatidylcholine, are oxidized without any transformation of the molecular structure of apolipoprotein B-100 (apo-B). In the second stage (stage of advanced

oxidation) lipids of LDL are further oxidized , while a simultaneous transformation of apo B-100 takes place. As a consequence, oxidized LDL is recognized by a particular group of receptors, the genetic expression of which is not regulated by intracellular levels of cholesterol, thus causing an increased uptake of oxidized LDL by subendothelially lying macrophages and further acceleration of "foam cells" formation.

Furthermore, oxidized LDL exerts on one side a chemotactic action on monocytes, by attracting these inflammatory cells on lesion sites, and on the other side a chemostatic action on macrophages, by impeding their entrance back to circulation (thus facilitating atherosclerotic process)[1]. Moreover, oxidized LDL induces adhesion of leukocytes (mainly monocytes) on endothelium in vivo[2], parallel to an enhanced vasoconstrictive action as a result of a reduced nitric oxide (NO) activity, which constitutes the main vasodilatory molecule produced by endothelium. Probably oxidised LDL promotes smooth muscle cells proliferation, parallel to a direct cytotoxic action as well. Finally, it seems that oxidized LDL facilitates intravascular thrombus formation by inhibiting production of prostacyclin by endothelial cells and inducing production of plasminogen activator's inhibitor-1 (PAI-1)[3].

However, besides their atherogenic effects, oxidized LDL particles possess parallel antigenic properties, thus inducing a corresponding antibodies production. Various recent studies have raised the concept that immune response to oxidized LDL may contribute to the pathogenetic process of atherosclerosis. It is true that many inflammatory cells , such as macrophages, T and B-lymphocytes, are placed inside atheromatous plaques, usually in an activated condition. It seems that some clones of T-lymphocytes are activated by oxidized LDL.

Serum titers of autoantibodies against oxidized LDL (aOxLDL) has been assessed in various studies, which included patients with severe atherosclerotic disease. Autoantibody titers against oxidized LDL was increased in patients with severe atheromatosis of coronary arteries[4], carotid arteries[5,6] and peripheral arteries[7]. It has also been found that serum titers of these autoantibodies are predictive of the progression of atherosclerosclerosis in carotids, and the outcome of a myocardial infarction. However, other studies have not confirmed these results. Moreover, reduced serum titers of autoantibodies against oxidized LDL have been documented in patients with familial hypercholesterolemia in a recent study. Very few studies have investigated autoantibody titers against oxidized LDL in a healthy population. These study populations consisted of middle-aged individuals, while in none of these studies were autoantibody titers against oxidized LDL assessed in young healthy adults.

The aim of our study was to compare serum titers of autoantibodies against oxidized LDL between two groups of general population: the group of young healthy adults and the group of middle aged healthy adults.

Subjects-Methods

Study Population

The population of our study included 243 healthy volunteers, of both sexes and a wide distribution of frequencies of age. The subjects of our study belonged into one of two groups: the group of young healthy adults aged <35 years (Group A, N=96, 30 males and 66 females, mean age 23.1±4.7 years) and the group of middle aged healthy adults aged >35 years (Group B, N=147, 56 males and 91 females, mean age 55.6±10.2 years). Group A completely consisted of students of Nursing School of "Evangelismos" Hospital (inclusion criterion in our study was age<35 years) while group B included healthy volunteers from personnel of the same hospital (inclusion criterion in our study was age>35 years).

Exclusion criteria in our study were the presence of known history of coronary artery disease, valvular disease, heart failure or other chronic disease (chronic renal failure, connective tissue disorder, hematological disease, chronic obstructive respiratory disease), diabetes mellitus and hypertension. The probability of a subclinical form of coronary artery disease in individuals of group B was very low, as all of them had a negative treadmill stress test (Bruce protocol).

Detailed medical history was obtained from all subjects of our study and the presence of any of the rest conventional coronary artery disease risk factors, such as hypercholesterolemia, smoking, family history of coronary artery disease and obesity was recorded as well. Individuals were considered to have hypercholesterolemia if they had a known history of the disorder documented by previous measurements, or if they were on a lipid-lowering drug therapy or if they were proved to have severe hypercholesterolemia (serum levels of cholesterol , assessed in our laboratory, >240 mg/dl). Obesity was

defined as evaluated Body Mass Index>30 kgr/m^2. Finally, family history of coronary artery disease was considered positive if there was a first degree relative (a parent, or a brother or a sister) with documented coronary artery disease aged <55 years regarding males, and <65 years regarding females.

Biochemical analyses

Venous blood samples were drawn from all individuals of our study, after 12 hours fasting and centrifuged at 4000 × g for 8 minutes. The separated serum was then stored and frozen at –80°C, until analysis was performed. Full lipid profile, as well as serum titers of autoantibodies against oxidized LDL, were assessed in a later time.

Total cholesterol, triglycerides and HDL cholesterol were measured with an automatic assay system using an enzymatic colorimetric method, while LDL cholesterol serum levels were evaluated using Friedwald's formula (if triglycerides serum levels were <400 mg/dl). In more details, the determination of total cholesterol was based on the complete enzymatic method, with reactions catalysed by cholesterol esterase and cholesterol oxidase (reagents by ROCHE co.). HDL cholesterol serum levels were determined applying the same method, after modification of the above mentioned enzymes by polyethylene glucole (PEG) and dextrane sulphate. Triglycerides measurement was based on their saponification by the enzymes lipoprotein lipase, glycerole kinase and 3-phosphate glycerole oxidase (reagents by ROCHE co.). End product of all reactions in the three mentioned biochemical methods is hydrogen superoxide (H_2O_2), which by taking part in a reaction catalyzed by a peroxidase (Trinder reaction), changes the color of a buffer (reagent by ROCHE co.). Finally, the color density of this buffer following Trinder reaction was determined with the use of a special spectrophotometric reader, thus reflecting hydrogen superoxide concentration and consequently serum levels of total cholesterol, HDL cholesterol and triglycerides respectively.

