

Lipoprotein(a) and Its Position among Other Risk Factors of Atherosclerosis

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Summary

Lipoprotein(a) [Lp(a)] comprises of an LDL particle and apolipoprotein(a) [apo(a)] and its elevated levels are considered a risk factor for atherosclerosis. The aim of our study was to find out whether elevated Lp(a) levels are associated with increased risk of atherosclerosis in patients with multiple other risk factors. We further tested the association of three polymorphisms of the apo(a) gene promoter with Lp(a) levels. No significant correlation was detected between Lp(a) levels and lipid and clinical parameters tested. The study demonstrated a significantly ($p=0.0219$) elevated Lp(a) level (mean 28 ± 35 mg/dl, median 0.14) in patients with coronary heart disease (CHD). In a group with premature CHD the correlation was not significant anymore. There was a significant correlation between polymorphic loci of the promoter region of apo(a) gene and Lp(a) levels (+93C>T, $p=0.0166$, STR, $p<0.0001$). Our study suggests that elevated Lp(a) level is an independent risk factor of CHD in carriers of other important CHD risk factors. Observed association of sequence variants of the promoter of apo(a) gene with Lp(a) levels is caused in part due to linkage to a restricted range of apo(a) gene length isoforms.

Key words

Lipoprotein(a) • Apolipoprotein(a) • Atherosclerosis • Risk factors
• Coronary heart disease • Gene polymorphisms

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Introduction

Lipoprotein(a) [Lp(a)] comprises of an LDL (low-density lipoprotein) particle covalently bound to a specific glycoprotein, apolipoprotein(a) [apo(a)], by apoB-100 (Hořejší and Češka 2000). Apolipoprotein(a) determines the structural and functional properties of the lipoprotein. A number of prospective and retrospective studies demonstrated that increased levels of Lp(a) are associated with atherosclerosis and Lp(a) is therefore considered an independent risk factor of atherosclerosis (Berglund and Ramakrishnan, 2004, Evans *et al.* 2001, Marcovina and Koschinsky, 2002). In numerous trials, patients with Lp(a) levels above 30 mg/dl had markedly increased risk of coronary heart disease (Foady *et al.* 2000, Paultre *et al.* 2000) and a susceptibility to occlusive complications after various interventions (percutaneous transluminal coronary angioplasty, stent placement) (Rifai *et al.* 2004).

Several mechanisms linking Lp(a) and development of atherosclerosis have been proposed. In arterial intima, Lp(a) is located only in atherosclerotic plaques but not in the intact tissue. Lp(a) captured in the atherosclerotic plaque stimulates smooth muscle cells proliferation and its binding to extracellular matrix enhances lipid accumulation. As a non-functional structural homologue of plasminogen it can also negatively affect the process of fibrinolysis (Koschinsky and Marcovina 2004, Shai *et al.* 2005a).

Plasma Lp(a) concentration is predominantly determined by genetic factors and is not affected by diet (Boerwinkle *et al.* 1992). Certain drugs (nicotinic acid

and its analogues) and sexual hormones (androgens, estrogens, progesterone) have a lowering effect on Lp(a) levels (Šulcová *et al.* 2001). No effects of statins and fibrates on the Lp(a) were demonstrated (Lippi and Guidi, 2003).

The amount of synthesized apo(a) is considerably different among individuals in a population. There is an apo(a) gene length polymorphism that accounts for about 40-60 % of the variance (Cohen *et al.* 1993, Kraft *et al.* 1992). Part of the variance could be attributed to polymorphic sites either in the coding sequence or in the transcription regulatory sequences of apo(a) gene. Four polymorphisms with possible relation to Lp(a) levels have been identified in the promoter region of the apo(a) gene. These are three single nucleotide substitutions (+121 G>A, +93 C>T, and -772 G>A) and one pentanucleotide TTTTA repetition (7-11 repeats) designated as the STR (short tandem repeat) locus (Trommsdorf *et al.* 1995, Zysow *et al.* 1995). In contrast to the other polymorphic sites the -772 G>A substitution was not reported to be functional.

