

# A new DNA polymorphism in the 5' untranslated region of the human SREBP-1a is related to development of atherosclerosis in high cardiovascular risk population

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## Abstract

Sterol-regulatory element binding proteins (SREBPs) are ubiquitous transcription factors that regulate the genes encoding key proteins in the control of cholesterol homeostasis. We looked for mutations or polymorphisms within the sequences of the SREBP-1a gene critical for the synthesis and/or activity of the protein in 204 asymptomatic men. A single G deletion at base pair –36 of the translation initiation site (designated G-) was found using single-strand conformation polymorphism (SSCP), in addition to three rare variants. This new marker was then assessed for its influence on the lipid parameters of 812 men at high cardiovascular risk, and on the presence of echographic atherosclerotic plaque in their peripheral arteries. The allelic frequency of the –36delG polymorphism was 0.58. At least one plaque was found in the carotid in 24% of subjects, in the femoral arteries of 48%, and in the aorta of 25%. There were significant associations between the –36delG polymorphism and mean total cholesterol ( $p = 0.02$ ) and LDL-cholesterol ( $P = 0.02$ ). There was a graded relationship between the G- allele and the presence of carotid plaque ( $r = 0.084$ ,  $P = 0.02$ ). In addition, there was a statistically significant interaction between the –36delG genotype and the apoE phenotype for plasma LDL-cholesterol ( $P = 0.04$ ) and apoB ( $P = 0.05$ ), suggesting a gene–gene interaction. Stepwise multiple regression analysis for lipid traits, risk factors, and apoE phenotype showed an independent association between carotid plaque and the –36delG polymorphism ( $\beta = 0.311$ ,  $P = 0.03$ ). Thus, we have identified a new polymorphism in the 5' untranslated region of the SREBP-1a gene, and demonstrated its association with an atherogenic lipid profile and echographic plaques. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** SREBP-1a; Polymorphism; SSCP; Atherosclerosis; Apolipoprotein E; Cholesterol

## 1. Introduction

Large epidemiological studies have established that elevated plasma lipid, lipoprotein, and apolipoprotein concentrations are risk factors for premature atherosclerosis. While cholesterol concentrations are strongly modulated by diet, the key steps in lipoprotein metabolism are under the control of a variety of genes. One powerful strategy for investigating candidate genes

likely to affect lipid metabolism and the atherosclerotic process is to conduct genetic and biochemical studies of their allelic variations [1]. The genes encoding a novel family of membrane-bound transcription factors, sterol-regulatory element binding proteins (SREBPs), can contribute to the variation between individuals in plasma cholesterol and in pathological processes including atherosclerosis. Cholesterol homeostasis is partly maintained by the sterol-regulated cleavage of three of these proteins: SREBP-1a, -1c and -2.

SREBP-1a and -1c are products of a single gene on chromosome 17; alternative splicing sites at the 5' and the 3' ends of the mRNA account for the existence of

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the two forms [2]. SREBP-2 is encoded by a separate gene located on chromosome 22; it has no known alternative form [2]. SREBP-1 and SREBP-2 have 47% identical amino acid sequences [3] and are tripartite proteins. The NH<sub>2</sub>-terminal segment of approximately 500 amino acids is a transcription factor of the basic-helix-loop-helix-leucine zipper (bHLH-Zip) family; there is also a hairpin membrane-anchor domain of approximately 80 amino acids comprising two transmembrane segments separated by a 31-amino-acid hydrophobic loop; and the carboxy-terminal segment has approximately 590 amino acids and forms a complex with the COOH-terminal domain of SREBP cleavage-activating protein (SCAP) [4–7]. The NH<sub>2</sub>-terminal segment of SREBP-1a and -2 begins with a cluster of negatively charged residues that form a putative transcriptional activation domain [3,4]. Mutational analysis has shown that this acidic region is the primary transcription activating domain [8]. A two-step proteolytic process releases the NH<sub>2</sub>-terminal fragment from the endoplasmic reticulum in cholesterol-depleted cells [9]. The first is catalyzed by site-1 protease (S1P), a membrane-bound subtilisin-related serine protease that cleaves the hydrophilic loop of SREBP [10]. This separation allows a second protease, designated site-2 protease (S2P), to cleave the NH<sub>2</sub>-terminal fragment at a point within its first membrane-spanning domain [11,12]. The NH<sub>2</sub>-terminal domain travels to the nucleus, where it activates the transcription of genes encoding the LDL receptor and several enzymes involved in cholesterol synthesis including 3-hydroxy-3-methylglutaryl coenzyme A [HMG CoA] synthase, HMG CoA reductase, farnesyl diphosphate synthase, squalene synthase and in fatty acid synthesis (fatty acid synthase) [4,13–15]. The family of SREBPs-responsive genes has been expanded to several genes controlling lipoprotein metabolism and the efflux of cellular cholesterol such as lipoprotein lipase [16,17], cholesteryl ester transfer protein [18], apolipoprotein AII [19,20], microsomal triglyceride transfer protein [21], and caveolin [22]. To date, no naturally occurring human mutations have been reported in either SREBP-1 or SREBP-2. Given the central role of SREBPs in regulating cholesterol, mutations or polymorphisms in SREBP-1 or SREBP-2 could influence the concentration of plasma and cellular cholesterol, and the early onset of atherosclerosis.

