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Available online at www.sciencedirect.com
ScienceDirect
Molecular Genetics and Metabolism 91 (2007) 79–84
Molecular Genetics and Metabolism
www.elsevier.com/locate/ymgme

LPA +93C>T and +121G>A polymorphisms detection by electronic microchip technology

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Received 27 October 2006; received in revised form 20 December 2006; accepted 20 December 2006
Available online 26 February 2007

Abstract

Lipoprotein(a) [Lp(a)] is a LDL-like particle containing a single copy of apolipoprotein B-100 (apoB-100), covalently attached to apolipoprotein(a) [apo(a)]. Apo(a) is encoded by *LPA* gene (6q26-27), and it has been hypothesized that *LPA* +93C>T and +121G>A polymorphisms in the 5′ flanking region could influence the apolipoprotein(a) synthesis, so affecting Lp(a) levels. In order to permit a rapid detection of *LPA* polymorphisms, we performed an analysis protocol for the SNPs detection through Nanogen Technology with the Universal Reporting System, and we compared our results with those obtained with a more conventional method, such as PCR-RFLP assay. Our experiments evidenced that Nanogen Technology may be used as a high-throughput tool in *LPA* +93C>T and +121G>A polymorphisms analysis, minimizing the hands-on time and the costs for the SNPs detection. In particular, this Technology allows the analysis of polymorphisms at the *LPA* locus, able to modulate the levels of Lp(a), a relevant marker of atherosclerosis.

Keywords: Lipoprotein(a); Polymorphism; Electronic microchip

Introduction

Lipoprotein(a) [Lp(a)] is a LDL-like particle containing a cholesteryl ester-rich core, a single copy of apolipoprotein B-100 (apoB-100), covalently attached to the distinctive molecule of apolipoprotein(a) [apo(a)]. Data from a large number of clinical studies evidenced an association between high Lp(a) levels and an increased risk for atherosclerotic disease including coronary artery disease and stroke [1–3].

Apo(a) is a high molecular weight glycoprotein of 300–700 kD [4], sharing homologous domains with plasminogen, kringle 4 (K4) and kringle 5 (K5) [5], and it is subjected to size heterogeneity because of the presence of a variable number of the K4 repeat unit (from 3 to more than 40 copies) [6].

Plasma Lp(a) concentration is characterized by a wide inter-individual variability (ranging from <0.1 to >200 mg/dl) [7,8], while it remains relatively constant throughout life [9,10]. It has been evidenced an inverse relationship between Lp(a) levels and the apo(a) size [11–13]: smaller apo(a) sizes seem to be related to higher Lp(a) plasma levels [6].

Apo(a) is encoded by *LPA* gene (6q26-27) [14] (NCBI Official Gene Name: lipoprotein, Lp(a); Accession No. NT_007422), hypothesized to be responsible for more than 90% of variability in Lp(a) levels and the varying number of K4 repeats seems to account for about 70% of these variations [15,16].

Nevertheless, it has been observed a significant variability in the Lp(a) levels among individuals having the same apo(a) size-isoform [12,17], so suggesting that polymorphisms

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doi:10.1016/j.ymgme.2006.12.011

other than K4 repeats could influence the protein synthesis. Several single nucleotide polymorphisms (SNPs) have been identified in the *LPA* gene 5′-flanking region and *in vitro* studies, by chloramphenicol acetyltransferase (CAT) assay, evidenced a role for *LPA* +93 C>T and +121 G>A SNPs in modulating the promoter activity [5]. It has been hypothesized that both sites may have significant effects on *LPA* gene expression, as confirmed by site-directed mutagenesis [5,18]. However, the +93C>T variation has been observed to reduce the efficiency in translation, rather than transcription. It has been shown that the C>T transition introduces an additional upstream ATG translation start codon, thus leading to a 60% reduction in protein translation [19]. Concerning the *LPA* +121G>A SNP, it has been hypothesized a positive regulation on gene expression, so contributing to the modulation of Lp(a) plasma levels [5,20].

In this study, we have considered DNA microarray technology in order to analyze *LPA* +93C>T and +121G>A polymorphisms. Medium to high-throughput techniques, that permit to carry out rapid, accurate and a highly reliable genotyping, are now available. In particular, a recently developed technology, based on the application of an electric field and allowing the rapid deposition of biotinylated PCR products on a streptavidin-coated array, is composed of 10×10 addressable test sites (Nanogen Technology) [21,22]. The addressed amplicons are then hybridized to fluorescent-labeled specific oligonucleotide reporters, so permitting the discrimination among homozygotes for the wild-type and the rare allele and heterozygous samples at a specific incubation temperature.

