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Summary of Ph.D. Thesis

VARIABILITY IN *LPA* GENE TRANSCRIPTION REGULATORY SEQUENCES

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
apo	apolipoprotein
C/EBP	CCAAT/enhancer binding protein
c-Ets	v-ets erythroblastosis virus E26 oncogene homolog
DGGE	denaturing gradient gel electrophoresis
dNTP	deoxynucletide triphosphate
ER	endoplasmic reticulum
ESR	estrogen receptor
HDL	high density lipoprotein
HGF	hepatocyte growth factor
HNF-1α	hepatocyte nuclear factor 1α
IDL	intermediate density lipoprotein
ICHS	ischaemic heart disease
IL-6	interleukin 6
K	kringle domain
K_{LPA}	kringle domain of *LPA* gene
LAL	lysosomal acid lipase
LD	linkage disequilibrium
LDL	low density lipoprotein
LDLR	LDL receptor
LF-A1	liver factor A1
LINE-1	long interspersed nuclear element type1
Lp(a)	lipoprotein(a)
LPA	gene for apolipoprotein(a)
LRP	LDL receptor-related protein
MDBP	methylated DNA-binding protein
NFκB	nuclear factor κB
Oct-1	octamer-binding transcription factor 1
PCR	polymerase chain reaction
PLG	gene for plasminogen
PPARα	peroxisome proliferator-activated receptor alpha
RXRα	retinoid X receptor alpha
SD	standard deviation
Sp1	specificity protein 1
STR	short tandem repeat
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TGF- β	transforming growth factor β
TGT3	thyroid transcription factor I
t-PA	tissue-type plasminogen activator
u-PA	urokinase-type plasminogen activator
VLDL	very low density lipoprotein

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I.INTRODUCTION

I.1.ATHEROSCLEROSIS

Atherosclerosis is a progressive disease of the arterial blood vessel wall. It is most often clinicaly manifested as myocardial and cerebral infarction or as ischemic heart disease but also other parts of the circulatory system could be affected. Many processes have been implicated including shear stress-related events, local adherence of platelets, lipoprotein oxidation and aggreration, macrophage chemotaxis, smooth muscle cell alterations and foam cell formation.

I.1.1.ATHEROSCLEROTIC LESIONS

Three main forms and stages at the same time of atherosclerotic lesions are distinguished: fatty streaks, fibrous and atheroma plaques, and complex advanced lesions (Češka,1999).

There are several hypothesis concerning the atherosclerotic lesions development. The response to injury hypothesis assumed that an injury to the endothelium might precipitate the atherosclerotic process. It is based on manifestations of the endothelium dysfunction caused by injury notably at branch points in the arterial tree. Increased lipoprotein plasma levels and their enhanced modification and entry in the vessel intima is the key process for the response to lipid infiltration hypothesis. On the contrary the response to retention hypothesis of early atheogenesis consider not the accelerated infiltration of lipoproteins but the enhanced retention of modified lipoproteins in the intima of blood vessel as the initial cause of lesion formation (Ross,1993; Augé *et al.*,1996; Inoue *et al.*,2001).

I.1.2.ATHEROSCLEROSIS RISK FACTORS

Several studies have proposed that abnormal lipids, smoking, hypertension, diabetes, abdominal obesity, high cholesterol diet and low physical activity account for most of the risk of premature atherosclerosis dvelopment (Maas a Boger,2003;

Yusuf *et al.*,2004). Besides age, male sex and genetic predisposition are the non modifiable risk factors. Elevated serum lipids are caused by disruption of lipoproteins structure and metabolism. The basic lipoprotein particles are chylomicrons, VLDL (Very Low Density Lipoprotein), IDL (Intermediate Density Lipoprotein), LDL (Low Density Lipoprotein), and HDL (High Density Lipoprotein). Lipoprotein(a), Lp(a), is the unique human plasma lipoprotein.

I.2.LIPOPROTEIN(a)

Lipoprotein(a), Lp(a), is a cholesterol-rich particle that is now recognized to be an independent risk factor for development of atherosclerosis (Djurovic *et al.*,1997; Luc *et al.*,2002; Emanuele *et al.*,2004). Lipoprotein(a) consists of LDL particle covalently bound to a specific glycoprotein, apolipoprotein(a), apo(a). By sequencing cloned human apo(a) cDNA, McLean *et al.* (1987) showed that apo(a) is very similar to human plasminogen. It is likely that the *LPA* gene arose from a duplication of the plasminogen gene, perhaps recently during mammalian evolution.

Studies have demonstrated that Lp(a) plasma concentrations are highly heritable and determined primarily by the gene for apo(a), so called *LPA* locus (Austin *et al.*,1992; de Meester *et al.*,1995).

I.2.1.*LPA* LOCUS

I.2.1.1.*LPA* locus structure

The size of apo(a) is highly variable ranging from 300 to 800 kDa (White *et al.*,1994). Apolipoprotein(a) is coded at *LPA* locus (GeneID:4018) that resides the 6q26-q27 chromosomal region. It contains a serin protease domain, which cannot be activated by tissue plasminogen activator (t-PA). Apolipoprotein(a) as well as plasminogen consists of tandemly arrayed kringle motifs (K). There are five different kringles, KI-KV, in the plasminogen sequence and only two different kringles,

K_{LPA}IV and K_{LPA}V, in the apo(a) protein. However apo(a) is much larger than plasminogen due to multiple copies of the K_{LPA}IV motif (type 1-10) that are 75-85% homologous to that of plasminogen. Moreover, while K_{LPA}IV type1 and type 3-10 are present as a single copy K_{LPA}IV type 2 occurs in a variable number of tandem repeats (Lackner *et al.*,1991; Guevara *et al.*,1993).

Variation in the number of the kringleIV type 2 motifs explains the genetically determined size polymorphism of apo(a) that is inversely correlated with plasma Lp(a) levels (Holmer *et al.*,2003).

I.2.1.2.Sequence variability in *LPA* locus

There is still a large variation in Lp(a) levels among carriers of a certain apo(a) length isoform (Cohen *et al.*,1993). It has been revealed that the abundance of apo(a) mRNA transcripts of the same size can vary greatly between individuals (Wade *et al.*,1991), proposing that some of the variation in Lp(a) concentrations can be related to differences in the rate of *LPA* gene transcription. Besides sequence variants from *LPA* gene coding regions can affect Lp(a) particle production rate.