This work was done to identify genetic variants in the SREBP-1a gene by studying, in particular, the regions involved in the synthesis, functional activity and maturation of this transcriptional factor: the known -562 bp 5' flanking region; the transcription domain located in the  $\alpha$  helical acidic region at the NH<sub>2</sub> terminus; the basic-helix-loop-helix-leucine zipper domain; the first membrane-spanning helix and the luminal loop where the two cleavage sites are located.

This report describes the first evidence of a common polymorphism in the 5' untranslated region of the SREBP-1a associated with an atherogenic lipid profile and the development of atherosclerosis.

## 2. Materials and methods

### 2.1. Subjects

The subjects were recruited from an ongoing risk factor screening program conducted by a group of occupational health physicians (PCVMETRA Groupe: Prévention Cardiovasculaire en Médecine du Travail) at the work sites of employees of companies in the Paris area. After their consent had been obtained, 1194 men were referred to Hôpital Broussais, between November 1994 and July 1999 if they had any of the following characteristics: hypercholesterolemia (LDL-cholesterol > 4.1 mmol/l); hypertension (systolic blood pressure  $\geq$  160 mmHg and/or diastolic blood pressure  $\geq$  90 mmHg) at the work site; and tobacco consumption. Exclusion criteria included: (1) treatment for hyperlipidemia; (2) secondary hypercholesterolemia or hypertension; (3) hypertriglyceridemia > 4.5 mmol/l; and (4) any symptomatic cardiovascular disease such as stroke, transient ischemia, coronary heart disease, congestive heart failure and intermittent claudication. From the initial population, 204 subjects were selected for screening for molecular variants in their SREBP-1a gene. The inclusion of 608 additional subjects led to a total of 812 genotyped subjects.

### 2.2. Evaluation of risk factors

Plasma lipids were measured after participants had fasted for 14 h. Venous blood samples were withdrawn after the subjects had been in the supine position for at least 10 min. Serum total triglycerides and cholesterol were measured using classical enzymatic methods [23]. HDL-cholesterol and -phospholipids were measured enzymatically in the supernatant after selective precipitation of apolipoprotein B-containing lipoproteins with a phosphotungstic acid and magnesium chloride reagent [24]. LDL cholesterol values were calculated according to Dahlen's modification of the Friedewald formula for triglyceride values below 4.5 mmol/l [ $LDL-C = TC - HDL - C - (TG/2.2) - Lp(a) \times 0.75$ ] (in mmol/L) [25]. Apolipoproteins AI and B were measured by means of immunonephelometry on a BNA analyzer using non-specific antibodies (Behring, Rueil-Malmaison, France) [26]. Blood pressure was determined as the mean of at least three measurements using the standard sphygmomanometric procedure after 10 min rest in the supine position. Hypertension was defined as a systolic blood pressure  $\geq$  160 mmHg and/or a diastolic blood pres-

sure  $\geq 95$  mmHg, the use of antihypertensive drug treatment, or a combination of these. Body mass index (weight/height<sup>2</sup>) was used as a measure of corpulence. The life-long smoking dose was defined as the mean number of cigarettes smoked daily multiplied by the number of years of smoking, and was expressed in pack-years. Diabetes was defined as a fasting blood glucose level  $\geq 7.1$  mmol/l, the use of anti-diabetic drugs, or both.

### 2.3. Detection and quantification of artery lesions

Three sites — the carotid arteries, the abdominal aorta, and the femoral arteries — were screened for atherosclerotic disease using real-time-B-mode ultrasonography (Ultramark 4, Advanced Technologies Laboratories, Les Ulis, France) with a 3.75-MHz probe for the aorta and a 7.5-MHz probe for the carotid and femoral arteries, as described elsewhere [27]. An echogenic structure encroaching into the vessel lumen was considered to be a plaque when a distinct area with an intimal plus media thickness at least 50% greater than that of neighboring sites could be identified. The presence of plaque at each site was considered positive regardless of the number. This classification based on absence or presence of plaque gives good reproducibility [27,28].