Here we have taken into account the NanoChip Technology in the detection of *LPA* +93C>T and +121G>A polymorphisms, thus comparing the accuracy of this high-throughput system to traditional assays (PCR-RFLP analysis). Nanogen Technology allows a rapid detection of *LPA* 5′ upstream polymorphisms and may be useful in the evaluation of the genotype–phenotype relationship on a large number of samples, in order to better define the role of these polymorphisms in the modulation of Lp(a) levels, in consideration of the relevant role of Lp(a) in atherosclerosis.

Methods

Study population

Blood samples are obtained from 647 clinically healthy subjects, living in Florence (Italy) and recruited from the population study "Progetto Nutrizione per la Salute e la prevenzione di Malattia" [23].

LPA +93C>T and +121G>A gene polymorphisms detection

Genomic DNA has been extracted from peripheral blood leukocytes by using the Flexigene DNA kit (Qiagen, GmbH, Germany).

PCR amplification and Nanochip platform analysis

LPA +93C>T and +121G>A polymorphisms have been evaluated through Nanogen Technology (NanoChip® Molecular Biology Worksta-

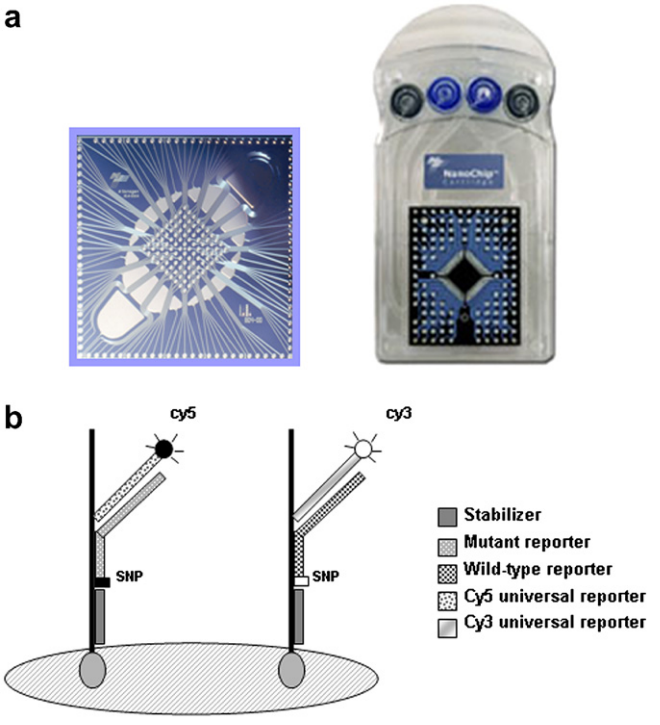

Fig. 1. (a) Electronic microchip characterized by the presence of 10×10 addressable sites (Nanogen Cartridge). (b) The Universal Reporting System is based on the use of a stabilizer and of mutant and wild-type reporters, consisting of a binding sequence, complementary to the gene, and of a tail sequence, unrelated to the gene sequence itself.

Table 1
LPA +93C>T and +121G>A Nanogen oligonucleotide sequences

SNP	Oligonucleotide	Sequence	T_m [a] (°C)	T_d [b] (°C)
LPA +93C>T	Wild-type reporter	5′-ctgagtccgaacattgagccGTAAGTCAACAAC-3′	34.0	37.0
	Mutant reporter	5′-gcagtatatcgcttgacattTGTAAGTCAACAAT-3′	35.4	
	Stabilizer	5′-GTCCTGGGATTGGGA-3′	50.3	
LPA +121G>A	Wild-type reporter	5′-ctgagtccgaacattgagttACACTTTCTGGG-3′	37.5	37.0
	Mutant reporter	5′-gcagtatatcgcttgacattACACTTTCTGGA-3′	35.2	
	Stabilizer	5′-CACTGCTGGCCAGTCCCAAAATGGAACATAAGGA-3′	65.6	
	TAIL-1	5′-ctgagtccgaacattgag-3′		
	TAIL-2	5′-gcagtatatcgcttgaca-3′		
	Universal reporter 1	5′-ctcaatgttcggactcag-3′		
	Universal reporter 2	5′-tgtcaagcgatatactgc-3′		