I.2.1.2.1.Transcription control regions

<u>I.2.1.2.1.1.Promoter</u>

The so called minimal promoter (from -98 bp to +141 bp upstream from the transcription start site) consists of transcription elements that are able to ensure the basal transcription activity (Wade *et al.*,1994). There are two polymorphic sites in this region, +121G/A and +93C/T. While the +121G/A substitution is rather without direct effect on apo(a) production rate (Brazier *et al.*,1999), the -93C/T polymorphism represents a functional change. The +93T allele is associated with 60% decrease in apo(a) expression resulting from a reduction in translation due to the creation of a novel upstream ATG start site (Zysow *et al.*,1995).

Several other polymorphisms have been described in the 5′untranslated region (1,5 kb upstream from the transcription start site) of *LPA* gene. There are

recognizable consensus sequences for nonspecific promoter elements (TATA, CAAT boxes), hepatocyte transcription elements (HNF-1α, CEBP, LF-A1), and sequence motifs for interleukin 6 (IL-6) (Wade *et al.*,1993). This is consistent with findings that the Lp(a) particle probably contributes to wound healing processes.
A polymorphic variant -772G/A was detected at this part of the promoter. It is not a functional change, however a minor -772A allele was significantly associated (p<0,001) with lower mean Lp(a) levels compared to the G variant in some studies (Brazier *et al.*,1999). Moreover such association persisted even after apo(a) isoform adjustment.

The pentanucleotide repeat polymorphism ($TTTTA_n$), referred as STR locus (Short Tandem Repeat), was described at a distance of -1231 bp upstream from the transcription start site, . In Caucasians the number of repeats range from 7 to 11. Several studies have been referred to significant association of differnt STR alleles and Lp(a) plasma levels (Kalina *et al.*,2001; Holmer *et al.*,2003). This polymorphic locus was able to explain from 5 to 14,3% of the Lp(a) plasma levels variability independently of apo(a) isoform size (Trommsdorff *et al.*,1995; Brazier *et al.*,1999). Such association is supposed to be due to linkage of certain STR alleles with a restricted range of $K_{1\,PA}$IV type 2 repeats and with functional variants from the coding sequences and transcription regulatory regions. In adition the work by Negi *et al.* (2004) proposed its possible direct effect on *LPA* gene transcription as a negative transcription binding site.

I.2.1.2.1.2.Enhancers

An intensive search for additional regulatory sequences of *LPA* gene uncovered two regions carrying properties of enhancers. Two different areas, 20 kb (DHIII enhancer) and 28 kb (DHII enhancer) upstream of the transcription start site, stimulate the transcription in an orientation independent manner (Wade *et al.*,1997; Huby *et al.*,2003; Puckey a Knight,2003). Neither of those enhancers appear to be cell type specific (Wade *et al.*,1997; Yang *et al.*,1998). It is possible that the chromatin structure at these sites is permissive for interaction with transcription

factors only in differentiated hepatic tissue. It is consistent with the observed position of these enhancers within DNaseI hypersensitive sites that are present only in hepatoma cell chromatin and absent in other cell types (Wade *et al.*,1997).

The DHII enhancer activity is colocalized to a 180 bp fragment and stimulates transription activity from the *LPA* gene promoter from 4 to 8 fold (Wade *et al.*,1997; Puckey a Knight,2002). Various members of the nuclear receptor family (PPARα, ESR) were reported to bind the DHII enhancer binding elements, suggesting to mediate drug and hormone responsiveness (Puckey a Knight,2002). No sequence variability was detected in this enhancer.

The DHIII enhancer activity was mapped to a 600 bp fragment that stimulates *LPA* gene expression from 10 to 15 fold (Wade *et al.*,1997). The region was revealed to be a part of LINE-1 retrotransposon, indicating regulation sequences of retrotransposons may alter the expression of nearby genes (Yang *et al.*,1998). Three base substitutions (1230A/G ; -1617C/A; -1712G/T) with a direct functional impact on enhancer activity were detected in this enhancer (Puckey and Knight,20003).

I.2.1.2.2.Kringle sequences

Only a few functional variants were discoverd in the coding sequence of *LPA* gene so far. In particular in kringleIV type 8 p.P52L substitution associated with lower mean Lp(a) levels and both carriers contained the STR allele with 11 repeats in their promoter (Ogorelkova *et al.*,2001). The p.T23P substitution is found in the same kringleIV type 8 and is correlated with lower risk of atherosclerosis development (Ogorelkova *et al.*,2001). The sequence change p.M75T in kringleIV type 9 was linked to increased Lp(a) levels and to higher risk of myocardial infarction (Simó *et al.*,2001). Finally a rare substitution p.W72R that has a direct effect on Lp(a) particle binding properties to extracellular and cellular surfaces was detected in the kringleIV type 10 (Simó *et al.*,2001).

Null alleles are defined by absence of apo(a) isoforms from immunoblots which depends on the method sensitivity. Such null alleles exist over the whole

spectrum of apo(a) isoform size but are more frequent within large apo(a) alleles (Gaw *et al.*,1994; Kraft *et al.*,1996). Only two transcript negative null alleles were detected so far. In the first exon of kringleIV type 8 a substitution 1G/A results in an alternative splice site activation and with truncated apo(a) protein (Ogorelkova *et al.*, 1999). The base change 61C/T resides in the repetitive kringleIV type 2 region and creates a termination codon (Parson *et al.*,2004).

I.2.2.LIPOPROTEIN(a) PARTICLE

I.2.2.1.Lipoprotein(a) structure

Apolipoprotein(a) is secreted from the hepatocyte as a free protein in a closed conformation (Demant *et al.*,2001; Becker *et al.*, 2003). Conformational status of apo (a) plays a significant role in determining the efficiency of covalent Lp(a) particle formation. The open conformation of apo(a) forms covalent Lp(a) particles more efficiently than closed form of the molecule (Becker *et al.*, 2004). There is an inverse relationship between apo(a) isoform size and the efficiency of transition from closed to open apo(a) conformation. Lipoprotein(a) assembly takes place extracellularly as a two-step process in which initial noncovalent interactions between apo(a) and apoB-100 precede the formation of a single disulfide bond (Trieu a McConathy,1995; Gabel a Koschinsky,1998). The noncovalent step is mediated by interactions between apo(a) kringleIV type 7-8 and amino-terminal lysine residues of apoB-100 (Brunner *et al.*,1993; Koschinsky *et al.*,1993).

Some of the apo(a) kringle IV domains are characterized by the presence of lysine-binding pockets. The kringleIV types 5-8 each contain a weak lysine-binding site, while apo(a) kringleIV type 10 contains a stronger lysine-binding site that has been proposed to mediate the binding of apo(a)/Lp(a) to ligands such as fibrin (Scanu *et al.*,1993; Ernst *et al.*,1995; Gabel *et al.*,1996).