### 2.4. DNA analysis

Genomic DNA was extracted from proteinase K-treated crude buffy coats by the salting-out method [29].

#### 2.4.1. PCR-SSCP analysis

Seven fragments of approximately 250 bp were enzymatically amplified using oligonucleotides designed according to published sequences [4,30] to cover the coding regions for the 5' flanking region, the transcription acidic domain, the bHLH-Zip, the first membrane-spanning domain, and the luminal loop.

Each amplification was carried out in a final volume of 20  $\mu$ l containing 100 ng of genomic DNA, 0.2 U of *Thermus aquaticus* DNA polymerase (ATGC, Biotechnologie, Noisy le Grand, France), 200  $\mu$ M dNTP (Boehringer Mannheim, Meylan, France), 10 pmol of each primer, and 2  $\mu$ l of the amplification buffer recommended by the manufacturer (10  $\times$  buffer: 500 mM KCl, MgCl<sub>2</sub> 15 mM, 100 mM Tris-HCl, 0.1% (w/w) gelatin; pH 9.0). The oligonucleotide primers and conditions used for thermal cycling with a automated Gene Amp PCR system 2400 (Perkin Elmer Cetus, Les Ulis, France) are described in Table 1. Some PCR fragments were digested by the addition of 5 U of the appropriate enzyme to yield fragments < 250 bp in length [31].

PCR products were diluted twofold in a solution containing 95% formamide, 0.025% bromophenol blue and 0.025% xylene cyanol, and denatured at 95°C for 5 min followed by rapid cooling on ice. Aliquots (7  $\mu$ l) were loaded on to non-denaturing 8% acrylamide gels (acrylamide *N-N'*-bisacrylamide 37.5:1) (Appligene Oncor, Illkirch, France) or a polyacrylamide-derived matrix, 0.5  $\times$  MDE gel (TEBU, Le Perray en Yvelines, France). Two electrophoresis conditions were tested: with or without 5% glycerol, and running at 20° or 4°C. Electrophoresis analysis was carried out on a Multi-

Table 1  
Oligonucleotides for SSCP analysis, studied regions and reaction conditions used to detect new variants of the SREBP-1a gene

Position <sup>a</sup>	PCR primers (5'–3')	Size (bp)	Annealing temperature (°C)	Studied region
–552	M9: GAT CCT GGT CTG TCT TGT TC	252	61	Promoter
–300	N: CCA CAA ATC TCC CCT CAG CC			
–398	M193: CGA GGC TGG ATA AAA TGA AT	464	51	Promoter
+91	B: cac acC TTC GAT GTC GGT CA			
–121	A: GAC ACG AAC GCG CGG AGC	213	58	Acidic domain (exon 1)
+91	B: cac acC TTC GAT GTC GGT CA			
+92	C: ttt cac agA CAT GCT TCA GC	153	56	Acidic domain (exon 2)
+236	D: AAT GTG GCA GGA GGT GGA			
+884	E: ACC ATC TTG GCA ACA GTC CC	212	50	Motif bHLH-Zip (exon 5)
+1068	F: ctc cac acC TTT GCC TCA GT			
+1069	G: ccg tgc agC TGA ATA AAT CT	131	56	bHLH-Zip (exon 6)
+1183	H: gga ctc acT GCT TTT GTG GA			
+1404	K: cca cca gGC AAA GCC AGA GC	382	68	First membrane spanning helix and luminal loop (exons 8 and 9)
+1669	L: CAT TGA GCA GCC AGA CCA CT			

<sup>a</sup> Nucleotides are numbered from the start of traduction. Small letters indicate intronic sequences.

phor II Electrophoresis Unit (Pharmacia Biotech, Orsay, France), equipped with a cryostat connected pump feed to the cooling coil of the unit. Gels were run in  $0.5 \times$  TBE at 600 V/30 mA/18 W for 1.5 h at 20°C or 200 V/23 mA/5W for 4 h at 4°C. After electrophoresis, gels were stained as follows: (1) fixation in 10% acetic acid (v/v) for 30 min; (2) washing three times in deionized water; (3) staining with 0.1%  $\text{AgNO}_3$  (w/v) containing 0.009% formaldehyde for at least 20 min; (4) removal of excess silver by washing in deionized water; (5) development in 0.236 M sodium carbonate, 0.019% formaldehyde (w/v) until stained bands of DNA fragments appeared (within a few minutes) and good contrast was obtained; (6) development was stopped by soaking the gels in 10% acetic acid (v/v) for 10 min; (7) gels were preserved in a 10% glycerol solution and air dried at room temperature.

