Journal of Clinical Laboratory Analysis 13:251–258 (1999)

Effects of *Ava* II and *Hinc* II Polymorphisms at the LDL Receptor Gene on Serum Lipid Levels of Brazilian Individuals With High Risk for Coronary Heart Disease

Luis A. Salazar,[1*] Mario H. Hirata,[1] Sérgio D. Giannini,[2] Neusa Forti,[2] Jayme Diament,[2] Jaqueline S. Issa,[2] and Rosario D.C. Hirata[1]

[1]*Department of Clinical and Toxicological Analysis, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil*

[2]*Heart Institute (InCor), University of São Paulo, Medical School, São Paulo, Brazil*

Coronary heart disease (CHD) has presented high prevalence in the Brazilian population. Nevertheless, studies of genetic risk factors for CHD in our country are insufficiently carried out. We have investigated the effects of *Ava* II (exon 13) and *Hinc* II (exon 12) polymorphisms at the low-density lipoprotein receptor (LDLR) gene on circulating lipids of 170 white unrelated individuals presenting a lipid profile with high risk for CHD (HRG) and 130 controls (CG) from São Paulo City, Brazil. *Ava* II and *Hinc* II polymorphic regions at the LDLR gene were amplified by PCR and analyzed by enzymatic isotyping. The frequency of the genotypes A+A+ (*Ava* II) and H+H+ (*Hinc* II) was greater in HRG group compared to that of the controls (32 vs. 16% and 32 vs. 18%, respectively). Moreover, in the HRG group, A+A+ and H+H+ genotypes were associated with high concentrations of total cholesterol and LDL-C in serum ($P = 0.0001$). Our results indicate that *Ava* II and *Hinc* II polymorphisms at the LDLR locus contribute to the variability of total cholesterol and LDL-C levels in HRG individuals. These data suggest that the LDLR polymorphism remains a useful genetic marker for predicting CHD risk. J. Clin. Lab. Anal. 13:251–258, 1999.
© 1999 Wiley-Liss, Inc.

Key words: DNA polymorphism; low density lipoprotein receptor gene; serum cholesterol levels; genetic markers; cardiovascular disease

INTRODUCTION

Coronary heart disease (CHD) is the leading cause of death in industrialized societies and is fast becoming the leading killer in the entire world (1,2). Hence, the early identification of subjects at risk of developing CHD is an important public health issue. Serum levels of cholesterol and cholesterol-rich lipoproteins are the main metabolic markers for atherogenesis and subsequent heart disease. Epidemiological and clinical studies have demonstrated that elevated circulating levels of low-density lipoproteins (LDL) are positively correlated with increased risk of atherosclerosis, whereas elevated levels of high density lipoproteins (HDL) are negatively correlated with risk and may be protective against atherosclerosis (3–7).

Genetic and dietary factors influence serum cholesterol levels, but detailed mechanisms of their interplay are not well known (8). Why increased dietary cholesterol intake raises serum cholesterol levels in some but not all subjects is poorly understood. Genetic variations of apolipoproteins, enzymes, and receptors, essentials in LDL metabolism, are involved, at least in part, in the regulation of serum total and LDL cholesterol levels (9). Common apolipoprotein E polymorphisms have been shown to contribute to the magnitude of cholesterol response in several dietary intervention studies (10–13). In addition, polymorphisms of the apolipoprotein B gene, evaluated by RFLP using the enzymes *Xba* I, *Eco*RI, or *Pvu* II, are associated, although inconsistently, with the variability of the serum cholesterol levels (14,15). Considering the crucial role of the LDL receptor (LDLR) in cholesterol homeostasis (16), common genetic alterations in this gene may also contribute to variation in plasma cholesterol levels in the general population.

The human LDLR gene was cloned, sequenced, and evaluated in many studies of patients with familial hypercholester-

Grant sponsor: CAPES, Brazil.

*Correspondence to: Luis Antonio Salazar Navarrete, Department of Clinical and Toxicological Analysis, Faculty of Pharmaceutical Sciences, University of São Paulo, Av. Lineu Prestes 580, CEP 05508-900, Brazil. E-mail: luisn@usp.br

Received 27 May 1999; Accepted 18 June 1999

© 1999 Wiley-Liss, Inc.

olemia (17–21). A large number of rare mutations in the LDLR were characterized at the molecular level (22–25), and they greatly contributed to our understanding of the structure-function relations of this protein. Other sequence changes in the LDLR gene have only a small effect on the function of the receptor and could influence the lipid levels within the normal population.