I.2.2.2.Plasma lipoprotein(a) levels

Despite nearly exclusive genetic determination to a unique locus Lp(a) concentrations are highly variable among individuals in all populations studied so far (Kraft *et al.*,1996; Paultre *et al.*,2002). Plasma levels vary over a 1 000-fold, ranging from 0.1 to over $6x10^2$ mg/dl. Lipoprotein levels are much less affected by age, gender, weight and diet than other classes of lipoproteins and remain relatively stable throughout life (Boerwinkle *et al.*,1992; Boomsma *et al.*,1993; Wild *et al.*,1997; Foody *et al.*,2000). As an exception menopause is associated with an 10-30% increase in Lp(a) concentration (Jenner *et al.*,1993; Wilmink *et al.*,2000).

The apo(a) circulating in plasma is synthesized exclusively in the liver (Hobbs a White,1999). Differences in plasma levels of Lp(a) are determined by differences in the rate of production, and not degradation. Individuals with diverse Lp(a) levels possess the same catabolised fraction of Lp(a) particle (Rader *et al.*,1993;1994). Multiple factors contribute to the characteristic rate of Lp(a) production. The limiting step is the amount of apo(a) secreted by hepatocytes. It could be influenced by the *LPA* gene transcription rate, mRNA stability and by posttranslation modification. The critical event is the time spent by apo(a) molecules of diffrent length in the endoplasmic reticulum. Larger apo(a) isoforms are retained for a prolonged time and thus greater proportion of these isoforms is degraded by proteasomes. Thus the apo(a) gene size heterogeneity (K_{LPA}IV type 2 repetition) accounting for 40-60% of the Lp(a) level variance (Kraft *et al.*,1992; Brazier *et al.*,1999).

I.2.2.3.Pathophysiology of lipoprotein(a)

Apo(a) is presnt in atherosclerotic but not normal vessel walls, which suggests that Lp(a) may play a role later in the atherosclerotic process, after lesions have developed. In the early plaque, most of the Lp(a) is located within endothelial cells (Jamieson *et al.*,1995). Lipoprotein(a) may get trapped in lesions by binding to components of the subendothelial matrix and cell surfaces (Beisiegel *et al.*,1990;

McConathy a Trieu,1991; d'Angelo *et al.*,2005; Tsurupa *et al.*,2006). In more advanced lesions, the apo(a) is predominantly situated extracellularly within the thickened intima (Jamieson *et al.*,1995). Lipoprotein(a) appears to accumulate preferentially to LDL within lesions (Beisiegel *et al.*,1990; Lawn *et al.*,1992). Localization of Lp(a) to the lesion will increase local cholesterol deposition and may have other effects that promote lesion development (Grainger *et al.*,1993; Buechler *et al.*,2003; Sotiriou *et al.*,2006).

Many of the potential atherogenic properties of Lp(a) can be attributed to apo(a) structural similarity with plasminogen and its inability to be modified to active protease and thus it possesses strong antifibrinolytic character (Rouy *et al.*,1992; Palabrica *et al.*,1995; Falco *et al.*,1998).

1.2.2.4.Physiological role of lipoprotein(a)

Perhaps the most plausible idea regarding a possible physiological role for Lp(a) is that it participates in wound healing (Hobbs a White,1999). Apolipoprotein (a) is present in lesions during the early stages of healing, when fibrin clots encase the wound and the wound is infiltrated with inflammatory cells (Yano *et al.*,1997; Mooser *et al.*,2000). Lipoprotein(a) clots the fibrous cap and is present within and surrounding the endothelial cells of newly infiltrating blood vessels (Mooser *et al.*,2000). After the epithelium has regenerated the apo(a) molecule is no more detected in the healing lesion, suggesting an early role for Lp(a) particle in the healing process (Yano *et al.*,1997).

II.AIMS OF THE STUDY

- to scan the variability in known *LPA* gene control regions (promoter, DHII and DHIII enhancer)
- to analyse the association of found polymorphic variants with quintiles of different range of Lp(a) levels (after the subjects were separated into quintiles and randomly chosen sample from each quintile was derived)
- to assign the rate of linkage disequilibrium (LD) between each pair of polymorphic sites
- to investigate if certain compound (5-polymorphic) genotypes are associated with narrow range of Lp(a) levels (at least in part due to linkage with restricted range of apo(a) gene K_{LPA}IV type 2 length variants)

III.MATERIALS AND METHODS

III.1.SUBJECTS

Two groups of subjects were used in this study. The DNA of 263 subjects from unrelated Caucasian individuals from Czech population was obtained from the Institute of Biology and Medical Genetics, 1st Faculty of Medicine, Charles University in Prague. These were apparently normal individuals from screening projects and volunteers and we used them as the population sample.

The study subjects were selected from the DNA pool comprising out-patients from the Lipid Clinic of the 3rd Medical Department, 1st Faculty of Medicine and General Teaching Hospital, Charles University in Prague. Individuals with renal insufficiency and endocrine disorders were excluded from the study. DNA and Lp(a) levels were available for 1 830 unrelated individuals, either healthy or with dyslipidemia. 82.2% of these were users of hypolipidemic therapy with statin further in 30.4% of them in combination with fibrates. The subjects were separated into quintiles and randomly chosen sample from each quintile was then derived having the total number of 713 individuals. 1st quintile Lp(a) levels from 0 to 8 mg/dl (134 subjects), 2nd quintile Lp(a) levels from 9 to 15 mg/dl (134 subjects), 3rd quintile Lp (a) levels from 16 to 24 mg/dl (120 subjects), 4th quintile Lp(a) levels from 25 to 64 mg/dl (146 subjects) and 5th quintile Lp(a) levels from 65 to 335 mg/dl (179 subjects). DNA from these individuals was analysed. The more detailed characteristics of each quintile is in Table.3.1.

The compound genotypes analyses were done on a restricted sample size of 664 individuals. In comparison with the previous part of the study we selected only 130 subjects from the 5th quintile to have a comparable sample sizes for all quintiles.

The study was approved with the ethics committee and all subjects were given informed consent.

Table.3.1.

Clinical characteristics and serum lipid parameters in each quintile.