#### 2.4.2. Direct sequencing

The PCR products in which variations were observed by SSCP were sequenced by cycle sequencing using the Taq DyeDeoxy Terminator Cycle Sequencing Kit and the ABI 310 Genetic Analyser (Perkin Elmer Cetus). The PCR product to be sequenced was purified on MicroSpin™ S-300 HR columns (Pharmacia Biotech). Sequencing in both directions was performed with the primers used for PCR-SSCP.

#### 2.4.3. Restriction fragment length polymorphism (RFLP) analysis

As the  $-36\text{delG}$  mutation in exon 1 of the SREBP-1a gene removes an *ApaI* restriction site, we were able to develop a rapid assay for screening a large number of DNA samples. PCR products were digested with 2.5 U of *ApaI* at 25°C for at least 3 h, in a buffer recommended by the supplier (Biolabs, New England). The restriction products were visualized after electrophoresis in 1.5% agarose gel (Life Technologies, Cergy Pontoise, France) at 80 V for 1 h.

Apolipoprotein E (apoE) genotyping was performed using the restriction isotyping procedure on polymerase chain-amplified fragments as described by Hixson and Vernier [32].

#### 2.5. Statistical analysis

Statistical analysis was performed on an Apple Macintosh computer with JMP software (SAS Institute, Cary, North Carolina). Quantitative variables are expressed as means  $\pm$  SD and ranges. Gene frequencies were determined by gene counting. Chi-square analysis was used to test for Hardy–Weinberg equilibrium. Alleles (coded  $G + G + = 0$ ,  $G - G + = 1$ , and  $G - G - = 2$ ) and arterial lesions (coded absence = 0 and presence = 1) were used as a continuous variable. Lipid and lipoprotein levels were compared between genotypes by an analysis of covariance. The tests were adjusted for

age, body mass index (BMI), systolic blood pressure, tobacco consumption, glycemia and apoE phenotype. As the distribution of triglyceride values was skewed, a logarithmic transformation was applied for the statistical test; but untransformed means are shown in the tables. Association between genotypes and the presence of arterial lesions were tested using linear regression. Stepwise multiple regression analysis was performed to confirm the results of univariate comparisons. Since very few subjects had the E2/2 ( $n = 1$ ) or E4/4 ( $n = 17$ ) phenotypes, subjects were divided into three groups: carriers of the  $\epsilon 2$  allele (E2/2 and E2/3 phenotypes); subjects with the E3/3 phenotype; and  $\epsilon 4$  carriers (E3/4 and E4/E4 phenotypes). Subjects with the E2/4 phenotype ( $n = 9$ ) and with apoE2 Christchurch ( $n = 1$ ) could not be assigned to any of the group and were therefore excluded. *P* values of  $< 0.05$  were considered as significant.

### 3. Results

#### 3.1. SSCP analysis

Two hundred and four asymptomatic men were screened for the presence of sequence variations in the regions coding for the expression or functional activity of SREBP-1a. Oligonucleotide primers were designed to amplify the promoter region and the translated exon sequences encoding the transcription domain, the bHLH-Zip, the first membrane-spanning helix, and the luminal loop.

Using PCR-SSCP analysis, four variant sites were identified. In the upstream flanking region of the gene, we found two bi-allelic variant sites at positions  $-186$  and  $-36$  relative to the translation initiation site. The variant at position  $-186$  was detected in one individual, who was heterozygous for a  $T \rightarrow C$  change; the variant at position  $-36$  was more frequent. It was caused by a single G deletion in the region containing one of the three G residues present at base pairs  $-36$ ,  $-35$  or  $-34$  of the translation initiation site. This deletion by the first G at base pair  $-36$  we arbitrarily designated ( $-36\text{delG}$  or G-); it abolishes an *ApaI* site. This restriction site was used to screen a large number of DNA samples.

Two others variant sites in the coding sequence were also found. In exon 5, a SSCP mobility shift was detected in one individual, who was heterozygous for a  $G892 \rightarrow A$  nucleotide change leading to a  $\text{Val298} \rightarrow \text{Ile}$  variation. Finally, we identified a silent  $G1638 \rightarrow G$  transition in exon 9 at codon 546.