The aim of our study was to test if Lp(a) levels are associated with coronary heart disease and premature manifestation of coronary atherosclerosis in patients with other important risk factors of atherosclerosis. We analyzed the relationship between Lp(a) levels and serum lipids (total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, apoB-100) and other risk factors of atherosclerosis (type 2 diabetes mellitus, arterial hypertension, smoking, and overweight). Finally, we investigated an association between selected polymorphic sites in the apo(a) gene promoter region and Lp(a) plasma levels.

Methods

Subjects

3915 out-patients followed at the Centre of Preventive Cardiology of the General Teaching Hospital, Charles University in Prague with dyslipidemia and other CHD risk factors (e.g. type 2 diabetes mellitus, hypertension, overweight, metabolic syndrome) were included in the study. Characteristics of the study group are shown in Table 1.

The study group was divided into quintiles according to Lp(a) level. We randomly selected a representative subgroup of 650 individuals (each sixth patient from each quintile). One hundred patients with inflammatory diseases, malignancies, renal insufficiency

or other diseases, which could affect the Lp(a) level, were excluded. The presence of type 2 diabetes mellitus, hypertension, smoking, overweight [defined as BMI (body mass index) over 25] and of CHD (defined as a documented myocardial infarction, revascularization procedure, angina, positive stress test or positive coronary angiography) were recorded in the final group of 550 individuals. The final group did not differ from the principal group in lipid parameters and it included 12.8 % of patients with type 2 diabetes mellitus, 35.4 % of patients with arterial hypertension, 26.5 % of smokers, 12.7 % patients with documented CHD and 7.8 % patient with premature CHD (men <55 years of age, women <65 years of age). When subgroups of diabetics and non-diabetics, hypertonics and non-hypertonics, smokers and non-smokers, males and females and those with CHD and without CHD were mutually compared, no significant differences were observed in selected lipid and clinical parameters (including age). The study was approved by the ethics committee of the General Teaching Hospital in Prague and all study participants gave their informed consent.

Biochemical analysis

Venous blood was collected after 12 h fasting and Lp(a) concentrations were measured using frozen serum (-20 °C), separated within two hours after blood collection, by the immuno LEIA[®] Lp(a) method (Technoclone GmbH, Vienna, Austria). We used internal control samples as well as control samples and standards provided by the manufacturer during each assay.

Plasma concentrations of total cholesterol, HDL [high-density lipoprotein] cholesterol, triglycerides, and apo-B were determined on automatic analyzers (Modular SWA, Roche). LDL cholesterol level was calculated by Friedewald equation ($LDL-c = TC - HDL-c - TG/2.2$).

DNA analysis

DNA was isolated from fresh or frozen whole blood using a salting-out method according to Miller *et al.* (1988). Subjects were then directly genotyped for +121 G>A, +93 C>T, and -772 G>A apo(a) gene polymorphisms. The sequence of the proximal promoter and the pentanucleotide microsatellite (STR locus) from the distal promoter were amplified using primers described in our previous study (Žídková *et al.* 2007). The promoter fragment was then restricted in two separated 10 µl reaction mixtures using 5 units of the *Hpy*188III (New England Biolabs GmbH, Frankfurt am

Table 1. Clinical and lipid characteristics of the principal out-patient group, serum lipid parameters and Pearson correlation coefficients to Lp(a).

Characteristics	N	Mean \pm SD	Median	Minimum	Maximum	Pearson ⁺
Age (years)	3915	50.95 \pm 16.34	53.00	19	94	0.14131
Total cholesterol (mmol/l)	3869	6.66 \pm 2.30	6.50	2.77	37.63	0.06036
LDL cholesterol (mmol/l)	3241	4.19 \pm 1.64	4.01	0.56	18.80	0.13002
HDL cholesterol (mmol/l)	3644	1.39 \pm 0.44	1.35	0.10	4.04	0.07100
Triglycerides (mmol/l)	3863	2.85 \pm 4.71	1.80	0.15	88.35	-0.08923
Apo-B (mmol/l)	3838	1.28 \pm 0.44	1.24	0.18	5.52	0.12770
Lp(a) (mg/dl)	3915	28 \pm 35	14	0.00	410	—

N – number of individuals with available data, ⁺value of Pearson correlation coefficient (r) to Lp(a), r value of 0.3 was considered as significant

Table 2. Clinical and lipid characteristics of the final group, serum lipid parameters and Pearson correlation coefficients to Lp(a).