	1st quintile	2nd quintile	3rd quintile	4th quintile	5th quintile
	Lp(a) 0-8 mg/dl	Lp(a) 9-15 mg/dl	Lp(a) 16-24 mg/dl	Lp(a) 25-64 mg/dl	Lp(a) 65-335 mg/dl
avarage age (years)	47	49	50	53	54
age range (years)	8-96	6-97	6-98	7-98	7-98
gender (% male)	49.9	50.4	45.0	47.6	41.2
Lp(a) (mg/dl)	2.4 ± 3.7	12.1 ± 3.4	20.2 ± 3.9	40.3 ± 13.1	110.8 ± 41.4
total cholesterol (mg/dl)	240.8 ± 101.4	249.2 ± 81.6	251.4 ± 75.8	257.3 ± 77.6	262.6 ± 69.4
LDL cholesterol (mg/dl)	116.2 ± 87.2	124.5 ± 82.3	132 6 ± 82.2	145.4 ± 83.9	154.2 ± 76 1
HDL cholesterol (mg/dl)	47.7 ± 22.9	48 4 ± 21.1	49.3 ± 21.9	52 1 ± 20.4	53.2 ± 19.8
triglycerides (mg/dl)	231.9 ± 293.5	239.3 ± 239.6	222.0 ± 225.5	211.7 ± 220 0	189.6 ± 157.5
BMI (kg/m^2)	27.2 ± 5.8	26.4 ± 3.7	27.0 ± 3.9	26.9 ± 4.5	26 3 ± 4.3
arterial hypertension (%)[1]	32.8	27.5	45 8	36.9	35.4
ischemic heart disease (%)	12.3	11.3	10.8	7.0	19.3
current and ex-smokers (%)[2]	23.1	31.0	31.7	28.3	24.5

[1] Defined as systolic blood pressure 140 mmHg or diastolic blood pressure 90 mmHg, or the use of antihypertensive medication.

[2] Subjects who have smoked more than 100 cigarettes in their lifetime.

III.2.LP(a) CONCENTRATION

Lipoprotein(a) concentrations were measured using frozen serum (-20°C), separated within two hours after venous blood collection, by 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. Lipoprotein(a) levels for the population sample were not available.

III.3.DNA ANALYSIS

III.3.1.AMPLIFICATION REACTION

DNA was isolated from fresh or frozen whole blood using a salting out method modified according to Miller *et al.* (1988). The promoter region and the DHII and the DHIII enhancer sequences were amplified. The 50 μl reaction mixture contained 120-250 ng of genomic DNA, 3.5 mM (promoter) or 2 mM (DHII enhancer) $MgCl_2$, 200 ng of each primer, 0.1 mM of each dNTP, 1% DMSO and 0.5 unit of Taq DNA Polymerase (recombinant) in the buffer provided by the manufacturer (Fermentas, Vilnius, Lithuania). Amplification conditions were 96°C for 2 min as a denaturation step followed by 35 cycles of 30 sec at 94°C, 30 sec at 62°C and 30 sec at 72°C, ending with 3 min at 70°C. The 1 142-bp unique fragment containing the whole DHIII enhancer sequence was produced according to Puckey and Knight (2003). As the DHIII core enhancer region is 600 bp long, we divided it in two separate amplified sequences with a 39-bp overlap. The first DHIIIA fragment is 486 bp long and the second DHIIIB fragment is 243 bp long. The 50 μl amplification reaction mixture for both fragments contained 1μl of the 1 142-bp PCR product as a template, 1.5 mM $MgCl_2$, 200 ng of each primer, 0.1 mM of each dNTP and 0.5 unit of Taq DNA Polymerase (recombinant) in the buffer provided by the manufacturer (Fermentas). Amplification conditions for both DHIII fragments were 96°C for 2 min as a denaturation step followed by 35 cycles of 30 sec at 94°C, 30 sec at 68°C and

Table.3.2.

Primer sequences used to amplify *LPA* gene transcription regulatory regions and to prepare the DGGE artificial positive controls

primer name	primer sequence	T_m °C	amplicon size
promotorF	[5] tatgttccattttgggactgg [3]	60	346 bp
promotorR[1]	[5] gatgaaggtctaggggtgagg [3]	60	
promotorMut[2]	[5] gatgaaggtctaggggtgagggaaggagtaaggatac [3]	-	
DHIIF	[5] tagcttttctgtgcatcggtctc [3]	63	266 bp
DHIIR[1]	[5] atattgggggttcaggccac [3]	63	
DHIIMut[2]	[5] atattgggggttcaggccaccactagcgga [3]	-	
DHIIIAF	[5] ctgctcaggggtcaggggtc [3]	67	486 bp
DHIIIAR[1]	[5] ctcccacccgaatactgcgc [3]	67	
DHIIIAMut[2]	[5] ctcccacccgaatactgcgcttttccgacaggct [3]	-	
DHIIIBF[1]	[5] tccgagccaggtgcgggata [3]	70	243 bp
DHIIIBR	[5] cgcccgccactgtgcgccagccgaagc [3]	72	
DHIIIBMut[2]	[5] tccgagccaggtgcgggatataaactcccggcgca [3]	-	
STRF (FAM)	[5] cagctactagggaggctgga [3]	60	164-184 bp
STRR	[5] ttcatttgcggaaagattga [3]	59	

[1] GC-clamped primer, sequence of the clamp: [5] gcgcccgcggccgcccggcccgcccgcccgcgccgccccg [3]

[2] mutation primer; t the site of introduced mutation

20 sec at 72°C, ending with 3 min at 70°C. All primers used to amplify *LPA* gene regulatory sequences are summarized in Table.3.2.

The pentanucleotide microsatelite from the distal promoter was amplified using one fluorescently (FAM) labelled primer (Table.3.2.). The 25 ml amplification mixture contained 120-250 ng of genomic DNA, 2.5 mM $MgCl_2$, 200 ng of each primer, 0.1 mM of each dNTP and 0.5 unit of Taq DNA Polymerase (recombinant) in the buffer provided by the manufacturer (Fermentas). Amplification conditions were 96°C for 2 min as a denaturation step followed by 25 cycles of 30 sec at 94°C, 30 sec at 62°C and 20 sec at 72°C, ending with 3 min at 70°C. The amplified product size

ranged from 164 bp (7 repeats) to 184 bp (11 repeats). The exact length was measured by fragmentation analysis conducted on ABI PRISM 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

The 263 subjects of the population sample were directly genotyped for six selected polymorphic sites from the regulatory regions of *LPA* gene (+121G/A; +93C/T; -1231 $TTTTA_n$; -1712G/T; -1617C/A; -1230A/G). The promoter region, DHIIIA, and DHIIIB enhancer fragments were amplified and the $TTTTA_n$ repetition was genotyped according to above-mentioned protocols. All RFLP assays were carried out in 10 ml reaction mixtures using 5 units of respective restriction enzyme and 5 ml of relevant amplified fragment. The respective fragments were restricted by *Hpy*188III (NEB, Inc., Ipswich, United Kingdom), *Hpy*CH4IV (NEB), and *Dpn*II (NEB) enzymes to get the +121G/A , +93C/T , and -1230G/A polymorphisms, respectively.