The polymorphic nature of the LDLR locus was demonstrated by the existence of several RFLPs within this gene (26–28). Until now 33 polymorphisms in the LDLR gene were reported using 25 different restriction enzymes. However, their significance in affecting plasma cholesterol levels is not well known. Recently, an association between plasma cholesterol levels and the genotypes for *Ava* II polymorphism (exon 13) at the LDLR gene was shown in normolipidemic individuals from Italy (26) and the United States (29). Similar observations were demonstrated in hypercholesterolemic patients from a London population (30). Another polymorphism at the LDLR gene, *Hinc* II (exon 12), was described showing association with differences in plasma cholesterol levels (13,31).

We have studied the distribution of two polymorphic loci at the LDL receptor gene detected with *Ava* II and *Hinc* II restriction enzymes, and evaluated their significance in affecting plasma lipid concentrations in individuals with high risk for CHD and controls residing in São Paulo City, Brazil.

MATERIALS AND METHODS

Subjects

The study group consisted of 170 white unrelated individuals (35 men and 135 women), ages 35–79 years (mean, 50 years). They were selected from among those attending the Heart Institute of the University of São Paulo (São Paulo City, Brazil) who presented a lipid profile suggesting high risk for CHD, a condition determined according to the National Cholesterol Education Program (NCEP) (32). The control group consisted of 130 white unrelated normolipidemic healthy individuals (55 men and 75 women), ages 30–78 years (mean, 44 years), from São Paulo City. None of them had gastrointestinal, thyroid, liver, or renal disease, or had diabetes mellitus. And, none of the patients were treated with lipid-lowering diets or drugs at the time of blood sampling for determination of lipoprotein parameters. The study protocol was approved by our hospital's ethical committee, and informed consent was obtained from each participant.

Lipid Measurements

Serum lipid levels were determined from blood samples collected after overnight (> 8 hours) fast. Triglycerides (TG) were determined by enzymatic assay (33), and total cholesterol (TC) was assayed by the esterase-oxidase method (34). High-density lipoprotein cholesterol (HDL-C) levels were measured by enzymatic assay after phosphotungstic acid and magnesium precipitation (35). Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald equation when the triglyceride levels did not exceed 4.8 mmol/L, and very low density lipoprotein cholesterol (VLDL-C) was calculated using the formula TG/5 (36).

DNA Isolation and PCR Amplification

Genomic DNA was extracted from blood leukocytes by a salting-out procedure modified in our laboratory (37). The *Ava* II and *Hinc* II polymorphic regions at the LDLR gene were amplified by polymerase chain reaction (PCR). We used two sets of primers. One set was derived from DNA sequences flanking an *Ava* II restriction site in exon 13 of the LDLR gene (29). The amplified fragment was 228 bp. The other set was from DNA sequences flanking a *Hinc* II restriction site in exon 12 (22). The amplified fragment was 210 bp.

Genomic DNA (500 ng) was used for a 50 μL PCR reaction containing 10 mmol/L Tris, pH 9.0, 50 mmol/L KCl, 1.5 mmol/L de $MgCl_2$, 0.2 mmol/L deoxynucleotide triphosphates, 100 nmol/L of each primer, and 1.25 U *Taq* DNA polymerase. Amplification of the *Ava* II and *Hinc* II polymorphic regions was performed for 28 cycles at 95°C for 1 minute, at 68°C for 2 minutes, and 72°C for 1 minute.

Restriction Isotyping

Amplified products were digested with *Ava* II or *Hinc* II, and the resulting fragments were separated on 2- or 4%-agarose gels stained with ethidium bromide, and visualized on UV light. The digestion of the 228-bp PCR-amplified products with the restriction enzyme *Ava* II revealed two fragments of 141 and 87 bp, indicating the presence of the restriction site (A+ allele). The digestion of the 210-bp PCR-amplified product with the restriction enzyme *Hinc* II yielded three fragments of 110, 62, and 38 bp when the *Hinc* II restriction site (T-to-C transition at base 1773) was present (H+ allele). The H– allele was identified by the presence of two fragments (148 and 62 bp) indicating C-to-T transition at base 1725.