[a] Melting temperature.
[b] Discrimination temperature.

tion; 10 × 10 array NanoChip cartridge; Nanogen, San Diego, CA, USA), accordingly to protocols extensively described in a previous paper [21]. Moreover, we used the Universal Reporting System in order to limit the necessity to purchase expensive labeled-reporters [24]. The amplification of the sequence containing the two SNPs has been performed through PCR reaction in a MJ thermocycler (MJ Research, Waltham, MA, USA) with the following settings: one denaturation cycle at 95 °C for 5′, 35 cycles with denaturation at 94 °C for 1′, annealing at 54 °C for 50″ and extension at 72 °C for 50″, followed by a final extension at 72 °C for 7'. The reaction has been performed in a final volume of 25 μl with 100 ng of genomic DNA, 0.2 mM of each dNTP, 1 μl of a 10 μM forward primer (5′-TGACA TTGCACTCTCAAATATTTT-3′), 1 μl of a 10 μM reverse primer (5′-biotin-AGAACCACTTCCTTATGTTCCA-3′) and 0.5 U of Taq polymerase (GoTaq, Promega Italia, Milano, Italy) in 1× PCR Buffer.

The amplicons have been desalted with Nucleo Fast System (96-well PCR plates) from Macherey–Nagel (Macherey–Nagel Gmbh & Co. KG, Düren, Germany).

A 30 μl volume from purified samples has been mixed with 30 μl of 100 mM histidine and placed in a 96-well Nunc plate (Nalge Nunc international, Rochester, NY, USA). A 0.3 M NaOH solution, requested for the denaturation of the PCR product, a 50 mM histidine solution and three heterozygous control samples, have also been added to the plate. The samples prepared as described previously, according to NanoChip guidelines, have been electronically addressed to the test sites (pads) on the cartridge. Histidine 50 mM without amplicons has been bound to two pads of the chip for background subtraction.

After the addressing of the amplicons on the array, the chip has been rinsed with 150 μl of High Salt Buffer (50 mM sodium phosphate and 500 mM sodium chloride, pH 7.4) for 3′ and next with 100 μl of hybridization mix, before loading the cartridge into the instrument for the fluorescence scanning at the specific discrimination temperature (Table 1).

The hybridization mix contains 200 pmol of stabilizer, 100 pmol of wild-type and mutant reporter probe and 100 pmol of both Cy3 and Cy5-labeled universal reporter [24] (Table 1). Fluorescent oligonucleotide probes, specific for the wild-type and for the mutant allele recognize the sequence containing the SNP site and its 3′-flanking region. Each reporter probe has a tail at the 5′-terminus that is not complementary to the gene sequence but it hybridizes in a specific way with the universal reporters (Fig. 1).

The probe complementary to the tail of the wild-type reporter is 3′-labeled with Cyanine 3 (Cy3), while the probe complementary to the tail of the mutant reporter is 3′-labeled with Cyanine 5 (Cy5). The difference in hybridization energy between the matched and mismatched reporters was enhanced by a stabilizer oligonucleotide, which hybridizes in a strict proximity of the reporter detection probe with the gene sequence surrounding the SNP site; thus providing a base-stacking energy in order to improve the allele discrimination.

The mutation analysis is performed with a software that calculates the fluorescence values from each sample. Recorded values from heterozygous control samples are used to normalize the Cy3/Cy5 signals to a value of 1. The mean values for Cy3 (green bar graph) and Cy5 (red bar graph) fluorescence are obtained by averaging the amount of fluorescence in each pad and the software assigns directly the genotype to each sample by considering the Cy5/Cy3 ratio both for *LPA* +93C>T and +121G>A gene polymorphisms (Fig. 2). Samples demonstrating a Cy3:Cy5 signal ratio >5:1 or >1:5 have been assigned as wild-type homozygotes or mutant homozygotes, respectively; while samples with a Cy3:Cy5 signal ratio between 1:3 or 3:1 have been assigned as heterozygotes, as a default. Test sites with a signal-to-noise ratio (SNR) <5 and Cy5:Cy3 fluorescence values between 1:3 and 1:5 or 3:1 and 5:1 have been excluded from the analysis.