Serum levels of apolipoprotein A-I (apo A-I), apolipoprotein B (apo B) and lipoprotein a (Lp(a)) were quantitatively determined using an automated immunonephelometric system. We used special liquid animal antisera (reagents by DADE BEHRING co., produced by immunization of rabbits) to achieve binding of apolipoproteins A-I and B, and a following corresponding immunocomplexes formation. These immunocomplexes cause scattering of a beam of light passing through the sample, a property which constitutes the basic principle of this method. The intensity of the scattererd light is proportional to the concentration of immunocomplexes and consequently to the corresponding concentration of apolipoprotein A-I and B. The intensity of scattered light was measured with a nephelometer. Assessment of serum levels of lipoprotein a was made according to the same method, except that the binding of lipoprotein was performed by a solution of polysteryl microparticles coated with monoclonal antibodies against lipoprotein, instead of liquid animal antiserum.

Serum titers of IgG-autoantibodies against oxidized LDL were determined with a sandwich enzyme-linked immunosorbent assay (ELISA) kit purchased from Biomedica Gesellschaft. According to this method, copper oxidized LDL (Cu^{++} oxidized LDL) was coated onto microtiter strips as antigen, which bound with IgG autoantibodies against oxidized LDL of 50μl dropped serum. The coated strip was immersed in buffer containing antihuman IgG, so as to detect bound autoantibodies. The concentration of IgG autoantibodies in the sample was quantified by an enzyme-catalyzed color change, detectable with a standard spectrophotometric ELISA reader in mU/ml. The whole assay was standardized with defined increased titers of autoantibodies against oxidized LDL.

Statistics

Statistical analysis of the data of our study was performed with the use of statistic software SPSS10.0. P values less than 0.05 were considered statistically significant. We must note that SPSS can estimate p values not less than one per thousand, so probabilities of lesser values are generally depicted in SPSS as 0.000. In such cases in our study we simply denoted this as p<0.0009.

Results

Characteristics of the study population

Our study aimed at comparing serum titers of autoantibodies against oxidized LDL between young and middle aged healthy adults. Although subjects of all ages were included in our study, it is remarkable that most of the individuals of group A were 18-25 years

Table 1. Characteristics of qualitative variables of the two groups of the study population.

	GROUP A N=96	GROUP B N=147	
Sex	F=66 M=30	F=91 M=56	X^2=1,38 p=0,24
Smoking	30	29	X^2=1,82 p=0,17
Hypercholesterolemia	2	59	X^2=49,5 p<0,0009
Family history of coronary artery disease	10	31	X^2=3,7 p=0,052
Obesity	0	9	X^2=5,2 p=0,02

F=Female, M=Male.

old (74 out of 96 subjects), while most of the individuals of group B were 45-65 years old (116 out of 147 subjects). From this point of view, age as a continuous variable of our study was not of normal distribution.

It is also noteworthy that most of the subjects included in our study were females: totally 156 (64.6%) versus 85 (33.4%) males. Both groups were sex-matched, although females were much more than males in both groups (X^2=1.38 p=0.24) (Table 1). Moreover, both groups consisted of similar percentages of smokers, so that the two groups were matched regarding this coronary artery disease risk factor (X^2=49.5 p<0.0009). Group A included 2 subjects with hypercholesterolemia, versus 59 that were included in group B (X^2=5.2 p=0.02). There were no subjects with severe obesity in group A, compared to 9 obese individuals that were included in group B (X^2=5.2 p=0.02). Finally, there was a borderline statistically significant difference between the two groups of our study population, regarding family history of coronary artery disease: 10 subjects in group A versus 31 in group B (X^2=3.7 p=0.052).

Regarding lipid profile (Table 2), subjects of group B compared to subjects of group A, had higher cholesterol serum levels (236±45 mg/dl vs 184±28 mg/dl respectively, p<0.0009), higher triglycerides serum levels (127±64 mg/dl vs 66±44 respectively, p<0.0009), higher LDL cholesterol serum levels (157±41 mg/dl vs 125±23 mg/dl respectively, p<0.0009), higher HDL cholesterol serum levels (52±14 mg/dl vs 46±10 mg/dl respectively, p=0.001), higher apo A-I serum levels (152±23 mg/dl vs144±21mg/dl respectively, p=0.02), and higher apo B serum levels (123±29 mg/dl vs 85±20 mg/dl respectively, p<0.0009). No difference between the two groups of subjects regarding Lp(a) serum levels was identified (20±25 mg/dl vs 15±17 mg/dl respectively, p=0.22).

Serum titers of autoantibodies against oxidized LDL

Subjects of group A had statistically significant higher serum titers of autoantibodies against oxidized LDL, compared to those of group B (592±490 mU/ml vs 456±366 mU/ml respectively, t=2.3 p=0.02). Serum titers of autoantibodies against oxidized LDL in group A, was not different between males and females - although males had higher titers compared to females,

Table 2. Characteristics of quantitative variable of the two groups of the study population (age, lipid-profile, autoantibodies against oxidized LDL).