Statistical Methods

Differences among lipid and lipoprotein concentrations in different groups of individuals were compared using the Student's *t*-test (38). Allele frequencies and genotype distribution for each polymorphic site were estimated by gene counting. Chi-square analysis was used to test Hardy–Weinberg equilibrium, and for comparison of allele frequencies and genotype distribution between the studied groups. The sampling distributions of all the quantitative variables were tested for normality, and were $\log_e$ transformed to obtain normal distribution. To evaluate the effect of each polymorphism on the variation of quantitative variables of lipid, one-way ANOVA or Tukey–Kramer was performed (38). Two-

Way ANOVA was used to evaluate the interaction between genotype-genotype, genotype-gender, genotype-age, and genotype-menopausal status. Component of variance analysis was used to determine the phenotypic variance on lipid traits (38). Men and women were analyzed separately within each group. Women were further categorized as either premenopausal and postmenopausal, and the data were reanalyzed. Significance was considered to be at the 5% level.

RESULTS

Lipids and Lipoproteins

Table 1 presents lipid and lipoprotein values (mean ± SD) in serum from 300 white unrelated individuals from Brazil. TC, TG, LDL-C, and VLDL-C levels were higher in the individuals with high risk for CHD ($P = 0.0001$). HDL-C levels were similar between both groups.

Allele Frequencies

Table 2 shows that the distribution patterns of the *Ava* II and *Hinc* II polymorphisms of the individuals with high risk for CHD (HRG) were different from those of the controls (CG). HRG subjects showed high frequency of the *Ava* II (A+) and *Hinc* II (H+) alleles compared with controls. The distribution of *Ava* II and *Hinc* II genotypes of the HRG group were also different from that of the control group. When Hardy–Weinberg equilibrium was evaluated we observed that *Ava* II and *Hinc* II genotypes were in equilibrium in HRG and CG subjects.

LDLR Genotypes and Lipid Profile

The influence of *Ava* II polymorphism at the LDLR gene on lipid profile of the HRG group is shown in Figure 1 (top). Individuals carrying the A+A+ homozygous genotype presented higher TC and LDL-C levels compared to other genotypes.

Table 3 shows the lipid levels in serum of individuals from the HRG group with *Ava* II genotypes (A+A+, A+A–, and A–A–) distributed by gender. As we can see, the effect of the A+A+ genotype on TC and LDL-C levels in women is simi-

TABLE 1. Serum lipid values of the Caucasian individuals with high risk for coronary heart disease (HRG) and controls (CG)

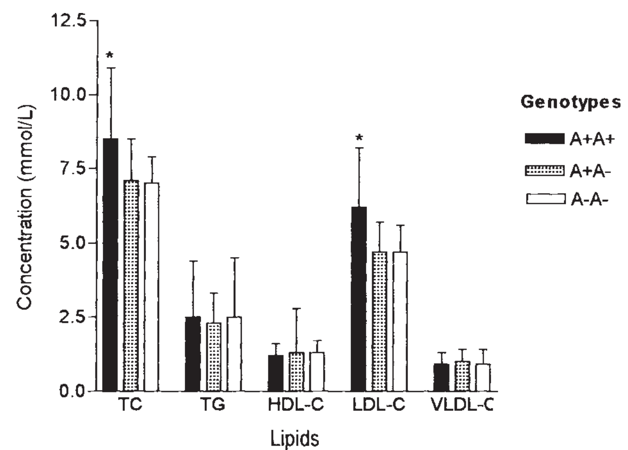
Lipids	HRG[a] (n = 170)	CG[a] (n = 130)	P[b]
Total cholesterol, mmol/L	7.5 ± 1.6	4.5 ± 0.6	0.0001
Triglycerides, mmol/L	2.4 ± 1.6	1.1 ± 0.4	0.0001
HDL-cholesterol, mmol/L	1.3 ± 0.4	1.3 ± 0.3	0.3160
LDL-cholesterol, mmol/L	5.2 ± 1.5	2.8 ± 0.6	0.0001
VLDL-cholesterol, mmol/L	0.9 ± 0.4	0.5 ± 0.2	0.0001

[a]Values are mean ± SD.
[b]P values from Student's t-test.