Among these different variant sites, only the frequent  $-36\text{delG}$  polymorphism could be considered in an association study to investigate the role of the SREBP-1a gene in atherosclerosis and lipid disorder.

Table 2  
Population characteristics

Variable	Value <sup>a</sup>
Number	812
Age (years)	47.8 ± 7.7 (28–65)
Body mass index (kg/m <sup>2</sup> )	25.2 ± 2.5 (17–30)
<i>Blood pressure (mmHg)</i>	
Systolic	136 ± 18 (98–210)
Diastolic	87 ± 12 (55–126)
Hypertension (%)	28
<i>Lipid levels</i>	
Total cholesterol (mmol/l)	6.25 ± 1.12 (3.23–11.80)
Triglycerides (mmol/l)	1.48 ± 0.80 (0.22–4.42)
HDL cholesterol (mmol/l)	1.21 ± 0.34 (0.37–3.72)
LDL cholesterol (mmol/l)	4.19 ± 1.03 (1.76–8.73)
apoAI (g/l)	1.41 ± 0.27 (0.79–2.62)
apoB (g/l)	1.30 ± 0.28 (0.44–2.64)
<i>Lipid profile groups (%)</i>	
Normal	35
IIa	33
IIb	19
IV	12
Fasting glucose (mmol/l)	5.75 ± 0.59 (4.30–8.20)
Diabetes (%)	4
Lifelong smoking (pack-years)	13 ± 14 (0–70)
Current smokers (%)	32
<i>Subjects with atherosclerotic plaque (%)</i>	
Carotid	24
Aortic	25
Femoral	48

<sup>a</sup> Values are means ± SD (range) or percentages. HDL, high-density lipoprotein; LDL, low-density lipoprotein; apo, apolipoprotein. Subjects were classified into four subgroups according to lipid levels: normal (LDL-cholesterol <4.1 mmol/l and triglycerides <1.7 mmol/l); IIa hypercholesterolemia (LDL-cholesterol ≥4.1 mmol/l and triglycerides <1.7 mmol/l); IIb hypercholesterolemia, (LDL-cholesterol ≥4.1 mmol/l and triglycerides ≥1.7 mmol/l); and IV triglyceridemia (LDL-cholesterol <4.1 mmol/L and triglycerides ≥1.7 mmol/l).

### 3.2. Population characteristics

Genetic, biochemical and clinical investigations were performed in a high cardiovascular risk population of 812 subjects. The mean levels of risk factors are summarized in Table 2.

### 3.3. Polymorphism frequency

The 812 men at high cardiovascular risk were genotyped for the –36delG polymorphism. The relative frequency was 0.58. There was no significant deviation

from the Hardy–Weinberg equilibrium in this population. When subjects were divided according to dyslipidemia type, we found no significant difference in the genotype frequencies ( $\chi^2 = 6.5$ ,  $df = 6$ , NS) (Table 3).

### 3.4. Effect of the –36delG polymorphism on lipid parameters

The association between –36delG polymorphism and plasma lipid and apolipoprotein levels is presented in Table 4. The values are adjusted for age, body mass index, tobacco consumption, systolic blood pressure and glycemia. Recognition of apoE polymorphism as one determinant of plasma lipid and lipoprotein level variability is now well established therefore, the data were analyzed including the apoE phenotype. There was evidence in this population of a significant –36delG genotype effect on total cholesterol and LDL-cholesterol levels. The G- allele was associated with the highest plasma concentration of total cholesterol ( $P = 0.02$ ) and LDL-cholesterol ( $P = 0.02$ ).

As expected, the apoE polymorphism was associated with total cholesterol ( $P = 0.02$ ), LDL-cholesterol ( $P = 0.003$ ) and apoB ( $P = 0.0003$ ) levels (Table 4). Significant interactions between –36delG genotype and apoE phenotype were observed for cholesterol-LDL ( $P = 0.04$ ) and apoB ( $P = 0.05$ ) levels, suggesting a

Table 3

Genotype distribution and relative frequency of the –36delG polymorphism according to dyslipidemia type in high cardiovascular risk men <sup>a</sup>