Characteristics	N	Mean \pm SD	Median	Minimum	Maximum	Pearson ⁺
Age (years)	550	49.56 \pm 12.54	51.50	30	81	0.15007
Diabetes mellitus	70	—	—	—	—	—
Arterial hypertension	195	—	—	—	—	—
Smoking	146	—	—	—	—	—
Overweight (BMI > 25)	215	—	—	—	—	—
CHD	70	—	—	—	—	—
Premature CHD	78	—	—	—	—	—
Total cholesterol (mmol/l)	550	6.44 \pm 1.94	5.90	3.04	20.78	0.05542
LDL cholesterol (mmol/l)	550	4.35 \pm 1.82	4.12	0.62	9.60	0.12788
HDL cholesterol (mmol/l)	550	1.30 \pm 0.37	1.33	0.40	3.12	0.1050
Triglycerides (mmol/l)	550	2.52 \pm 5.01	1.95	0.45	45.35	-0.09024
apo-B (mmol/l)	550	1.29 \pm 0.34	1.25	0.19	5.48	0.13040
Lp(a) (mg/dl)	550	27 \pm 56	13.5	0.00	340	—

N – number of individuals with available data, ⁺value of Pearson correlation coefficient (r) to Lp(a), r value of 0.3 was considered as significant

Main, Germany) enzyme for the +121G>A variant detection and 5 units of the *Hpy*CH4IV (NEB) enzyme for the +93C>T variant detection. Restricted fragments were then subjected to 2 % agarose gel electrophoresis and visualized in UV light after ethidium bromide staining. The STR locus product varied in size according to the number of TTTTA repeat units from 164 bp (7 repeats) to 184 bp (11 repeats) and was measured by fragmentation analysis conducted on ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Correlation between Lp(a) levels and serum lipid

concentrations, apo-B plasma levels, risk factors of atherosclerosis, and prevalence of CHD was evaluated using Pearson's correlation coefficient (r). The Lp(a) plasma levels in subgroups of diabetics and non-diabetics, hypertonics and non-hypertonics, smokers and non-smokers, and those with heart disease and without heart disease were compared by Mann-Whitney test. The logistic regression analysis was used to establish predicted probability. The ANOVA test was employed to reveal an association of polymorphic loci with Lp(a) levels. Each polymorphic locus was tested for significant deviation from Hardy-Weinberg equilibrium using the χ^2 test ($p < 0.05$, 1 d.f.).

Results

No significant correlation was observed between Lp(a) levels and serum lipids and age in the overall patient group of 3915 individuals (for relevant values of Pearson's correlation coefficient see Table 1). The same correlation analyses were conducted in the final group of 550 individuals (Table 2), which had correlation coefficients not markedly different from those in the basic group (data not shown).

When we compared the mean Lp(a) plasma levels between the subgroup of type 2 diabetics and non-diabetics ($p=0.3935$), hypertonics and non-hypertonics ($p=0.2329$), and smokers and non-smokers ($p=0.2682$), no significant differences were detected.

We found a significant increase of mean Lp(a) levels in the subgroup of patients with CHD ($p=0.0219$). In the logistic regression analysis we utilized the Lp(a) plasma level as a predictor of CHD. Lipoprotein(a) levels significantly affected the probability of CHD ($p=0.0234$). The predicted probability represents the likelihood of CHD development among patients with a given Lp(a) level (Fig. 1). When we analyzed the individuals with premature manifestation of CHD (78 individuals) the association of Lp(a) levels with the development of CHD was not significant ($p=0.1471$).