TABLE 2. Comparison of genotype distribution and relative allele frequencies of *Ava* II and *Hinc* II polymorphisms at the LDLR gene in Caucasian Brazilian individuals with high risk for coronary heart disease (HRG) and controls (CG)

Polymorphism	Genotype distribution[a]			Allele frequency[a]	
Ava II	A+A+	A+A–	A–A–	A+	A–
HRG (n = 170)	32%	47%	21%	0.56	0.44
CG (n = 130)	16%	58%	26%	0.45	0.55
		$\chi^2 = 9.58$[b]		$\chi^2 = 6.55$[d]	
Hinc II	H+H+	H+H–	H–H–	H+	H–
HRG (n = 170)	32%	47%	21%	0.56	0.44
CG (n = 130)	18%	55%	27%	0.45	0.55
		$\chi^2 = 7.72$[c]		$\chi^2 = 6.55$[d]	

[a]+/– indicates presence/absence of restriction site.
[b]$P = 0.0083$, 2 degrees of freedom (df).
[c]$P = 0.0211$, 2 df.
[d]$P = 0.0105$, 1 df.
Hardy–Weinberg Equilibrium: *Ava* II Genotypes: HRG, $\chi^2 = 0.34$ (1 df, P = NS) and CG, $\chi^2 = 3.53$ (1 df, P = NS); *Hinc* II Genotypes: HRG, $\chi^2 = 0.34$ (1 df, P = NS) and CG, $\chi^2 = 1.79$ (1 df, P = NS); NS, not significant.



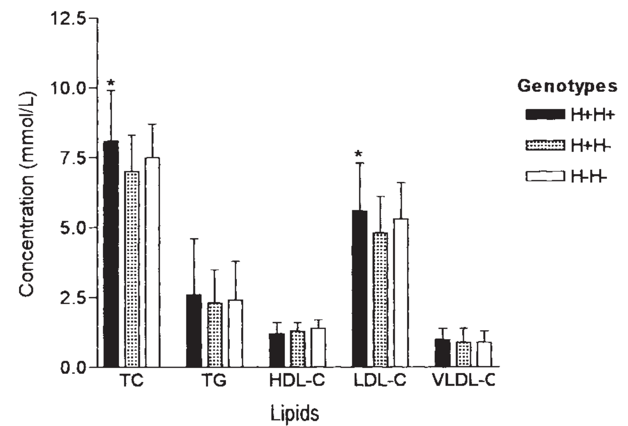


Fig. 1. Serum lipid values (mean ± SD) from Brazilian individuals with high risk for coronary heart disease grouped in *Ava* II genotypes (top) and grouped in *Hinc* II genotypes (bottom). *$P < 0.05$; TC, total cholesterol; TG, triglycerides; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol; VLDL-C, VLDL cholesterol.

TABLE 3. Serum lipid levels among LDL receptor/*Ava* II genotype in Brazilian individiuals with high risk for coronary heart disease separated by gender (n = 170)

	Men				Women			
Lipids[a]	A+A+ (n = 10)	A+A– (n = 20)	A–A– (n = 5)	P[b]	A+A+ (n = 44)	A+A– (n = 60)	A–A– (n = 31)	P[b]
TC, mmol/L	8.4 ± 1.8	6.9 ± 0.7	6.9 ± 0.7	0.0088	8.6 ± 2.3	7.2 ± 0.9	7.0 ± 0.9	0.0004
TG, mmol/L	2.9 ± 1.5	2.5 ± 1.1	2.7 ± 1.2	0.6469	2.3 ± 2.0	2.2 ± 1.2	2.4 ± 2.1	0.9961
HDL-C, mmol/L	1.2 ± 0.4	1.2 ± 0.2	1.2 ± 0.5	0.2139	1.2 ± 0.4	1.3 ± 0.4	1.3 ± 0.4	0.3456
LDL-C, mmol/L	6.4 ± 1.9	4.4 ± 1.0	4.6 ± 0.8	0.0060	6.1 ± 2.0	4.9 ± 1.0	4.7 ± 1.0	0.0006
VLDL-C, mmol/L	1.1 ± 0.5	1.1 ± 0.5	1.2 ± 0.5	0.9094	0.9 ± 0.4	0.9 ± 0.4	0.9 ± 0.5	0.9199

Lipids	A+A+ vs. A+A–	A+A+ vs. A–A–	A+A– vs. A–A–	A+A+ vs. A+A–	A+A+ vs. A–A–	A+A– vs. A–A–
TC[c]	$P < 0.05$	$P < 0.05$	NS[d]	$P < 0.05$	$P < 0.05$	NS
LDL-C	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$	NS

[a]Values are mean ± SD.
[b]*P* values are one-way ANOVA.
[c]*P* values from Tukey–Kramer method.
[d]NS, not significant.

lar to that in men (*Ava* II × gender interaction, $P > 0.05$ for all lipid traits). This effect is also similar in both menopausal status in women from HRG group (Table 4). To evaluate the influence of age in lipid traits, we have categorized individuals with age risk (men ≥ 45 years and women ≥ 55 years, according to NCEP) (32) and individuals with non-age risk. Table 5 shows that are no differences between individuals with age risk and non-age risk for the effect of A+A+ genotype on lipid profile in the HRG group (*Ava* II × age interaction, $P > 0.05$ for all lipid traits).