Population frequencies of two variants, -1712G/T and -1617C/A, were evaluated by allele specific amplification. Both 25 µl reaction mixtures contained 120-250 ng of genomic DNA, 1.0 mM $MgCl_2$, 0.08 mM of each dNTP, and 0.3 unit of Taq DNA Polymerase (recombinant) in the buffer provided by the manufacturer (Fermentas, Vilnius, Lithuania). The -1712G/T variant was detected using 70 ng of allele-specific 1712alA primer, and 50 ng of 1712com primer. As for the -1617C/A polymorphism, the appropriate amplification fragments were produced using combination of 70 ng of 1617alA primer, 90 ng of 1617alC primer, and 50 ng of 1617com primer in each amplification reaction. All primers used for allele specific amplification are summarized in Table.3.3. Amplification conditions were 96°C for 2 min as a denaturation step followed by 35 cycles of 30 sec at 94°C, 20 sec at 58°C (-1712G/T) or at 50°C (-1617C/A), and 20 sec at 72°C, ending with 3 min at 70°C. Products of amplification reactions were then subjected to 2% agarose gel electrophoresis and visualized by following ethidium bromide staining. The -1712T allele carriers were subsequently proved by sequencing reaction of DHIIIB fragment on ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Table.3.3.

Primer sequences used to detect -1712G/T, and -1617C/A polymorphisms in the *LPA* gene DHIII enhancer region using allele-specific amplification

primer name	primer sequence	T_m °C	amplicon size
1712alA[1]	5' gccagccgaatcatggt 3'	56	181 bp
1712com	5' gtgcgggatataaactcc 3'	55	
1617alC[1]	5' acctggaaaatcgagtc 3'	50	160 bp
1617alA[1,2]	5' *atgcgaaacggaatgcgttaaatgctaa*acctgga aaatcgagta 3'	50	188 bp
1617com	5' ccttgcagtttgatctc 3'	50	

[1] allele specific primer; **t** is the site of introduced change

[2] *atgcg* the non complementary part of primer

III.3.2.DGGE ANALYSIS

To find unknown changes, the DGGE analysis of amplified fragments was used (BioRad Laboratories Inc., Hercules, CA, USA; Hoefer, Amersham Biosciences Corp., San Francisco, CA, USA) combined with sequencing. Moreover, a specific mutation primer was designed for each analysed fragment to obtain artificial positive controls (Table.3.2.). The change was introduced to a domain with the highest melting temperature in the vicinity of GC-clamped primer. It is supposed to be the site with the most difficult substitution detection. Mutated PCR products were used in the course of condition optimisation and as positive controls during each run. Heteroduplexes were formed by denaturation of amplified products for 5 min at 96°C, followed by incubation for one hour at annealing temperature. The DGGE conditions for the minimal promoter fragment were 6% PAD gel, 16 cm long with, denaturing gradient 20-80%, run for 8 hours at 150 V and 60°C. The DHII amplicon was run for 8 hours at 150 V and 60°C on 8% PAD gel, 10 cm long with denaturing gradient 30-80%. The DHIIIA fragment was analysed in 6% PAD gel, 16 cm long with denaturing gradient 30-90%, and run for 16 hours at 150 V and 60°C.

The conditions for the DHIIB fragment were 8% PAD gel, 10 cm long with denaturing gradient 40-80%, run for 2.5 hours at 150 V and 60°C. All novel SNPs were verified by two different methods (sequencing, DGGE) and in two independent PCR reactions and DGGE runs.

III.4.STATISTICAL ANALYSIS

Allele frequencies were obtained by allele counting and standard errors were assigned. Each polymorphic locus was tested for significant deviation of genotype distributions from Hardy-Weinberg equilibrium using the χ^2 test ($p<0.05$; 1d.f.) for di-allelic sites and the exact test for the STR locus. Significance of allele frequencies differences between quintiles was evaluated using the χ^2 test. Non-random association between each pair of sites was measured by the standardized disequilibrium statistic (Δ) (Chakravarti *et al.*,1984), after haplotype frequencies were estimated by maximum likelihood procedure. The statistical significance of Δ statistic was tested applying a χ^2 test of independence. Association of compound genotypes with Lp(a) levels was evaluated using the 1-way ANOVA test (STATISTICA, version 7.1). Haplotypes were reconstructed using Haploview (version 32.2) programme (www.broad.mit.edu/mpg/haploview) (Barrett *et al.*,2005).

IV.RESULTS

IV.1.POLYMORPHISMS IN THE PROMOTER

The 346 bp fragment harbouring sequence of the promoter was analysed. Except for the two previously described polymorphisms, +121G/A; +93C/T, we have observed three novel substitutions +62T/C (8 subjects); -70C/T (1 subject); -93G/A (1 subject). The two latter variants were present only once in the same individual suggesting the cis position. Allele frequencies of the two polymorphic sites are summarized in Table.4.1. The genotype frequencies distribution for both promoter polymorphic sites was in Hardy-Weinberg equilibrium in all quintiles. There is a bimodal distribution of the +121A allele across quintiles having the highest frequency in the 2nd and 5th quintile, that was significantly ($p=0.020$) increased comparing to the 4th quintile. The +93T allele frequency was comparable in all quintiles with an exception of the 5th quintile where was the frequency significantly ($p=0.005$) decreased. The +62T/C variant was found in four carriers from the 3rd quintile, in three carriers from the 4th quintile and in one carrier from the 5th quintile [mean Lp(a) level in carriers 33.5 ± 5.3 mg/dl]. All carriers of the +62C variant, except one with the 9-STR allele, possess the STR allele with 10 repetitions and +121G/G and +93C/C promoter genotypes.

The pentanucleotide microsatelite at the distal promoter of *LPA* gene was amplified. The PCR product varied in size and cover from 7 to 11 TTTTA repeats. Allele frequencies of the STR locus in each quintile are summarized in Table.4.1. The most common allele had 8 repeats. There was a significantly ($p=0.003$) higher joint frequency of short alleles (7- and 8- repeats) in the 5th quintile comparing to other quintiles. The 9-STR allele frequency was significantly ($p=0.030$) decreased in the 5th quintile comparing to other quintiles. The STR allele with 10 repeats was underrepresented in quintiles of both extremes reaching the statistical significance when compared to the 2nd and 3rd quintile ($p=0.020$ for the 5th quintile and $p=0.040$ for the 1st quintile). The highest frequency of the 11-repeat allele was observed in

Table.4.1.