PCR-RFLP assay

LPA +93C>T and +121G>A polymorphisms have also been detected with PCR-RFLP analysis. The sequence surrounding the two SNPs has been amplified through PCR reaction at an annealing temperature of

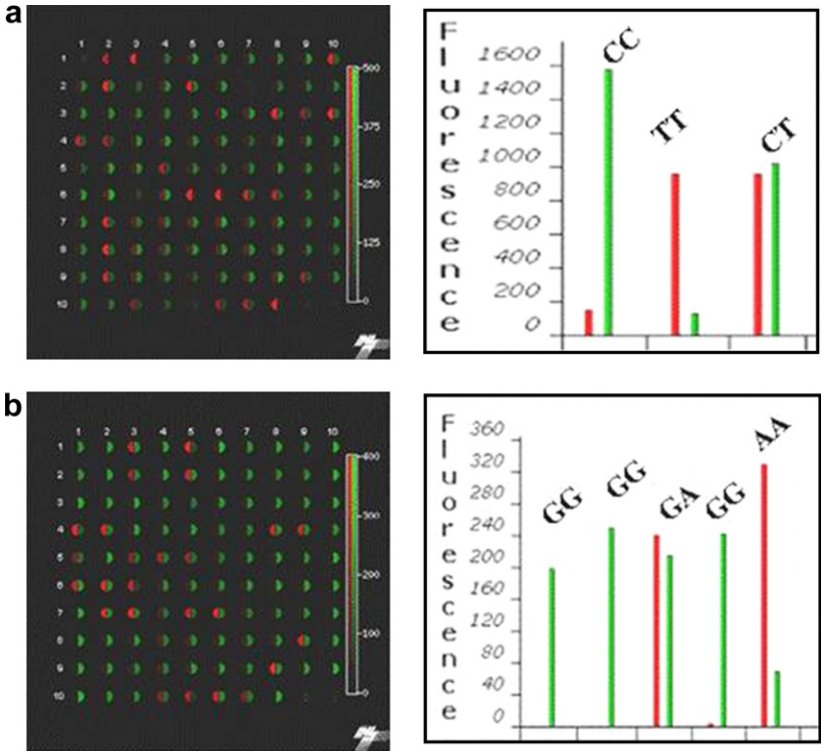

Fig. 2. The mean values for Cy3 (green bar graph) and Cy5 (red bar graph) fluorescence are obtained by averaging the amount of fluorescence in each pad and the software assigns directly the genotype to each sample by considering the Cy5/Cy3 ratio. If Cy5/Cy3 fluorescence ratio lies between 3:1 and 1:3, samples are designated as heterozygous, if Cy5/Cy3 ratio is >5:1, they are considered wild-type homozygous and if Cy5/Cy3 ratio is <1:5 mutant homozygous. (a) *LPA* +93C>T genotyping results (fluorescence histograms and array image). (b) *LPA* +121G>A genotyping results (fluorescence histograms and array image). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

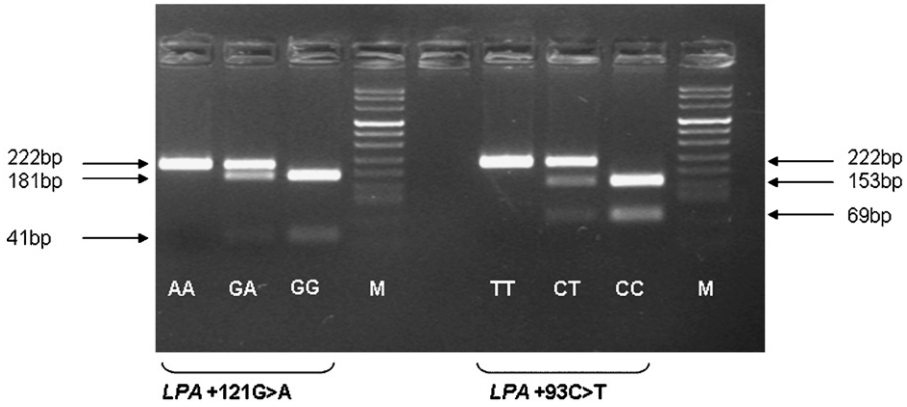

Fig. 3. *LPA* +93C>T and +121G>A gel electrophoresis on 3.5% agarose gel. **M** = DNA ladder (19–1114bp): 19bp, 26bp, 34bp, 37bp, 67bp, 110bp, 124bp, 147bp, 190bp, 242bp, 320bp, 404bp, 489bp, 501bp, 692bp, 900bp, 1114bp.