Similar results were observed for *Hinc* II polymorphism at the LDLR gene. Figure 1 (bottom) shows that the H+H+ genotype is associated with greater serum levels of total cholesterol and LDL-C in HRG subjects. Age, gender, and menopausal status of these individuals do not modify the effect of *Hinc* II polymorphism at the LDLR gene on total cholesterol and LDL-C levels (data not shown). Interestingly, the effect of both *Ava* II and *Hinc* II polymorphisms on serum lipid profile is not observed in individuals of the control group.

LDLR Genotypes Interaction and Lipid Profile

We also examined the possibility of joint effects (a genotype-genotype interaction) of two loci, *Ava* II and *Hinc* II, in determining serum lipid levels (Table 6). When the information of *Ava* II and *Hinc* II polymorphisms was combined, we can verify that A+A+/H+H+ genotype shows a significant association with higher levels of TC and LDL-C in the HRG group (Table 7). The interaction of both polymorphisms observed in the HRG group contributes with 16% of the pheno-

TABLE 4. Serum lipid levels among LDL receptor/*Ava* II tenotype in women with high risk for coronary heart disease separated by menopausal status (n = 135)

	Premenopausal			Postmenopausal			
Lipids[a]	A+A+ (n = 17)	A+A– (n = 28)	A–A– (n = 12)	A+A+ (n = 27)	A+A– (n = 32)	A–A– (n = 19)	*Ava* II × status[b]
TC, mmol/L	10.6 ± 3.7	7.0 ± 1.0	6.4 ± 0.8	8.3 ± 1.9	7.2 ± 0.9	7.2 ± 0.9	0.0412
TG, mmol/L	2.9 ± 1.3	1.6 ± 1.0	1.0 ± 0.5	2.3 ± 2.1	2.4 ± 1.3	2.7 ± 2.2	0.0247
HDL-C, mmol/L	0.9 ± 0.3	1.4 ± 0.3	1.6 ± 0.4	1.3 ± 0.4	1.3 ± 0.4	1.3 ± 0.3	0.0194
LDL-C, mmol/L	8.5 ± 3.5	4.7 ± 1.3	4.3 ± 0.6	5.8 ± 1.4	4.9 ± 0.9	4.8 ± 1.0	0.0234
VLDL-C, mmol/L	1.2 ± 0.6	0.7 ± 0.4	0.4 ± 0.2	0.8 ± 0.4	1.0 ± 0.4	1.0 ± 0.5	0.0073

Lipids[c]	A+A+ vs. A+A–	A+A+ vs. A–A–	A+A– vs. A–A–	A+A+ vs. A+A–	A+A+ vs. A–A–	A+A– vs. A–A–
TC	$P < 0.05$	$P < 0.05$	NS[d]	$P < 0.05$	$P < 0.05$	NS
TG	NS	$P < 0.05$	NS	NS	NS	NS
HDL-C	$P < 0.05$	$P < 0.05$	NS	NS	NS	NS
LDL-C	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$	NS
VLDL-C	$P < 0.05$	$P < 0.05$	NS	NS	NS	NS

[a]Values are mean ± SD.
[b]*P* values are two-way ANOVA.
[c]*P* values from Tukey–Kramer method.
[d]NS, not significant.

TABLE 5. Serum lipid levels among LDL receptor/*Ava* II genotype in Brazilian individiuals with high risk for coronary heart disease separated by age risk[a] (n = 170)

	Age risk				Non-age risk			
Lipids[b]	A+A+ (n = 23)	A+A– (n = 37)	A–A– (n = 12)	P^c	A+A+ (n = 31)	A+A– (n = 43)	A–A– (n = 24)	P^c
TC, mmol/L	8.7 ± 1.8	7.1 ± 0.8	7.2 ± 0.9	0.0001	8.4 ± 2.5	7.0 ± 0.9	6.9 ± 0.9	0.247
TG, mmol/L	2.4 ± 2.2	2.4 ± 0.9	2.4 ± 1.1	0.6912	2.6 ± 1.5	2.2 ± 1.6	2.6 ± 2.5	0.5724
HDL-C, mmol/L	1.3 ± 0.4	1.3 ± 0.3	1.2 ± 0.3	0.8600	1.2 ± 0.4	1.3 ± 0.4	1.4 ± 0.4	0.3067
LDL-C, mmol/L	6.1 ± 1.3	4.7 ± 0.9	4.8 ± 1.1	0.0001	6.4 ± 2.6	4.7 ± 1.1	4.6 ± 0.9	0.0135
VLDL-C, mmol/L	0.8 ± 0.3	1.0 ± 0.4	1.0 ± 0.5	0.2269	1.0 ± 0.5	0.8 ± 0.5	0.9 ± 0.5	0.4607