Allele frequencies and standard deviation of polymorphic sites from the apo(a) gene regulatory regions in quintiles.

polymorphism[1]		1st quintile	2nd quintile	3rd quintile	4th quintile	5th quintile
		Lp(a) 0-8 mg/dl frequency ± SD	Lp(a) 9-15 mg/dl frequency ± SD	Lp(a) 16-24 mg/dl frequency ± SD	Lp(a) 25-64 mg/dl frequency ± SD	Lp(a) 65-335 mg/dl frequency ± SD
+121	G/A	0.795/0.205 ± 0.025	0.776/0.224 ± 0.025	0.817/0.183 ± 0.025	0.853/0.147 ± 0.021	0.777/0.223 ± 0.022
+93	C/T	0.854/0.146 ± 0.021	0.858/0.142 ± 0.021	0.838/0.162 ± 0.024	0.853/0.147 ± 0.021	0.927/0.073 ± 0.014
STR	7	0.007 ± 0.005	0.000 ± 0.000	0.004 ± 0.004	0.003 ± 0.003	0.022 ± 0.008
	8	0.716 ± 0.028	0.668 ± 0.029	0.654 ± 0.031	0.709 ± 0.027	0.802 ± 0.021
	9	0.149 ± 0.022	0.160 ± 0.022	0.183 ± 0.025	0.140 ± 0.020	0.087 ± 0.015
	10	0.086 ± 0.017	0.142 ± 0.021	0.146 ± 0.023	0.130 ± 0.020	0.084 ± 0.015
	11	0.041 ± 0.012	0.030 ± 0.010	0.013 ± 0.007	0.017 ± 0.008	0.006 ± 0.004
-1617	C/A	0.892/0.108 ± 0.019	0.869/0.131 ± 0.021	0.829/0.171 ± 0.024	0.880/0.120 ± 0.019	0.793/0.207 ± 0.021
-1230	A/G	0.541/0.459 ± 0.033	0.463/0.537 ± 0.030	0.525/0.475 ± 0.032	0.568/0.432 ± 0.029	0.578/0.422 ± 0.026
number of individuals		N=134	N=134	N=120	N=146	N=179

[1] Nucleotide positions in the proximal promoter are expressed relative to the transcription start site. The STR alleles are discriminated according to the TTTTA repeat number and the locus is mapped at the position -1231 from the transcription start site. In the DHIII enhancer region is the nucleotide position designed to be numbered from the 3'-end of the 1.8-kb KpnI fragment as was outlined by Wade *et al.* (1997).

quintile including two homozygotes that were not detected in other quintiles and in the 1st quinitile which affected the Hardy-Weinberg equilibrium. The STR genotype frequencies distribution in other quintiles was in Hardy-Weinberg equilibrium. The difference in 11-repeat allele frequency in the 1st and in the 2nd quintile comparing to the 5th quintile was statistically significant ($p=0.002$ and $p=0.017$, respectively).

Group of 263 subjects, considered as the population sample, was genotyped four five selected single nucleotide polymorphism and one STR locus from the *LPA* gene transcription regulatory sequences. Ascertained allelic frequencies of these polymorphic sites in the population sample were as follows: +121G/A (0.814/0.186 ± 0.017); +93C/T (0.882/0.118 ± 0.014); -1231 $TTTTA_n$ STR-7 (0.006 ± 0.003), STR-8 (0.705 ± 0.020), STR-9 (0.120 ± 0.014), STR-10 (0.150 ± 0.016), STR-11 (0.019 ± 0.006), -1712 G>T (0.996/0.004 ± 0.003). The genotype frequencies distribution for all polymorphic sites was in Hardy-Weinberg equilibrium.

IV.2.POLYMORPHISMS IN THE ENHANCERS

The 266 bp fragment encompassing the entire DHII enhancer was almost fully conserved with only two mutations detected, -213C/T and -114G/T, each of them observed only once in individuals from the 5th and 2nd quintile, respectively. Neither of them reside DNase-I footprints located close to them (Puckey and Knight,2003; Wade *et al.*,1997).

The 600 bp core DHIII enhancer was divided into two separately analysed PCR products with a short overlap. We detected three already reported variants: -1712G/T (2 subjects); -1617C/A; -1230A/G, and eleven novel rare base substitutions: -1711C/T (1 subject); -1586G/A (1 subject); -1557A/G (4 subjects); -1540G/A (1 subject); -1521G/A (1 subject); -1408C/T (1 subject); -1357C/T (1 subject); -1301 C/T (1 subject); -1274G/A (1 subject); -1253G/T (2 subjects); -1219G/A (1 subject). Allele frequencies for DHIII enhancer polymorphic sites in quintiles are summarized in Table.4.1. The genotype frequencies distribution for

polymorphic sites was in Hardy-Weinberg equilibrium in all quintiles. The -1617A allele frequency seems to have a bimodal distribution across quintiles reaching the peak in the 3rd and 5th quintile, in the latter one being significantly (p=0.013) increased comparing to the 1st, 2nd and 4th quintile. The highest frequency of the -1230G allele was observed in the 2nd quintile that was significantly (p=0.014) different from the frequency in the 4th and 5th quintile.

Ascertained allelic frequencies of these polymorphic sites in the population sample were as follows: -1617C/A (0.856/0.144 ± 0.015); -1230A/G (0.532/0.468 ± 0.022).

IV.3.LINKAGE DISEQUILIBRIUM

Linkage disequilibrium was measured in the population sample for each pair of polymorphic sites in the promoter (+121G/A; +93C/T; STR) and for all pairs between these sites and two polymorphic DHIII enhancer variants (-1617C/A; -1230A/G). We considered three alleles for the STR locus. The allele with 7- and 8- repeats were designated as S (short), the allele with 9- repeats as 9, and the alleles with 10- and 11- repeats as L (long). The linkage disequilibrium for each STR allele (S, 9, L allele) was calculated as for di-allelic locus using the relevant allele frequency opposing to all other STR alleles pooled together. The -1712T variant was too rare to conduct LD calculation and was thus excluded from consequent analyses. Values of standardized disequilibrium statistics (Δ) are given in Table.4.2.