	Group A N=96		Group B N=147		t, p
	Range	Mean values ± standard deviation	Range	Mean values ± standard deviation	
Age (years)	17-35	23.1±4.7	36-75	55.6±10.2	
aOxLDL (mU/ml)	22-1428	592.2±490.7	30-1400	456.6±366.1	t=2.3, p=0.02
Chol (mg/dl)	133-291	184.2±28.2	134-377	236.4±46	t=8.9, p<0.0009
TGL (mg/dl)	21-363	66.3±44.9	11-372	127.9±64.1	t=7.3, p<0.0009
HDL (mg/dl)	20-68	46.2±10.9	25-107	52.5±14.4	t=3.2, p=0.001
LDL (mg/dl)	79-236	125,5±23.9	54-279	157.7±41.7	t=6.1, p<0.0009
ApoA (mg/dl)	91-196	144,1±21.1	98-205	152.5±23.7	t=2.2, p=0.02
ApoB (mg/dl)	30-162	85.8±20.6	70-230	123.9 ± 29.5	t=9.1, p<0.0009
Lp(a) (mg/dl)	2.5-118	15.8±17.4	2.5-127	20.2 ± 25.6	t=1.2, p=0.22

aOxLDL: Serum titers of autoantibodies against oxidized LDL cholesterol, Chol: serum levels of cholesterol, TGL: serum levels of triglycerides, HDL: serum levels of HDL cholesterol, LDL: serum levels of l LDL cholesterol, ApoA: serum levels of apolipoprotein A, ApoB: serum levels of apolipoprotein B, Lp(a):serum levels of lipoprotein a.

this difference was not statistically significant-(714 ± 515 mU/ml vs 528 ± 469 mU/ml respectively, t=1.7 p=0.09), neither between smokers and non-smokers (542 ± 482 mU/ml vs 642 ± 505 mU/ml respectively, t=-0.85 p=0.39), nor between subjects with positive and negative family history of coronary artery disease (761 mU/ml ± 544 mU/ml versus 582 mU/ml ± 489 mU/ml respectively, t=1.03 p=0.31). On the contrary, the two subjects in group A with hypercholesterolemia had very high serum titers of autoantibodies against oxidized LDL (1400 mU/ml both of them), which were found statistically significantly higher than those of the rest subjects of group A without hypercholesterolemia (1400 mU/ml vs 586±489 mU/ml respectively, t=2.3 p=0.02).

Regarding group B, serum titers of autoantibodies against oxidized LDL, were not different between males and females (503 mU/ml ± 404 mU/ml vs 423 mU/ml ± 335 mU/ml respectively , t=1.2 p=0.23), neither between subjects with and without hypercholesterolemia (388 mU/ml ± 368 mU/ml vs 435 mU/ml ± 349 mU/ml respectively, t=-0.59 p=0.59), nor between subjects with positive and negative family history of coronary artery disease (345 mU/ml ± 308 mU/ml vs 436 mU/ml ± 368 mU/ml respectively, t=-0.98 p=0.32), and nor between smokers and non smokers (406 mU/ml ± 399 mU/ml vs 420 mU/ml ± 340 mU/ml respectively, t=-0.16 p=0.87). On the contrary, obese subjects in group B had higher autoantibody titers against oxidized LDL (767 mU/ml ± 369 mU/ml vs 390 mU/ml±343 mU/ml respectively, t=2.5 p=0.01).

Nine out of 59 subjects of group B with hypercholesterolemia were on lipid-lowering drug therapy with statins, and compared to the rest who were not on drug therapy had lower cholesterol serum levels (192,3 mg/dl ± 33 mg/dl vs 265 mg/dl ± 41 mg/dl, respectively p<0,0009), and lower LDL cholesterol serum levels (122.5 mg/dl ± 21 mg/dl vs 171 mg/dl ± 42 mg/dl, respectively p<0,0009). However, no difference regarding serum titers of autoantibodies against oxidized LDL was identified between these nine subjects and either the rest who were not on drug therapy (395 mU/ml ± 363 mU/ml vs 379 mU/ml ± 387 mU/ml, respectively P>0.05), or subjects without hypercholesterolemia of group B (395 mU/ml ± 363 mU/ml έναντι 435 mU/ml ± 349 mU/ml, respectively p>0.05).

In all cases, we compared the mean values of serum titers of autoantibodies against oxidized LDL using student's t test. However, even after logarithmic transformation of the values of serum titers of autoantibodies against oxidized LDL (a normalization procedure), comparisons of the mean values of the normalised data between subgroups of our population study using t-test, reached to similar results. Moreover, serum titers of autoantibodies against oxidized LDL in subjects of group A were found statistically significantly higher than in subjects of group B, even after performing comparisons of the respective mean values using non-parametric tests Mann Whitney and Kolmogorov (p=0.04).

Correlations - Multivariate regression analyses

Pearson correlation of serum titers of autoantibodies against oxidized LDL in group A with the variables of lipid profile revealed a positive relationship with LDL cholesterol (R=0.285 p=0.01) (Figure 1) and a negative relationship with apo A-I (R=-0.239 p=0.039) (Figure 2). No correlation was identified between serum titers of aOxLDL and the rest of the lipid profile parameters in group A. On the contrary, regarding group B no correlation was found between serum titers of aOxLDL and any of the lipid parameters.