Lipids[d]	A+A+ vs. A+A–	A+A+ vs. A–A–	A+A– vs. A–A–	A+A+ vs. A+A–	A+A+ vs. A–A–	A+A– vs. A–A–
TC	$P < 0.05$	$P < 0.05$	NS[e]	$P < 0.05$	$P < 0.05$	NS
LDL-C	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$	NS

[a]Age risk for CHD: mean ≥ 45 years and women ≥ 55 years (32).
[b]Values are mean ± SD.
[c]*P* values are one-way ANOVA.
[d]*P* values from Tukey–Kramer method.
[e]NS, not significant.

typic variance in the total cholesterol and 10% in LDL-C serum levels. On the other hand, the effect of the A+A+/H+H+ genotype combination on lipid profile is not observed in control group.

DISCUSSION

The early development of CHD is largely attributed to elevated plasma LDL-C levels (1–7). However, it is likely that other lipid and nonlipid factors also influence CHD risk. Common DNA polymorphisms of genes related with lipid metabolism are potentially important genetic markers of variation in the plasma lipid profile, thus determining susceptibility or resistance to CHD in the general population.

In this study, we determined for the first time in a Brazilian population the relative allele frequency of *Ava* II and *Hinc* II polymorphisms at the LDL receptor gene. The frequencies of *Ava* II and *Hinc* II polymorphisms found in the Caucasian Brazilian subjects are different from other Caucasian individuals. The frequency of the A+ allele in the HRG group is greater than that observed in hypercholesterolemic patients from London, Italy, Spain, Switzerland, and Germany (21,39,40). On the other hand, control subjects present allele frequencies similar to that of control individuals from Switzerland, North America, Italy, and Spain (39,40). The frequency of the H+ allele in HRG subjects is similar to that found in Swiss, German, and Spanish hypercholesterolemic patients (39–41). However, in the controls, the frequency of the H+ allele is lower than in control individuals of these studied populations (39–41). These results suggest that there is a heterogeneity of frequencies of LDLR gene polymorphisms among samples from the Brazilian population. Further support for this hypothesis requires enlargement of the Brazilian sample size and analysis of additional polymorphisms. Nevertheless, the finding that *Ava* II (A+) and *Hinc* II (H+) allelic frequencies are greater in HRG individuals indicates an important correlation between these alleles and high risk for CHD.

TABLE 6. *P* values from two-way ANOVA (*Ava* II × *Hinc* II interaction) in HRG subjects

Lipids	*Ava* II	*Hinc* II	*Ava* II × *Hinc* II
TC	0.0001	0.0001	0.0027
TG	0.9025	0.7301	0.7945
HDL-C	0.4638	0.1264	0.3990
LDL-C	0.0001	0.0001	0.0319
VLDL-C	0.6403	0.9277	0.4630

The strong association between A+A+ (*Ava* II) and H+H+ (*Hinc* II) genotypes with a higher total cholesterol and LDL-C circulating levels found in the HRG group provides important evidence that LDLR polymorphisms affect cholesterol levels in individuals with high risk for CHD. Although previous studies failed to detect a significant association between LDLR genotype and serum lipid levels (42), a number of recent reports have indicated that common polymorphism in the LDLR gene is associated with interindividual differences on plasma LDL-C levels in normo and hypercholesterolemic subjects (8,26,30,31,43). Similar results were also found for *Ava* II polymorphism in normolipidemic Caucasian individuals (29), but this effect was gender specific and confined to postmenopausal women. Interestingly, in our study the effects of *Ava* II and *Hinc* II on serum lipids are not modified when the individuals of the HRG group were categorized by gender, age risk for CHD, or menopausal status.