Variable	Lipid profile groups			
	N (n = 288)	IIa (n = 271)	IIb (n = 152)	IV (n = 101)
Age (years)	48.2 ± 8.3	48.2 ± 7.0	46.8 ± 7.3	46.8 ± 8.0
<i>LDL-cholesterol</i>				
Mean (mmol/l)	3.35	4.95	4.96	3.38
Range (mmol/l)	1.76–4.09	4.10–8.73	4.10–8.40	1.77–4.09
<i>Triglycerides</i>				
Mean (mmol/l)	0.96	1.14	2.42	2.45
Range (mmol/l)	0.22–1.69	0.34–1.69	1.70–4.23	1.70–4.42
<i>Genotype</i>				
G–G–	102	109	51	29
G–G+	129	119	67	51
G+G+	57	47	34	21
<i>Allele</i>				
G– (%)	57.8	62.2	55.6	54.0
G+ (%)	42.2	37.8	44.4	46.0

<sup>a</sup> Pearson's  $\chi^2$  test was used to test for differences in distribution of genotypes and alleles between dyslipidemia groups. None of the differences were statistically significant.

Table 4  
Lipid and apolipoprotein concentrations (mean  $\pm$  SE) in  $-36\text{delG}$  genotypes in men at high cardiovascular risk<sup>a</sup>

	$-36\text{delG}$			apoE	$-36\text{delG}^* \text{apoE}$
	G-G-	G+G-	G+G+	Main effect <sup>c</sup>	Interaction <sup>e</sup>
TC (mmol/l)	6.37 $\pm$ 0.07	6.22 $\pm$ 0.06	6.13 $\pm$ 0.09	0.02	NS
TG (mmol/l) <sup>b</sup>	1.48 $\pm$ 0.05	1.48 $\pm$ 0.04	1.47 $\pm$ 0.06	NS	NS
HDL-C (mmol/l)	1.23 $\pm$ 0.02	1.20 $\pm$ 0.02	1.18 $\pm$ 0.03	NS	NS
LDL-C (mmol/l)	4.31 $\pm$ 0.06	4.15 $\pm$ 0.05	4.10 $\pm$ 0.08	0.02	0.003
apoAI (g/l)	1.41 $\pm$ 0.02	1.41 $\pm$ 0.01	1.41 $\pm$ 0.02	NS	NS
apoB (g/l)	1.32 $\pm$ 0.02	1.30 $\pm$ 0.02	1.28 $\pm$ 0.02	NS	0.0003

<sup>a</sup> Alleles (coded: G+G+ = 0; G'G+ = 1; G-G- = 2) are used as a continuous variable and tested in a regression model.

<sup>b</sup> Test performed on log-transformed values. SE: standard error, TC: total cholesterol; TG: triglycerides; HDL-C: HDL-cholesterol; LDL-C: LDL-cholesterol; apoAI: apolipoprotein AI; apoB: apolipoprotein B; BMI: body mass index.

<sup>c</sup>  $-36\text{delG}$  genotype main effect.

<sup>d</sup> apoE phenotype main effect.

<sup>e</sup> Interaction coefficient between  $-36\text{delG}$  genotype and apo E phenotype. All values were adjusted on age, BMI, tobacco consumption, systolic blood pressure, blood glucose and apoE phenotype.

gene-gene interaction. To specify this interaction, LDL-cholesterol and apoB levels were assessed in relation to  $-36\text{delG}$  genotype and apoE phenotype (Fig. 1). This analysis showed no difference in the mean value between  $-36\text{delG}$  genotypes in the apoE 3/3 phenotype and in the carriers of the  $\epsilon 2$  allele. By contrast, in the carriers of the  $\epsilon 4$  allele, the mean levels of LDL-cholesterol ( $P = 0.006$ ) and apoB ( $P = 0.01$ ) were higher in individuals carrying the G- allele with a dose allele effect.

### 3.5. Effect of the $-36\text{delG}$ polymorphism on extra-coronary atherosclerosis

Forty two percent of the population had no plaque at any site, while 24% had at least one carotid plaque, 25% had aortic plaque, and 48% had femoral plaque. Plaque at the carotid site were detected in 27% of the carriers of G-G- genotype, 24% of the G-G+ and 16% of the G+G+ ( $r = 0.084$ ,  $P = 0.02$ ) (Fig. 2). No significant association was observed between the presence of plaque at other arterial sites and the  $-36\text{delG}$  polymorphism.

When we used a stepwise regression analysis to create a model of predictors of atherosclerotic change in the carotid arteries, the  $-36\text{delG}$  polymorphism remained significantly and independently associated with the presence of carotid plaque ( $P = 0.03$ ) in addition to the well-established risk factors: age ( $P < 0.0001$ ); systolic blood pressure ( $P = 0.001$ ); and tobacco consumption ( $P = 0.01$ ) (Table 5). All other variables, including BMI, fasting blood glucose, smoking status, lipid parameters and apoE phenotype did not enter the model.