54 °C, by using the forward and reverse primers (without biotin-modification) and the same experimental procedure taken into account in the Nanochip analysis. In order to detect *LPA* +93C>T SNP, 10 μl of the PCR products (222 bp) are subjected to digestion with TaiI restriction enzyme (Fermentas International Inc., Burlington, Canada) while the evaluation of *LPA*+121G>A transition requires an enzymatic digestion with *Sdu*I (Fermentas International Inc., Burlington, Canada). Both the reactions are carried out at 37 °C for 16 h and the digestion fragments are separated on 3.5% agarose gel (Fig. 3).

Automated sequencing

The DNA sequencing has been performed by using the BigDye chemistry (Applied Biosystem, Warrington, Cheshire, UK).

Results and Discussion

To date *LPA* +93C>T and +121G>A polymorphisms have been detected through PCR-RFLP assay. Here we have developed an analysis protocol for the detection of *LPA* polymorphisms through Nanogen Technology with the Universal Reporting System. We have designed discriminator oligonucleotides, necessary for the hybridization reaction, and we applied specific discrimination temperatures to analyze the two SNPs. Afterwards, we have compared results from this technique with those obtained with a more conventional method. In order to perform this comparison we used both the assays to genotype 647 subjects (Table 2).

The Nanogen analysis evidenced a "call rate" for *LPA* +93C>T and *LPA* +121G>A SNPs of about 88% and 87%, respectively. The decreasing in genotyping efficiency may be due to both PCR failure or sample loss during purification and insufficient amount of genomic DNA in the PCR reaction mix. Samples producing a "no-call" have been re-amplified and retested after purification of the PCR products in a new experiment. After being performed the new analysis, it has been noted that only 0.02% of the samples do not produce a result, maybe due to a low DNA template concentration in the amplification reaction mixture.

Table 2
Genotype distribution and allele frequency of *LPA* polymorphisms

SNP	Genotype	Allele	Healthy subjects, $N = 647$ (%)
LPA +93C>T	CC		458 (70.8)
	CT		176 (27.2)
	TT		13 (2.0)
		T	0.16
LPA +121G>A	GG		524 (81.0)
	GA		120 (18.5)
	AA		3 (0.5)
		A	0.10

Moreover, we have used the Universal Reporting System in the DNA microarray analysis, thus reducing the cost of the original Nanogen procedure. In order to design the PCR primers for both microarray and PCR-RFLP assays, genomic DNA sequence (GenBank Accession No. NT_007422) surrounding the polymorphic sites has been submitted to BLAST search (Basic Local Alignment Sequence Tool; http://www.ncbi.nlm.nih.gov/BLAST/). So it has been possible to evidence regions with high homology to other genes and to avoid aspecific amplifications. BLAST search revealed a great similarity of *LPA* gene to the gene encoding for plasminogen (*PLG*), in particular in relation to the 5′-upstream regions.

Our results evidenced a 100% concordance between NanoChip analysis and PCR-RFLP assay, so supporting the use of the Nanogen Technology in the evaluation of *LPA* polymorphisms. The samples used as heterozygous controls in the Nanogen System have been sequenced (ABI310 automated sequencer) (Fig. 4), so contributing to produce more accurate results, and to minimize the risk of sample error.

The sequencing results evidenced the specificity of the amplification products; the primers used in the PCR reaction, annealing to regions showing a lower sequence homology to *PLG* gene (Fig. 4), and the high stringency PCR cycling conditions prevent the obtainment of aspecific products.

Moreover, the accuracy of our results has been further confirmed by the comparison between the electronic microchip analysis and the restriction enzyme digestion, and a complete agreement has been observed.