A cumulative effect on the lipid profile resulting from the interaction of both *Ava* II and *Hinc* II polymorphisms is shown by the significant association between the A+A+/H+H+ genotype and higher total cholesterol and LDL-C levels in the HRG group. Our study also indicates that this association contributes with about 16 and 10% of the phenotypic variance of

TABLE 7. Effects of association of the *Ava* II and *Hinc* II polymorphisms at the LDL receptor gene on serum lipid levels of Brazilian individuals with high risk for coronary heart disease (n = 170)

Genotypes		Lipids[a] (mmol/L) TC	TG	HDL-C	LDL-C[b]	VLDL-C
A+A+	H+H+ (n=11)	10.6 ± 2.1[b]	3.2 ± 3.0	1.1 ± 0.4	8.1 ± 2.0[b]	1.0 ± 0.4
	H+H– (n=20)	7.7 ± 1.8[c]	2.2 ± 1.1	1.2 ± 0.4	5.4 ± 1.7	1.0 ± 0.5
	H–H– (n=23)	7.9 ± 1.2[d]	2.3 ± 1.6	1.4 ± 0.4	5.9 ± 1.0[c]	0.8 ± 0.3
A+A–	H+H+ (n=22)	7.5 ± 0.6[e]	2.4 ± 1.0	1.2 ± 0.4	5.0 ± 0.8	1.0 ± 0.4
	H+H– (n=35)	6.8 ± 0.9	2.2 ± 1.3	1.3 ± 0.3	4.5 ± 1.0	0.9 ± 0.5
	H–H– (n=23)	7.5 ± 0.9	2.6 ± 0.3	1.1 ± 0.3	5.0 ± 0.9	1.1 ± 0.1
A–A–	H+H+ (n=16)	7.2 ± 1.0[f]	2.5 ± 2.4	1.2 ± 0.4	5.0 ± 0.8	0.9 ± 0.4
	H+H– (n=8)	6.9 ± 0.7	2.4 ± 1.3	1.3 ± 0.4	4.5 ± 0.9	1.0 ± 0.6
	H–H– (n=12)	6.2 ± 0.5	2.5 ± 1.7	1.5 ± 0.3	3.7 ± 1.2	1.1 ± 0.8

[a]Values are mean ± SD.
Tukey–Kramer method: [b]Different from all other genotypes ($P < 0.05$); [c] Different from A+A+/H+H– genotype ($P < 0.05$); [d]Different from A–A–/H–H– genotype ($P < 0.05$); [e]Different from A+A–/H+H– genotype ($P < 0.05$); [f]Different from A–A–/H–H– genotype ($P < 0.05$).

total cholesterol and LDL-C levels in HRG subjects. In an earlier study (29), the genetic variation at the LDLR/*Ava* II polymorphism explained about 4% of the variance in total cholesterol and about 2% of the variance in LDL-C in Hispanic and non-Hispanic white women from the United States. The finding of lower TC and LDL-C levels in HRG subjects with A–A–/H–H– genotype suggests that the absence of a restriction site for both polymorphisms is probably associated with a lower risk for CHD.

The *Ava* II and *Hinc* II polymorphisms, located in exons 13 and 12, respectively, do not involve an amino acid substitution. These exons code for the second domain of the LDLR, which is 33% homologous with part of the extracellular domain of the epidermal growth factor precursor (EGF), a peptide hormone involved in growth stimulation (44). It remains open, however, by which mechanisms LDLR gene polymorphisms affect plasma cholesterol levels. Some authors described an association of *Ava* II polymorphism with the *Lebanese* mutation, a functional alteration responsible for familial hypercholesterolemia in Jewish Israeli and Christian Arab families (28,45). Other reports have shown a strong linkage disequilibrium between *Ava* II polymorphic site with the *Pvu* II polymorphism (P-allele), located in intron 15 at the LDLR gene (19,26,29), that is related to higher levels of total cholesterol and LDL-C levels in the general population (27,42,46,47). These studies suggest that the *Ava* II site is linked to other LDLR polymorphisms, additionally to the *Hinc* II site, and that this affects the level of LDLR gene expression. In addition, the effect of *Ava* II (A+) and *Hinc* II (H+) alleles on total cholesterol and LDL-C levels of HRG individuals demonstrated in our study can be mediated by a functional mutation in the LDLR gene that is linked with these polymorphic sites. Therefore, future studies will be necessary to identify the molecular relations between *Ava* II and *Hinc* II and other polymorphisms at the LDLR locus.

The early diagnosis of hypercholesterolemia is important for delaying the onset of CHD by means of suitable diet, lifestyle, and cholesterol-lowering drugs. Appropriate diet and specific treatment of patients with hypercholesterolemia carrying specific genotypes can reduce the risk of future myocardial infarction. Therefore, the identification of the LDLR genetic profile of affected subjects before the onset of clinical symptoms is important to the evaluation of CHD risk.