## 4. Discussion

PCR-SSCP analysis and direct sequencing of double-stranded DNA from a series of 204 asymptomatic men revealed a new polymorphism: a single G deletion at

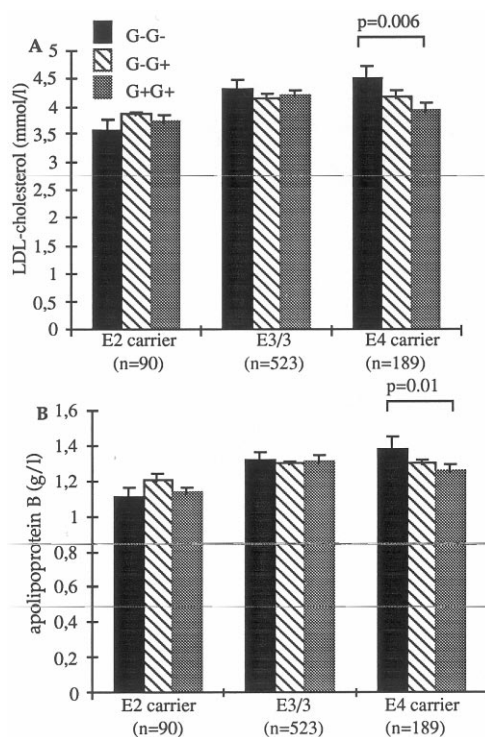


Fig. 1. LDL-cholesterol (A) and apoB (B) levels according to  $-36\text{delG}$  polymorphism stratified by apoE phenotype. Means are adjusted for age, BMI, tobacco consumption, systolic blood pressure, and blood glucose. Allelic (coded G+G+ = 0, G-G+ = 1, G-G- = 2) are used as continuous variables and tested in a regression analysis.

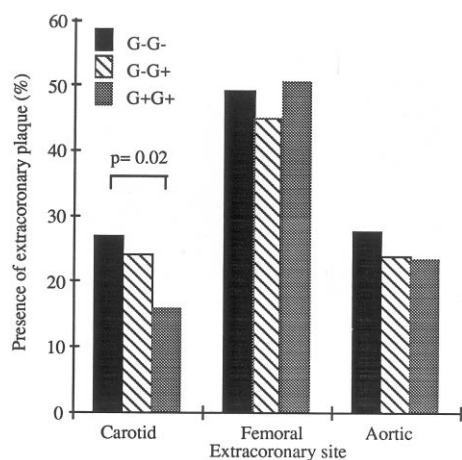


Fig. 2. Percentage comparison of subjects with plaque at carotid, aortic, and femoral sites according to the genotypes of the  $-36\text{delG}$  polymorphism. Allelic (coded  $G + G + = 0$ ,  $G - G + = 1$ ,  $G - G - = 2$ ) and arterial lesions (coded absence = 0 and presence = 1) are used as continuous variables and tested in a regression analysis.

base pair  $-36$  of the translation initiation site (the transcription start site has not been yet determined). This single G deletion was established from the published sequence of human SREBP-1 cDNA [4].

The influence of the  $-36\text{delG}$  polymorphism, located in the untranslated region of exon 1 of SREBP-1a, was analyzed in relation to lipid variables and the presence of extra-coronary atherosclerosis in a total population of 812 men at high cardiovascular risk.

SREBPs play a key role in integrating intracellular pathways with the expression of genes coding for proteins not only in cholesterol and triglyceride metabolism, but also in the development of cardiovascular risk factors, including obesity and insulin resistance [33]. We addressed the question of a potential effect of the SREBP-1a on lipoprotein metabolism by looking for a possible association between the  $-36\text{delG}$  polymorphism and circulating lipoprotein levels. The results of the study demonstrated a significant association between the  $-36\text{delG}$  polymorphism and total cholesterol and LDL-cholesterol acting in a co-dominant manner.