Stepwise multivariate linear regression analysis (a dependent variable entered and remained in the model if probability F was low enough <0.05) revealed that both LDL cholesterol serum levels (b=5.77 SE=2.28 beta= 0.285 t=2.52 p=0.01) and apo A-I serum levels (part. correl.=-0.22 t=1.96 p=0.05) are predictors of serum titers of autoantibodies against oxidized LDL in group A, independently from sex, history of smoking, family history of coronary artery disease, and serum levels of cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, apo B and Lp(a) (Table 4).

Another stepwise multivariate linear regression analysis which investigated whether age is an independent predictor of serum titers of aOxLDL in the whole study population, revealed that only apo A-I serum levels is an independent predictor (b=-3.79 SE=1.6 beta= -0.197 t=2.26 p=0.025) of the titers of these autoantibodies, comparing with age (both as a categorical variable with cut off point <35 years, as well as a continuous variable), sex, history of smoking, family history of coronary artery disease, presence of severe obesity and serum levels of cholesterol, triglycerides, HDL cholesterol, LDL

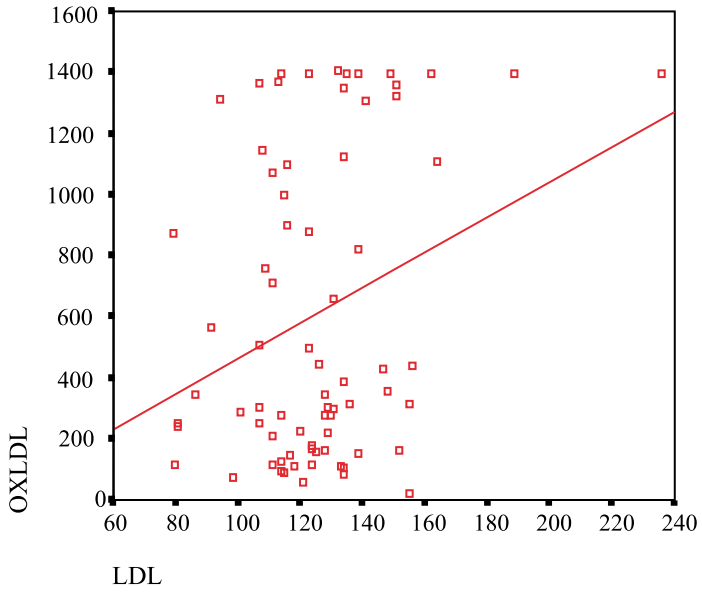

Figure 1. Diagram of serum titers of autoantibodies against oxidized LDL (OXLDL) and serum levels of LDL cholesterol in group A of the study population.

cholesterol, apo B and Lp(a) (Table 5). It is remarkable that in the same multivariate regression model, the categorical variable of age remains the only independent predictor (p=0.007) of serum titers of aOxLDL, until the variable of apo A-I serum levels enters the model and proved to be the only dominating independent predictor. It is noteworthy that results from stepwise multivariate linear regression analyses applied both in population of group A as well as in the whole study population, remains unchanged, even after using a different multivariate linear regression method, such as remove, forward or backward.

Finally, it is noteworthy that although serum titers of aOxLDL were found to be statistically significantly different between the two groups of our study population, the group of young and the group of middle aged healthy adults, after considering age as a continuous variable no correlation between age and serum titers of these autoantibodies was identified (even after normalization of data of age). This finding is further

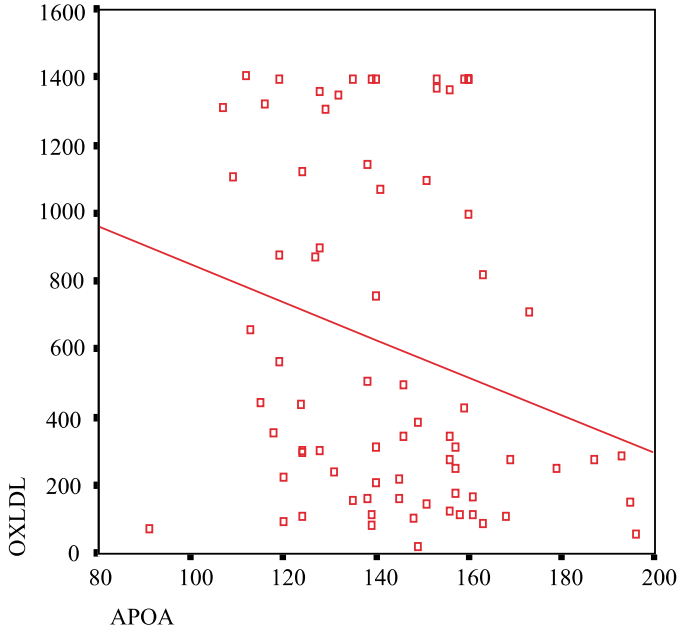

Figure 2. Diagram of serum titers of autoantibodies against oxidized LDL (OXLDL) and serum levels of apolipoprotein A-I (APOA) in group A of the study population.

Table 3. Serum titers of autoantibodies against oxidized LDL cholesterol in different subgroups of the study population.

	GROUP A N=96			GROUP B N=147		
	+	–	t, p	+	–	t, p
Hypercholesterolemia	1400±0	586±489	*	388±368	435±349	t=–0.6, p=0.59
Smoking	542±482	642±505	t=–0.85, p=0,39	406±399	420±340	t=–0.16, p=0.87
Family history of coronary artery disease	761±544	582±489	t=1.03, p=0,31	345±308	436±368	t=–0.98, p=0.32
Obesity				767±369	390±343	t=2.5, **p=0.01**
Sex	M=714±515	F=528±469	t=1.7, p=0,09	M=503±404	F=423±335	t=1.2, p=0.23

F=Female, M=Male.
* We did not perform statistical comparison of the mean value of the serum titers of autoantibodies against oxidized LDL cholesterol between subjects of group A with and without hypercholesterolemia, because only two subjects of group A had hypercholesterolemia.

explained by non-homogenous distribution of age in our study population.