IV.4.COMPOUND GENOTYPES

We have detected 91 different 5-polymorphic (5-pol) genotypes (+121G/A; +93C/T; -1231 TTTTA$_n$; -1617C/A; -1230A/G) in the outpatient group. Only fifteen of them have overall quintile frequency above 0.01. These fifteen genotypes have a combined frequency of 0.689 in the population sample. Frequencies and relevant

Table.4.2.

Values of standardized disequilibrium statistic (Δ) between pairs of polymorphic sites from the *LPA* gene regulatory regions measured in the population sample

		promoter		STR[1]			DHIII enhancer	
		+121 G/A	+93 C/T	S/nonS	non9/9	nonL/L	-1617 C/A	-1230 A/G
promoter	+121 G/A	-	-0.127	-0.307‡	-0.143	-0.212‡	-0.102	0.441‡
	+93 C/T	-0.127	-	0.566‡	0.643‡	0.058	-0.105	0.211‡
STR[1]	S/nonS	-0.307‡	0.566‡	-	-	-	0.525‡	0.403‡
	non9/9	-0.143	0.643‡	-	-	-	-0.088	0.143
	nonL/L	-0.212‡	0.058	-	-	-	0.652‡	0.310‡
DHIII enhancer	-1617 C/A	-0.102	-0.105	0.525‡	-0.088	0.652‡	-	0.438‡
	-1230 A/G	0.441‡	0.211‡	0.403‡	0.143	0.310‡	0.438‡	-

[1] S allele corresponds to the 7- and 8-repeat allele, and L allele corresponds to the 10- and 11-repeat allele of the TTTTA repeat polymorphism from the apo(a) gene distal promoter (STR locus).

‡ statistically significant value of Δ statistic; $P < 0.001$

Table.4.3.

The most common 5-pol compound genotypes of *LPA* gene regulatory sequences, frequency in quintiles, relevant mean Lp(a) levels ± standard deviation (SD), and population frequency.

Genotypes[1 2]	Quintile frequency [3]	Mean Lp(a) (mg/dl)[4] ± SD	Population frequency
GGCC88CCAA	0.190	48.6 ± 51.4	0.183
GACC88CCAG	0.164	32.9 ± 42.0	0.133
GGCC810CAAG	0.065	47.6 ± 42.8	0.087
GGCT89CCAG	0.075	27.3 ± 31.6	0.065
GGCC88CCAG	0.032	35.0 ± 33.0	0.061
GGCC89CCAA	0.023	21.6 ± 33.0	0.030
AACC88CCGG	0.030	23.4 ± 35.8	0.027
GACT89CCGG	0.015	24.0 ± 37.1	0.027
GGCT810CCAG	0.017	12.8 ± 17.3	0.019
GGCT89CCAA	0.020	50.5 ± 56.5	0.015
GACC7/88CAAG	0.032	147.0 ± 49.1	0.011
GGCC7/88CAAG	0.015	29.8 ± 36.7	0.011
GGCT810CCAA	0.017	30.6 ± 42.4	0.008
GGCT910CAGG	0.015	23.3 ± 17.0	0.008
GACC89CCAG	0.014	11.6 ± 15.4	0.004

[1] genotypes are designed from the 3´- end to the 5´- end of the apo(a) gene locus

[2] 7/8 indicate that compound genotypes carrying both the 7 allele and the 8 allele in combination with the 8 allele of the STR locus were included

[3] compound genotype frequency in all quintiles (outpatient sample group)

[4] mean Lp(a) level and standard deviation associated with compound genotype in all quintiles

mean Lp(a) levels of the fifteen 5-pol genotypes in the outpatient group and frequencies in the population group are summarized in Table.4.3. There is a statistically significant association of mean Lp(a) levels with compound 5-pol genotypes (d.f.14/464; $F=10.54$; $p<0.0001$).

Particularly, one genotype GACC7/88CAAG was seen 20 times in the 5th quintile [mean Lp(a) level in carriers 147.0 ±49.1 mg/dl] and was not present in other quintiles and only three times in the population group. The genotype consists from the most common haplotype GC8CA and from a rare AC7/8AG haplotype. The AC7/8AG haplotype carriers were significantly ($P<0.0001$) associated with Lp(a) levels over 65 mg/dl. Several other 5-pol genotypes were also associated with a narrow range of Lp(a) levels. In detail, the GACC89CCAG (11.6 ±15.4 mg/dl), the GGCT810CCAG (12.8 ±17.3 mg/dl), and the GGCT910CAGG (23.3 ±17.0 mg/dl) genotype carriers were linked to low and middle mean Lp(a) concentrations. However, all these 5-pol genotypes were quite rare in the population group.

V.DISCUSSION

Microvariants *LPA* gene regulatory sequences are supposed to contribute to the high variability of Lp(a) plasma concentrations. We separated the subjects into quintiles to detect the distribution of polymorphic variants across the range of Lp(a) levels. The so called minimal promoter of *LPA* gene houses essential transcriptional and hepatic tissue specific regulatory elements (Wade *et al.*,1994). In our study we detected two reported polymorphisms (+121G/A; +93C/T) and three novel rare substitutions. The role of +121G/A substitution is somehow ambiguous. Study by Suzuki *et al.* (1997) had indicated its potentially positive role in *LPA* transcription what was not clearly reproduced by others (Wu and Lee,2003). The +121A allele was associated with elevated Lp(a) levels when compared to the major G allele in several studies (Puckey *et al.*,1997; Prins *et al.*,1999) but contrary data are also available (Brazier *et al.*,1999). It could be the linkage to other functional variants what can explain such heterogeneous data. This is supported by the strong linkage disequilibrium between the +121A allele and the DHIII -1230G variant as well as with short STR alleles (7- and 8-repeats) observed in our study. The +121A allele was also reported to be associated rather with middle sized (26-35 $K_{LPA}IV2$ repeats) and large apo(a) isoforms (Puckey *et al.*,1997).

Lower frequency of the +93T promoter variant in the 5th quintile is consistent with its previously described linkage disequilibrium with 9-STR allele and its direct negative effect on apo(a) translation efficiency (Zysow *et al.*,1995). The +93T variant was probably evolved on chromosome with 9 repeats and expanded to the other STR alleles during the locus evolution. The 9-STR allele was reported to be associated with apo(a) middle sized isoforms, $K_{LPA}IV2$ 24-34 repeats (Mooser *et al.*,1995). The three novel promoter variants do not reside in known transcription factors binding sites and are too rare to reveal a significant association with Lp(a) concentration. However, the +62C variant was only found among individuals with Lp(a) levels over 16 mg/dl.