Bearing in mind that SREBPs regulate LDL receptor gene expression and that the apoE phenotype modulates lipoprotein binding to lipoprotein receptors, it was interesting to look for possible effect of the  $-36\text{delG}$

genotypes and apoE phenotypes. Although subdivision of the subjects according to apoE phenotypes and  $-36\text{delG}$  genotypes greatly reduced the size of some groups, the following results merit consideration. The association between the G- allele and an atherogenic lipid profile was found only in carriers of the  $\epsilon 4$  allele, suggesting that in humans SREBP-1a and apoE may interact to regulate lipoprotein metabolism. The existence of severe hyperlipidemia in double transgenic mice that overexpress the nuclear form of SREBP-1a and which are knocked out for the LDL receptor, but not in single transgenic mice with the nuclear form of SREBP-1a, provides further support for the emerging concept that LDL receptors are able to compensate for large increases in cholesterol input from either dietary sources or endogenous synthesis [34]. Significant hypercholesterolemia occurs only when the function of the LDL receptor is impaired. The synergic effect of the apoE and phenotype  $-36\text{delG}$  polymorphism on lipid levels could be the result of a negative feedback regulation of the LDL receptor due to increased affinity of its ligand ( $\epsilon 4$  allele) [35] and dysfunction of the SREBP pathway.

Atherosclerotic plaques, which can develop long before the occurrence of clinical cardiovascular events, were detected using high-resolution B-mode echography at the carotid, abdominal aortic, and femoral sites. Ultrasound detection of atherosclerotic plaque showed that the majority (58%) of the studied population had extra-coronary plaque, with a higher prevalence of femoral lesions. In this study, we found a graded relationship between the allelic dose of the  $-36\text{delG}$  polymorphism and carotid plaque, but not with other arterial sites. The presence of carotid plaque was almost twice as high in individuals homozygous for the presence of the G- allele than in those homozygous for the absence of the G- allele. Moreover, the association between the  $-36\text{delG}$  polymorphism and the presence of carotid plaque was independent of other cardiovascular risk factors (age, BMI, systolic blood pressure, tobacco consumption, glycemia, total cholesterol, triglycerides, and apoE phenotype). These results provide support for the concept that SREBP-1a has a more direct role in atherogenesis. Moreover, our findings showed that the atherogenic effects of the  $-36\text{delG}$  genotypes were different according to the arterial site. In previous reports, we showed that the risk factor profile related to arterial plaque location is different according to the location of the plaque in the arterial tree [27,36]. Unlike aortic lesions, carotid plaques are influenced by total and LDL-cholesterol levels; femoral plaques show an intermediate pattern. Taking the association between the  $-36\text{delG}$  polymorphism and total and LDL-cholesterol into account, it was not surprising that this polymorphism was related only to the presence of carotid plaque. It would be interesting to study the association between this poly-

Table 5

Stepwise regression analysis of risk factors influencing the presence of carotid plaque in men at high cardiovascular risk

Variable	$\beta$	df	F-value	P
Age	0.013	1	42.6	<0.0001
Smoking	0.080	1	6.2	0.01
Systolic blood pressure	0.003	1	10.3	0.001
$-36\text{delG}$	0.043	1	4.5	0.03

morphism and the progression of early pre-intrusive atherosclerosis as determined by quantitative measurements of common carotid artery intima-media thickness (IMT), because it has been reported that about 30% of IMT variation is the result of genetic influences [37].

The result of this study should not be applied to screening of the general population because subjects who underwent vascular evaluations were a selected population in whom the prevalence of dyslipidemia and hypertension was greater than in the general population. Furthermore, the present study involved only men, and the results cannot therefore be extrapolated to women.

Although the role of untranslated sequences is not well defined in gene expression, this region has been shown in some specialized systems (5′ untranslated region of beta-globin, tobacco-mosaic-virus, and ferritin) to contain sequences which are involved in the regulation of translation [38–40]. Genetic variations in this region could therefore affect the translational efficiency of the mRNA of SREBP-1a. In vitro expression studies are required to determine if this nucleotide deletion has any effect on SREBP-1a synthesis. However, it must be kept in mind that this polymorphism could be non functional and in linkage disequilibrium with other functionally important molecular variants of the gene. Because of the limited sensibility of the SSCP method, some functional variants of the SREBP-1a gene may have remained undetected. The efficiency of SSCP in detecting point mutations depends on the length of the fragment studied and the electrophoretic conditions applied. These conditions including temperature, glycerol, and the two different polyacrylamide matrices which were used to maximize the resolution of SSCP.

In conclusion, we have identified a new polymorphism in the 5′ untranslated region of the SREBP-1a gene, and shown that this –36delG polymorphism is associated with an atherogenic lipid profile in apparently healthy asymptomatic men at risk of cardiovascular disease. An interaction effect of SREBP-1a and apoE polymorphisms on lipids and lipoproteins was demonstrated. Moreover, we found a significant dose-dependent relationship between the –36delG polymorphism and the presence of carotid plaque.

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