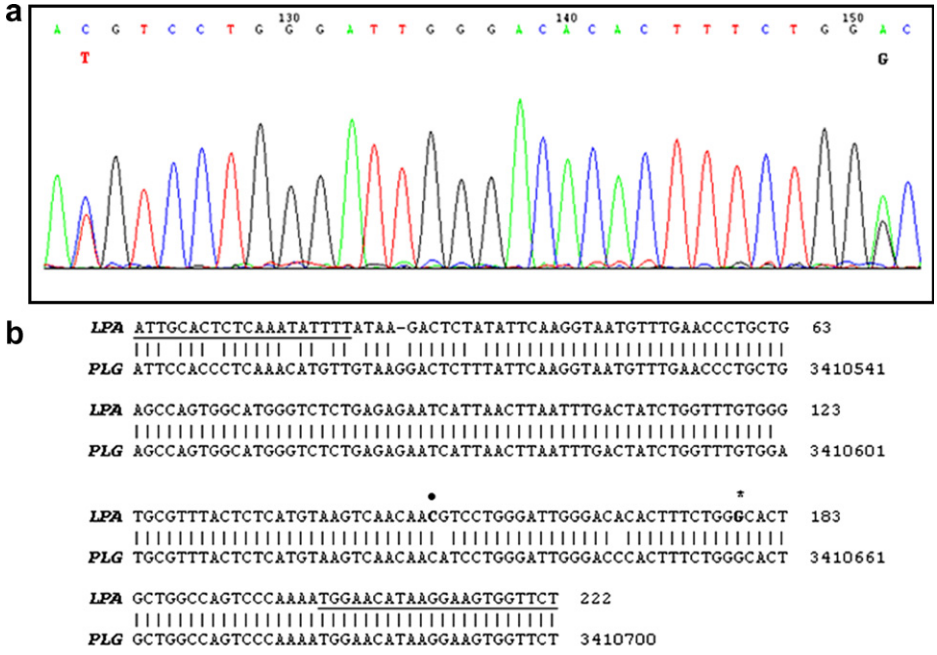

Fig. 4. (a) *LPA* +93C>T and +121G>A polymorphisms: automated sequencing performed by using the BigDye chemistry (Applied Biosystem, Warrington, Cheshire, UK). BLAST search (Basic Local Alignment Sequence). (b) Homology between *LPA* and *PLG* genes; forward and reverse *LPA* primers are underlined; ● *LPA* +93C>T; ∗ *LPA* +121G>A.

Table 3
Evaluation of costs and time consumed per assay

Method	Supplies[a] (€)	Technician			Total running cost per SNP (€)
		Total time consumed[b] (h)	Hands-on time (h)	Wages (€)	
RFLP	3	39	5	0.31	3.31
Microelectronic chip	2	8	1	0.06	2.06

H, hour.

[a] Supplies included all materials common to the different genotyping methods (thermowell plates, DNA polymerase, deoxynucleotides, primers, thermowell seals, tips) and specific materials for the two methods: (1) RFLP (agarose gel, tris-acetate-EDTA buffer, DNA molecular weight marker, polaroid film to photograph DNA fragments under UV illumination and restriction endonucleases); (2) microelectronic chip (Macherey–Nagel 96 well plates, Nanogen cartridges, universal reporters, histidine buffer, high and low salt buffers).

[b] Time consumed to genotype 96 samples for two SNPs.

NanoChip technology approach shortens the time required for the detection of each polymorphism; the hands-on time necessary for the analysis of the two SNPs at the *LPA* locus in 96 samples has been reduced of about 4 h with the electronic microchip strategy, in comparison to PCR-RFLP assay (Table 3). Another possible advantage of Nanogen technology is the reduction of the costs per assay: it has been observed a lowering of the total running cost per SNP of about 38% (Table 3). Therefore, Nanogen Technology allows to analyze a large number of subjects and in particular to get information regarding polymorphisms at the *LPA* locus observed to be involved in the modulation of Lp(a) levels. This study provides evidence of two possible aspects, a methodological and a clinical one. The methodological advantage is due to the rapid *LPA* +93C>T and +121G>A SNPs genotyping, and the clinical relevance lies in the possible characterization of subjects with high Lp(a) levels, which are known to modulate the vascular disease.

Nevertheless, a limit of our study is to have not taken into account the comparison between Nanogen Technology and "Real-time SNPs detection systems", such as TaqMan Probe method, which permits the direct genotyping of PCR reactions and is the preferred method in many laboratories.

In conclusion, our experiments evidence that the electronic microchips (Nanogen Technology) may represent a useful tool in the analysis of *LPA* +93C>T and +121G>A polymorphisms as a high-throughput system in the SNPs detection. It permits a high-speed genotyping and it reduces not only the technician time and the cost for the SNPs detection, but also the use of hazardous reagents (ethidium bromide), in comparison to conventional methods.

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