Cluster analysis - Discriminant analysis

The aim of our study was to compare serum titers of aOxLDL between two different, according to age, groups of healthy subjects from the general population. However, we performed cluster and discriminant analysis in our study population, in order to further clarify the relationship between lipid parameters and age on one side, and serum titers of aOxLDL on the other side. The aim of these analyses was neither to divide our study population in groups (cluster analysis), nor to identify any variables which, as a part of a formula, could predict whether a subject of this population belong to a certain group according to age or serum titers of aOxLDL (Discriminant analysis). On the contrary, we performed such analyses in order to confirm the results of the above mentioned multivariate linear regression analyses.

We performed Cluster analysis of K means, in order to divide the whole study population into two groups, according to age, sex, lipid profile and serum titers of aOxLDL. We demonstrated that there was a statistically significantly difference between the two formed groups , only regarding age, serum titers of aOxLDL and serum levels of apo A-I. In more details, the centers of the groups 1 and 2 that were statistically significantly different were: regarding age:38.1 years vs 31.5 years respectively (ANOVA: F=3.6 p=0.03), regarding apolipoprotein A-I: 152

Table 4. Multivariate linear analysis of serum titers of autoantibodies against oxidized LDL cholesterol and the different qualitative and quantitative variables of the group A of the study population.

GROUP A N=96

	Partial correlation	t	P
Sex	0.12	1.1	0.27
Smoking	–0.11	–0.94	0.34
Family history of coronary artery disease	0.12	1.03	0.3
Cholesterol	–0.17	–1.48	0.14
Triglycerides	–0.11	0.32	0.32
HDL	–0.12	–1.02	0.31
LDL	0.285	2.52	0.01
ApoA	–0.22	–1.95	0.05
ApoB	–0.15	–1.32	0.19
Lp(a)	–0.41	–0.34	0.72

Table 5. Multivariate linear analysis of serum levels of antibodies to oxidized LDL cholesterol and the different qualitative and quantitative variables of the whole study popoulation.

	Partial correlation	t	P
Age <35 years	–0.09	–1.1	0.26
Age (as a continuous variable)	–0.06	–0.67	0.97
Sex (males)	0.038	0.42	0.67
Smoking	–0.14	–1.6	0.11
Family history of coronary artery disease	–0.039	–0.43	0.66
Obesity	0.1	1.2	0.22
Cholesterol	–0.029	–0.32	0.74
Triglycerides	–0.065	–0.73	0.46
HDL	0.005	0.05	0.95
LDL	0.000	0.003	0.99
ApoA	–0.197	2.2	0.025
ApoB	–0.03	–0.42	0.67
Lp(a)	–0.02	–0.29	0.76

mg/dl vs 141mg/dl respectively (ANOVA: F=3.6 p=0.02), and regarding aOxLDL 298.7 mU/ml vs 1197mU/ml (ANOVA: F=777.6 p<0.0009).

We also performed a stepwise Discriminant analysis in order to find out if there was any variable that could divide the study population into two groups according to a cut-off point of serum titers of aOxLDL (cut-off point =700 mU/ml). Age proved to be the only variable that has been included in a formula (y=–2.22 + 0.06x, z=–8.5), which can statistically significantly (Wilks' Lamda=0.96, F=4.74, p=0.03) predict correctly in 68% of cases.

Discussion

This study was the first to investigate serum titers of autoantibodies against oxidized LDL, in a group of patients from general population, with age so widely ranging. In all previous studies that autoantibodies against oxidized LDL were measured, both subjects from studies that included exclusively healthy population without severe atherosclerosis, and subjects from control groups of studies that were consisted of patients with severe atherosclerosis, were all middle-aged. The distribution of frequencies of age in our study population was not normal, because we intended beforehand to focus our investigation on two different group of individuals, regarding age: young healthy adults versus middle aged subjects. From this point of view, although age in subjects of our study population was quite widely ranging, as a matter of fact the distribution of frequencies of age is two-headed, as our study population is consisted of two homogenous groups, regarding age. Another characteristic of our study is the high percentage of females included in both groups of our study.

Regarding the method for assessing autoantibodies against oxidised LDL that we applied in our study it is remarkable that copper oxidised LDL (Cu^{++} oxLDL) was used as an antigen of a relevant ELISA. In previous studies in which serum titers of aOxLDL were assessed, other antigens were used, such as aldeydes, as byproducts of advanced LDL oxidation and malondialdeyde-LDL (MDA-LDL). It is probable that using Cu^{++} oxLDL as an antigen of LDL oxidation represents a model that may simulate adequately biological reality.

There are split views regarding pathophysiological role of autoantibodies aOxLDL and what actually they represent. Some studies in healthy population have revealed a positive[8,9] , while some others a negative[10,11] correlation between aOxLDL and intima-media thickness of common carotid artery, as an index of asymptomatic atherosclerosis.

There are three significant prospective studies [12,13,14] in healthy population , which showed a positive correlation between serum titers of aOxLDL and the risk for developing myocardial infarction in a follow up period of 5, 10 and 20 years respectively, independently from traditional coronary artery disease risk factors. These findings were confirmed by another study[15].