Allelic frequencies of the two single nucleotide substitutions were as follows: +121G > A ($0.794/0.206 \pm 0.017$), +93C > T ($0.865/0.135 \pm 0.010$). Genotypes and relevant mean Lp(a) levels are summarized in Table 3. The most frequent allele of the STR locus had 8 repeats (0.711 ± 0.019), allelic frequencies of other alleles were: STR with 7 repeats (0.008 ± 0.003), STR with 9 repeats (0.151 ± 0.015), STR with 10 repeats (0.111 ± 0.013), and STR with 11 repeats (0.019 ± 0.006). All genotypes detected in the final group and related Lp(a) levels are given in Table 4. Distribution of the genotype frequencies were in Hardy-Weinberg equilibrium.

No effect of the +121G>A polymorphism on Lp(a) level was observed ($p=0.2515$), but there was a significant association of the +93C>T polymorphism with Lp(a) levels ($p=0.0166$). There was a highly significant association of different length of the STR locus with Lp(a) levels ($p<0.0001$). Our results indicate that mean Lp(a) levels increase with a decreasing number of TTTTA repeats. Moreover, when we analyzed a combined effect of all three polymorphic sites on Lp(a) levels the association of the +93C>T polymorphism became attenuated and was no more significant

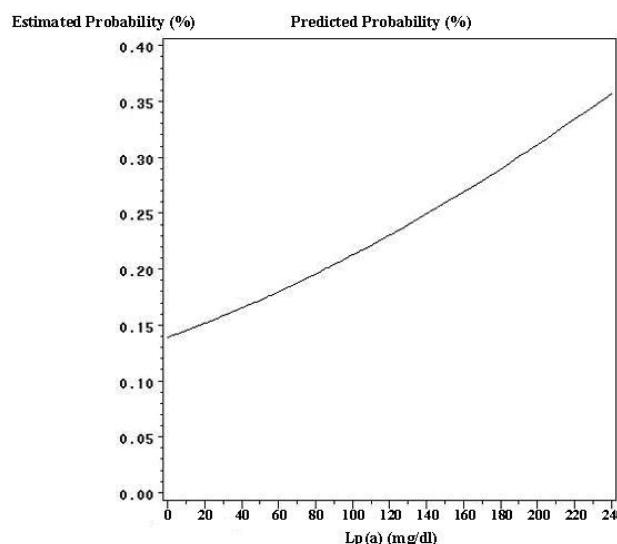


Fig. 1. The predicted probability represents likelihood of coronary heart disease development among patients with a given Lp(a) level.

($p=0.6001$), whereas the effect of the STR locus was still persistent ($p=0.0029$).

Discussion

The study was carried out in a group of patients of the Centre of Preventive Cardiology and thus it includes individuals with different risk factors of atherosclerosis. The purpose of the study was to find out whether Lp(a) levels are still associated with CHD in patients with other cardiovascular risk factors (type 2 diabetes, arterial hypertension, smoking, obesity) and if Lp(a) levels are independent of these risk factors. We also tested the association of three polymorphic loci from the apo(a) gene promoter region with Lp(a) levels. The polymorphic sites were chosen for the analysis based on the data from previous studies (Brazier *et al.* 1999, Kalina *et al.* 2001).

No correlation was detected between Lp(a) levels and risk factors of atherosclerosis examined. Our results are in agreement with other studies (Shai *et al.* 2005b), but in other studies is demonstrated a positive correlation of Lp(a) and diabetes mellitus. Major limitation of this study is that it included patients with renal complications (albuminuria and renal insufficiency), which increase Lp(a) levels. Thus we support the independent role of Lp(a) on studied risk factors of atherosclerosis.

In our study there is a lack of dependence of Lp(a) levels and apo-B, which forms a part of Lp(a), but

Table 3. Number of observed genotypes, mean Lp(a) plasma levels and standard deviation (SD) for the +121G>A and +93C>T polymorphic sites from the apo(a) gene promoter region in quintile based group.