Several studies have reported a correlation between the alleles of STR locus

at a distal *LPA* 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 5 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 distinct range of apo(a) isoforms and to other functional changes influencing apo(a) production rate. Moreover, recent work by Negi *et al.* (2004) proposed its possible direct effect as a transcription factor-binding site.

There was a limited incidence of the 11-repeat allele on short apo(a) isoforms in previous studies (15-24 repetic $K_{LPA}IV2$) (Mooser *et al.*,1995). However, this STR variant was very rare in the 4th and 5th quintile in our study, having the highest frequency on the opposite end of Lp(a) range. Ogorelkova *et al.* (2001) proposed a linkage between the P52L polymorphism and the 11-repeat allele. The P52L substitution in *LPA* gene coding region ($K_{LPA}IIV8$) may negatively modify protein folding. Deviation from the Hardy-Weinberg equilibrium observed in the 1st quintile was created because of selection criterion, since genotypes that are responsible for extremely low Lp(a) levels are expected to be overrepresented in this quintile.

An intensive search for additional *LPA* gene regulatory sequences uncovered two upstream regions having properties of enhancers (Wade *et al.*,1997; Huby *et al.*,2003; Puckey and Knight,2003). We investigated these sites for presence of common variants. The previous in vitro mutation analyses revealed sensitiveness of DHII enhancer activity to mutations (Wade *et al.*,1997). However, sequence of this enhancer exhibited a strong conservation and showed no common variability in our study. The DHII enhancer is the likely site of estrogen and other hormones and drugs operation (Puckey and Knight,2002). Our results indicate there is no common population variability in responsiveness to these molecules mediated by this *LPA* gene regulatory element. On the other hand, the DHIII enhancer possesses much higher diversity and could, at least to some extent, explain the high variability of Lp(a) levels. Except for three previously reported changes (Puckey and Knight,2003), -1712G/T; -1617C/A; -1230A/G, we have detected eleven novel variants.

The -1586G/A; -1408C/T; -1253G/T and -1219G/A base substitutions reside in distinct DNase-I protected sequences (Wade *et al.*,1997). The second one altered the core Ets transcription factor recognition element with significant effect on enhancer activity in previously reported mutagenesis attempts (Yang *et al.*,1998). Nevertheless, all novel variants are very rare, playing no significant role in Lp(a) plasma variability at a population level.

Selected polymorphic sites were genotyped in the population based sample group. Allele frequencies of these variants were comparable to frequencies in other Caucasian populations (Trommsdorff *et al.*,1995; Puckey *et al.*,1997; Kfaft *et al.*,1998) or to frequencies in the Puckey's study (Puckey and Knight,2003) with an exception of the -1712T variant that was quite rare in our population sample group.

Several studies have pointed out the low recombination rate in the whole *LPA* gene region and high linkage between observed polymorphic sites (Boerwinkle *et al.*,1992; Puckey *et al.*,1997; Puckey and Knight,2003). We have calculated the rate of genetic linkage between each pair of polymorphic sites involved in our study (with an exclusion of the -1712G/T base substitution). Our results confirmed the strong linkage disequilibrium between the 9-STR allele and the +93T variant detected also by others (Puckey *et al.*,1997; Kfaft *et al.*,1998). There was a persistent strong linkage even between regulatory sites more than 20 kb far-away.

In contrast with the persistent LD throughout the *LPA* locus, all major 5-pol compound genotypes were distributed in a broad range of lipoprotein levels. Only several rare 5-pol genotypes were restricted to a narrow range of Lp(a) concentrations. Each polymorphic site evolved as an independent event during the *LPA* gene locus evolution on certain apo(a) isoform background. Unequal sister chromatid exchange and gene conversion could be possible mechanisms of a new apo(a) isoform evolvement and redistribution of polymorphic sites without disrupting linkage disequilibrium. Thus, major 5-pol compound genotypes are not associated with restricted range of Lp(a) plasma levels.

VI.CONCLUSION

Increased levels of Lp(a) are supposed to be an independent risk factor for atherosclerosis. Apolipoprotein(a) determines structural and functional characteristics of the Lp(a) particle. The Lp(a) concentration is almost entirely genetically determined with an exclusive linkage to a unique locus, the gene for apo(a), so called *LPA* locus. Nevertheless Lp(a) concentrations varies widely between individuals in all populations studied so far. There is a *LPA* gene size heterogeneity (K_{LPA}IV type 2 repetition) accounting for 40-60% of the variance. Some of the variance could be additionally related to polymorphic sites either in the coding sequence or in transcription regulatory regions of *LPA* gene.

Only a few functional variants were discovered in a coding part of *LPA* gene so far. Moreover *LPA* gene transcription regulatory regions we had analysed, have not been revealed to be extremely polymorphic. However significant linkage disequilibrium was detected even between polymorphic sites from far regulatory sequences. In contrast with the persistent genetic linkage all major 5-polymorphic compound genotypes were distributed in a broad range of Lp(a) levels.

Considering the low recombination rate that tends to preserve the ancestral chromosome segment composition we suggest the unequal sister chromatid exchange and gene conversion as possible mechanisms of a new apo(a) isoform evolvement and redistribution of polymorphic sites without disrupting linkage disequilibrium. Thus, combined effect of all polymorphic sites from the whole *LPA* gene locus, including the gene length polymorphism, should be considered when dealing with high population variability of Lp(a) levels.

Besides, minor impact of other locuses and modulation by non-genetic factors should be considered.

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VIII.LIST OF PUBLICATIONS

1) **Zidkova K, Zlatohlavek L, Ceska R.** Variability in apo(a) gene regulatory sequences, compound genotypes, and association with Lp(a) plasma levels. In press. doi:10.1016/j.clinbiochem.2007.03.012
IF: 2,35
2) **Zidkova K, Kebrdlova V, Zlatohlavek L, Ceska R.** Detection of variability in apo(a) gene transcription regulatory sequences using the DGGE method. Clin Chim Acta 2007;376(1-2):77-81.
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3) **Zídková K.** Variabilita v regulačních oblastech genu pro apo(a) a její vztah k hladině Lp(a). Sborník 1.Královéhradeckých lékařských postgraduálních dnů, 2004:204-207.
4) **Zídková K**. Detekce variability v regulačních oblastech genu pro apo(a). Sborník 4. studentské vědecké konference (UK, 1.LF, Praha), 2003:91-93.
5) Zlatohlávek L, **Zídková K,** Vrablík M. Lipoprotein (a). In press. Vnitř Lék.
6) **Zídková K,** Zlatohlávek L, Češka R. Genetické aspekty velkého rozpětí hladin lipoproteinu(a). Submitted. Čas Lék Čes.