In our study, we demonstrated that young healthy adults, have high serum titers of autoantibodies aOxLDL which actually are statistically significantly higher than those of middle aged subjects, after comparing with parametric student's t-test. This difference is still statistically significant, even after normalisation of the values of serum titers of autoantibodies aOxLDL (logarithmic transformation) and then comparing with t- test or even after performing comparisons of the mean values using non-parametric tests. We note that the fact that the variance of the variable of the values of serum titers of autoantibodies aOxLDL is high, does not prove that this variable is not normally distributed in the general population as our sample was neither large nor representative enough. Safe conclusions regarding the distribution of the values of serum titers of autoantibodies aOxLDL in the general population can only be extracted, after conducting a prolonged epidemiological survey. Our study aimed simply at comparing serum titers of aOxLDL between young and middle aged healthy adults.

Provided that individuals from the group of young adults have probably limited atherosclerosis regarding severity, high serum titers of autoantibodies aOxLDL must not reflect increased quantity of oxidised LDL inside arterial walls. Consequently, this finding is conflicting with findings from other studies which are evident of positive relationship between serum titers of aOxLDL and severity of atherosclerosis. However, our findings are confirmative of other studies, that not only reached similar conclusions with the previous ones, but also deduced that autoantibodies aOxLDL may represent an expression of a defence against atheromatosis.

Although there is not any report in the literature having included young healthy adults, an apparent conclusion of our study was that serum titers of aOxLDL and probably the ability of defending against atherogenic factors declines with aging. The

fact of not revealing any relationship between serum titers of autoantibodies aOxLDL and age as a continuous variable after applying linear regression analysis in our study, must be attributed to the non-homogeneity of study population. On the contrary, by performing the same statistical methods we demonstrated that age, as a categorical variable (cut-off point 35 years), is a significant predictor of serum titers of autoantibodies aOxLDL. From this point of view, our study is the first revealing a link between age and autoantibodies against oxidised LDL. Moreover, both cluster analysis and discriminant analysis, which performed for reasons of indirect statistical confirmation of the results of multivariate analyses, evidenced an association between age and autoantibodies aOxLDL.

The mechanisms through which autoantibodies aOxLDL may contribute to atherosclerotic process are various. Firstly, these autoantibodies may be linked with their antigens, i.e. particles of oxidised LDL, which circulate freely in the blood, thus inhibiting their entrance inside the arterial wall. Many clinical and experimental studies[16-18] are supportive of the hypothesis that humoral immune response to oxidatively modified lipoproteins may increase their clearance. Secondly, autoantibodies against oxidised LDL may facilitate removal of oxidised LDL from the arterial wall and its re-entrance back to circulation. Horrko et al[19] have demonstrated that uptake of oxidised LDL by macrophages may be inhibited by autoantibodies against oxidised LDL, which in this way are opposing transformation of macrophages to foam cells[20,21]. Moreover, Ling et al[22] showed that when oxidised LDL can not be uptaken by macrophages, it may follow other metabolic alternative routes. Thirdly, the theory of antiatherogenic action of humoral immune response is further supported by experimental studies, which revealed that immunization of animals against oxidised LDL caused a significant increase of IgG autoantibodies aOxLDL and a simultaneous inhibition of atherosclerosis progression. Finally, another point that should be kept in mind, is the role of cellular immune response in the pathophysiologic process of atherosclerosis, in which T-lymphocytes are of vital importance. The activation of T lymphocytes is also triggered by oxidised LDL. On the other hand, these cells present oxidised LDL as an antigen to B-lymphocytes, thus provoking the production of autoantibodies by the latter. The significance of the role of T and B lymphocytes on atherosclerosis has been demonstrated in many studies[23,24].

Taking into consideration these concepts, the finding of our study regarding reduced immune response of middle-aged individuals to antigenic stimulus of oxidised LDL, which are expected to be of higher concentration in this group of patients, is surprising. An interesting study[25] showed that system of immunity exerts a significant role on atherosclerosis under normal conditions, while in a severe atherogenic environment –such as in hypercholesterolemia– this role is overshadowed by prevailing atherogenic factors. These findings have also been confirmed by clinical studies[26,27]. It is probable that humoral immunity[8,10] play a significant role in the very initial stages of atherosclerotic process, when various relevant risk factors have not yet caused severe disease. Consequently, we can deduce that immune response to oxidized LDL is of variable importance, regarding pathophysiologic process in the various stages of atherosclerosis. The findings of these studies are in accordance with findings of our study, according to which individuals from group B with hypercholesterolemia who were in lipid lowering drug therapy with statins and having achieved reduction of total cholesterol and LDL serum levels to normal range, had though similar serum titers of autoantibodies aOxLDL with individuals without hypercholesterolemia or hypercholesterolemic subjects without drug therapy. It seems that modification of an atherogenic environment, such as hypercholesterolemia (by administering statins which also have an antioxidant activity, due to their pleiotropic properties), does not affect immune response to oxidized LDL cholesterol in middle aged subjects. However, on the other side there is experimental evidence that autoantibodies aOxLDL contribute to atherosclerosis progression, either by increasing foam cells formation[28], or inducing arterial wall injury[29].