Genotypes		N	Lipoprotein(a) (mg/dl)		
			Mean ± SD	Minimum*	Maximum
+121	G/G	348	39 ± 37	0.0	205
	G/A	177	45 ± 46	0.0	190
	A/A	25	54 ± 66	0.0	268
+93	C/C	412	44 ± 43	0.0	268
	C/T	128	34 ± 36	0.0	131
	T/T	10	19 ± 18	0.0	50

* the lowest non-zero detected Lp(a) level was 1 mg/dl

Table 4. Number of observed genotypes, mean Lp(a) plasma levels (\pm SD) for the STR polymorphic site from the apo(a) gene promoter region in quintile based group.

Genotype	N	Lipoprotein(a) (mg/dl)		
Number of TTTTA repetition		Mean \pm SD	Minimum*	Maximum
7/10; 7/8	9	76 \pm 47	5.0	143
8/8	282	44 \pm 43	0.0	268
8/9	115	38 \pm 38	0.0	131
8/10	82	43 \pm 40	0.0	160
8/11	13	27 \pm 28	0.0	81
9/9	16	23 \pm 35	0.0	132
9/10; 9/11	19	23 \pm 19	0.0	67
10/10; 10/11; 11/11	14	10 \pm 7	0.0	22

* the lowest non-zero detected Lp(a) level was 1 mg/dl

such correlation was also missing in other trials (Lippi and Guidi 2003). In most individuals, the Lp(a) concentration is very low in comparison with the abundance of other lipoproteins containing apo-B. It seems that even markedly decreased apo-B plasma levels are still not limiting for the rate of Lp(a) synthesis

Significantly increased mean Lp(a) level (mean 0.49 \pm 0.54 mg/dl) was found in a subgroup of patients suffering from CHD. However, such significant correlation was absent when only patients with premature CHD were included. An insufficient sample size of the subgroup with premature CHD could be the possible reason for our results, because a positive correlation was demonstrated in other trials (Stein and Rosenson 1997, Berlung and Ramakrishnan 2004). Nevertheless, our results support the notion that increased Lp(a) levels are associated with CHD in patients having other CHD risk factors.

Gene variants affecting apo(a) gene transcription may contribute to the high variability of Lp(a) plasma concentrations. Suzuki *et al.* (1997) reported a potentially positive role of the +121 G>A substitution on apo(a) gene transcription, but this was not reproduced by Wu and Lee (2003). We did not detect any association of the +121 G>A polymorphism with Lp(a) levels. A significant association of the +93 C>T polymorphism with Lp(a) levels is in accordance with its direct negative effect on apo(a) production (Zysow *et al.* 1995). Nevertheless, in Caucasians the effect is usually masked by the strong linkage disequilibrium with intermediate apo(a) gene length isoforms (Brazier *et al.* 1999).

Several studies have reported a correlation between the STR locus of apo(a) gene promoter and plasma Lp(a) concentrations (Brazier *et al.* 1999, Kalina *et al.* 2001). The correlation was independent of apo(a) size isoforms and it accounted for up to 14 % of Lp(a)

level variation (Brazier *et al.* 1999, Trommsdorff *et al.* 1995). Our findings are consistent with these data. It is supposed to be due to the linkage disequilibrium of different STR alleles to the distinct range of apo(a) isoforms and to other functional changes influencing apo(a) production rate. The STR allele with 9 repeats was reported to be in a linkage disequilibrium with the +93 T promoter variant (Holmer *et al.* 2003) and with apo(a) middle-sized isoforms (Puckey *et al.* 1997). It seems that the association of +93 C>T polymorphism with Lp(a) levels was actually caused by the STR allele with 9 repeats at least in part due to the linkage with restricted apo(a) gene length isoforms. Such a conclusion is in agreement with results of the combined analysis where the effect of +93 C>T polymorphism lost its significance and the effect of the STR locus was preserved. Thus, our

results are in compliance with previously published data (Kraft *et al.* 1998, Puckey *et al.* 1997).

Our study suggests that elevated Lp(a) level is independently associated with CHD in patients with other important risk factors of atherosclerosis. Sequence variants of the regulatory regions of apo(a) gene are associated with Lp(a) plasma levels, in particular due to the linkage to a restricted range of apo(a) gene length isoforms.

Conflict of Interest

There is no conflict of interest.

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