Another significant finding of our study was the inverse relationship between serum levels of Apo A-I and serum titers of autoantibodies aOxLDL, which was demonstrated in the group of young healthy adults. We have to note that no study in the past has examined the relationship between serum titers of autoantibodies and serum levels of Apo A-I. Performing multivariate analysis, serum levels of both LDL cholesterol and Apo A-I were recognized as independent predictors of serum titers of autoantibodies against oxidized LDL in young healthy adults.

The antiatherogenic activity of HDL cholesterol has been documented by a large number of experimental and both prospective and randomized clinical

studies. It seems that activity of HDL cholesterol can not be evaluated exclusively and solely by a quantitative assessment of this lipoprotein, although the reported guidelines regarding the management of hyperlipidemias and the relevant charts for the assessment of cardiovascular risk, approved by both European Society of Cardiology and American Heart Association / American College of Cardiology, have included algorithms containing only the quantitative assessment of HDL. It also seems that the antiatherogenic properties of HDL are not always proportional to serum levels of HDL cholesterol[30], but it is possible on one hand, low levels of HDL to be associated with significant antiatherogenic activity[31] and vice versa. A remarkable part of HDL cholesterol's antiatherogenic avtivity must be attributed to its antioxidant properties, owing to two enzymes with antioxidant action, paraoxonase and PAF-acetylhydrolase[32-34]. These two antioxidant enzymes[32,33] are incorporated mainly into HDL particle, and they protect both HDL and LDL particles from oxidation. However, paraoxonase is particularly linked to apolipoprotein A-I[32] (and to apolipoprotein J). Although HDL particle contains apolipoproteins Apo A of various types (I-IV), Apo A-I accounts for more than 70% of total apolipoprotein content, so it actually reflects the quantity of paraoxonase on HDL particle.

Accordingly, the group of young healthy adults in our study with high serum levels of Apo A-I had lesser quantity of oxidized LDL. However this population had also low serum titer of autoantibodies against oxidized LDL. These findings in conjunction with the identified positive correlation between serum levels of LDL cholesterol and serum titers of autoantibodies against oxidized LDL in the same group of subjects, evidence that autoantibodies aOxLDL reflect the quantity of oxidized LDL (provided that levels of oxidized LDL under normal conditions parallel to levels of circulating LDL cholesterol). Reviewing the literature, there is only one study[36] which identified a negative correlation between serum titers of autoantibodies against oxidized LDL and serum levels of HDL cholesterol in middle aged healthy subjects, but measurements of apo A-I serum levels were not performed.

In conclusion, there were two significant findings in our study: Firstly, young healthy adults, who by definition have limited atheromatosis, have higher serum titers of autoantibodies against oxidized LDL, compared to those of a middle-aged population. And secondly, serum levels of LDL cholesterol are positively correlated, while serum levels of Apo A-I are negatively correlated, with serum titers of autoantibodies against oxidized LDL in the group of young adults. It seems that in the very initial steps of atherosclerotic process, oxidation of LDL cholesterol induces the production of protective autoantibodies against oxidized LDL, whose serum titers are proportional to the quantity of oxidized LDL cholesterol. It seems that the contribution of this mechanism on atherosclerosis, declines with aging. The inverse relationship between serum titers of autoantibodies against oxidized LDL and serum levels of Apo A-I, confirms the antioxidant properties of the latter and its significant role on antiatherogenic defense.

References

1. Singal PK, Petkau A, Gerrard JM, et al: Free radicals in health and disease. Mol Cell Biochem 1988; 84: 121-122.
2. Kako KJ: Free radical effects on membrane protein in myocardial inschemia/reperfusion injury. J Mol Cell Cardiol 1987; 19: 209-211.
3. Paradies G, Ruggiere FM, Petrosillo G, et al: Peroxidative damage to cardiac mitochondria: cytochrome oxidase and cardiolipin alterations. FEBS Lett 1998; 424: 155-158.
4. Virella G, Virella I, Leman RB, et al: Anti-oxidized low-density lipoprotein antibodies in patients with coronary heart disease and normal healthy volunteers. Int J Clin Lab Res 1993; 23: 95-101.
5. Salonen JT, Yla-Herttuala S, Yamamoto R, et al: Autoantibody against oxidized LDL and progression of carotid atherosclerosis. Lancet 1992; 339: 883.
6. Maggi E, Finardi G, Poli M, et al: Specificity of autoantibodies against axidized LDL as an additional marker for atherosclerotic risk. Coronary Artery Disease 1993; 4: 1119-1125.
7. Bergmark C, Wu R, De Faire U, et al: Patients with early-onset peripheral vascular disease have increased levels of autoantibodies against oxidized LDL. Arterioscler Thromb Vasc Biol 1995; 15: 441-445.
8. Hulthe J, Bokemark L, Fagerberg B, et al: Antibodies to oxidized LDL in relation to intima-media thickness in carotid and femoral arteries in 58-year-old subjectively clinically healthy men. Arterioscler Thromb Vasc Biol 2001; 21: 101.
9. Iribarren C, Folsom AR, Jacobs DR, et al: Association of serum vitamin levels, LDL susceptibility to oxidation, and autoantibodies against MDA-LDL with carotid atherosclerosis. Arterioscler Thromb Vasc Biol 1997; 17: 1171.
10. Fukumoto M, Shoji T, Emoto M, et al: Antibodies against oxidized LDL and carotid artery intima-media thickness in a healthy population . Arterioscler Thromb Vasc Biol 2000; 20: 703-710.
11. Hulthe J, Wiklund O, Hurt-Camejio Eva, et al: Antibodies to oxidized LDL in relation to carotid atherosclerosis, cell adhesion molecules and phospholipase A2. Arterioscler Thromb Vasc Biol 2001; 21: 269.

12. Puurunen M, Manttari M, Manninen V, et al: Antibody against oxidized low-density lipoprotein predicting myocardial infarction. Arch Intern Med 1994; 154: 2603-2605.
13. Musfafa A, Nituyanand S, Berglund L, et al: Circulating immune complexes in 50-year-old men as a strong and independent risk factor for myocardial infarction. Circulation 2000; 102: 2576-2581.
14. Wu R, Nityanand S, Berglund L, et al: Antibodies against cardiolipin and oxidatively modified LDL in 50-year-old men predict myocardial infarction. Arterioscler Thromb Vasc Biol 1997; 17 (11): 3159-3163.
15. Ejaz A, Trifunovic J, Stegmayr B, et al: Autoantibodies against oxidatively modified LDL do not constitute a risk factor for stroke. Stroke 1999; 2541-2546.
16. Itabe H, Yamamoto H, Imanaka T, et al: Sensitive detection of oxidatively modified low density lipoprotein using a monoclonal antibody. J Lipid Res 1996; 37: 45-53.
17. Shoji T, Nishzawa Y, Fukumoto M, et al: Inverse relationship between circulating oxidized low density lipoprotein (oxLDL) and anti-oxLDL antibody levels inhealthy subjects. Atherosclerosis 2000; 148: 171-177.
18. Wiklund O, Witztum JL, Carew TE, et al: Turnover and tissue sites of degradation of glucosylated low density lipoprotein in normal and immunized rabbits. J Lipid Res 1987; 28: 1098-1109.
19. Horkko S, Bird DA, Miller E, et al: Monoclonal autoantibodies specific for oxidized low-density lipoproteins. J Clin Invest 1999; 103: 117-128.
20. Griffith RL, Virella, GT, Stevenson HC, et al: Low density lipoprotein metabolism by human macrophages activated with low density lipoprotein immune complexes: a possible mechanism of foam cell formation. J Exp Med 1988; 168: 1041-1059.
21. Khoo JC, Miller E, Pio F, et al: Monoclonal antibodies against LDL further enhance macrophage uptake of LDL aggregates. Arterioscler Thromb Vasc Biol 1992; 12: 1258-1266.
22. Ling WH, Lougheed M, Suzuki H, et al: Oxidized or acetylated low density lipoproteins are rapidly cleared by the liver in mice with disruption of the scavenger receptor class A type I/II gene. J Clin Invest 1997; 100: 244-252.
23. Hansson GK, Holm J, Holm S, et al: T lymphocytes inhibit the vascular response to injury. Proc Natl Acad Sci USA. 1991; 94: 2516-2520.
24. Rosellar SE, Schonfeld G, Daugherty A: Enhanced development of atherosclerosis in cholesterol-fed rabbids by suppression of cell-mediated immunity. J Clin Invest 1995; 96: 1389-1394.
25. Dansky HM, Charlton SA, Harper MM, et al: T and B lymphocytes play a minor role in atherosclerotic plaque formation in the apolipoprotein E-deficient mouse. Proc Natl Acad Sci 1997; 94: 4642-4646.
26. Hulthe J, Wikstrand J, Lidell A, et al: Antibody titers against oxidized LDL are not elevated in patients with familiar hypercholesterolemia. Arterioscler Thromb Vasc Biol 1998; 18: 1203-1211.
27. Uusitupa M, Niskanen L, Luoma J, et al: Autoantibodies against oxidized LDL do not predict atherosclerotic vascular disease in non-insulin-dependent diabetes mellitus. Arterioscler Thromb Vasc Biol 1996; 16: 1236.
28. Wu R, Lefvert AK: Autoantibodies against oxidized low density lipoproteins (oxLDL): characterization of antibody isotype, subclass, affinity and effect on the macrophage uptake of oxLDL. Clin Exp Immunol 1995; 102: 174-180.
29. Libby P, Hansson GK: Biology of disease: involvement of the immune system in human atherogenesis: current knowledge and unanswered questions. Lab Invest 1991; 64: 5-15.
30. Zannis V, Kardasis D: The revolution of HDL. Atheroma 2002; 6: 1-2.
31. Navab M, Berliner J, Watson A, et al: The yin and yang of oxidation in the development of the fatty streak. Arterioscler Thromb Vasc Biol 1996; 16: 831-838.
32. Shah P, Kaul S, Nilsson J, et al: Exploiting the vascular protective effects of high-density lipoprotein and its apolipoproteins: an idea whose time for testing is coming, part I. Circulation 2001; 104: 2376-2383.
33. Shah P, Kaul S, Nilsson J, et al: Exploiting the vascular protective effects of high-density lipoprotein and its apolipoproteins: an idea whose time for testing is coming, part II. Circulation 2001; 104: 2498-2502.
34. Navab M, Van Lenten B, Reddy S, et al: High-density lipoprotein and the dynamics of atherosclerotic lesions. Circulation 2001; 104: 2386-2387.
35. Tsimikas S, Palinski W, Witztum J, et al: Circulating autoantibodies to oxidized LDL correlate with arterial accumulation and depletion of oxidized LDL in LDL receptor-deficient mice. Arterioscler Thromb Vasc Biol 2001; 21: 95-101.
36. Van de Vijver L, Steyger R, Van Poppel G, et al: Autoantibodies against MDA-LDL in subjects with severe and minor atherosclerosis and healthy population controls. Atherosclerosis 1996; 122: 245-253.