In summary, this study demonstrates that *Ava* II and *Hinc* II polymorphisms at the LDLR locus affect serum cholesterol levels in white individuals from Brazil with high risk for CHD, indicating that the LDLR polymorphism remains a useful genetic marker to study CHD risk.

ACKNOWLEDGMENTS

The authors thank Dr. Carlos Sannazzaro and Francesca C. Theobaldo for their assistance in collecting the samples. The expert technical assistance of Marcos Machado, Selma Cavalli, and Elizabeth Rodriguez is gratefully acknowledged. We thank Dr. Nga Y. Nguyen (CBER, FDA, Bethesda, MD) for providing the primers used in the amplification of the LDLR gene. We thank especially Creusa Maria Roveri Dal Bó for excellent statistical assistance. This study was supported by grants from the CAPES. L. A. Salazar was the recipient of fellowship from the CNPq-Brazil.

REFERENCES

1. Simons LA. Interrelations of lipids and lipoproteins with coronary artery disease mortality in 19 countries. Am J Cardiol 1986;57: 5G–10G.
2. Kane JP. Regression of coronary atherosclerosis during treatment of familial hypercholesterolemia with combined drug regimens. J Am Med Assoc 1990;264:3007–3012.
3. Castelli WP. Epidemiology of coronary heart disease: the Framingham study. Am J Med 1984;76:4–12.
4. Stamler J, Wentworth D, Neaton JD. Is relationship between serum cholesterol and risk of premature death from Coronary Heart Disease continuous and graded? Findings in 356.222 primary screenees of the Multiple Risk Factor Intervention Trial (MRFIT). J Am Med Assoc 1986;256:2823–2828.

5. Kannel WB. Range of serum cholesterol values in the population developing coronary artery disease. Am J Cardiol 1995;76:69C–77C.
6. Grundy SM, Veja GL, Kesáániemi Y. Abnormalities in metabolism of low density lipoproteins associated with coronary heart disease. Acta Med Scand 1985;701:23–37.
7. Haberland ME, Fogelman AM. The role of altered lipoproteins in the pathogenesis of atherosclerosis. Am Heart J 1987;79:1712–1716.
8. Gylling H, Kontula K, Koivisto UM, Miettinen HE, Miettinen TA. Polymorphisms of the genes encoding apoproteins A-I, B, C-III and E and LDL receptor, and cholesterol and LDL metabolism during increase cholesterol intake . Common alleles of the apoprotein E gene show the greatest regulatory impact. Arterioscler Thromb Vasc Biol 1997;17:38–44.
9. Galton DJ. Genetic determinants of atherosclerosis-related dyslipidemias and their clinical implications. Clin Chim Acta 1997;257:181–197.
10. Babaev VR, Dergunov AD, Chenchik AA, et al. Localization of apolipoprotein E in normal and artherosclerotic human aorta. Atherosclerosis 1990;85:239–247.
11. Davignon J, Gregg RE, Sing CF. Apoliprotein E polymorphism and artherosclerosis. Arteriosclerosis 1988;8:1–21.
12. Ferrieres J, Sing C, Roy M, Davignon J, Lussier-Cacan S. Apolipoprotein E polymorphism and heterozygous familial hypercolesterlemia. Sex-specific effects. Arterioscler Thromb Vasc Biol 1994;14:1553–1560.
13. Friedlander Y, Berry EM, Eisenberg S, Stein Y, Leitersdorf E. Plasma lipids and lipoproteins response to a dietary challenge: analysis of four candidate genes. Clin Genet 1995;47:1–12.
14. Berg K. DNA poymorphism at the apolipoprotein B locus associated with lipoprotein levels. Clin Genet 1986;30:515–520.
15. Demant T, Houlston RS, Caslake MJ, et al. The catabolic rate of low density lipoprotein is influenced by variation in the apoliprotein B gene. J Clin Invest 1988;82:797–802.
16. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science 1986;232:34–47.
17. Yamamoto T, Bishop RW, Brown MS, Goldstein JL, Russell DW. Deletion in cysteine-rich protein region of LDL receptor impedes transport to cell surface in WHHL rabbit. Science 1986;232:1230–1237.
18. Südhof TC, Goldstein JL, Brown MS, Russell DW. The LDL receptor gene: a mosaic of exons shared with different proteins. Science 1985;228:815–822.
19. Humphries SE, Coviello DA, Masturzo P, Balestreri Jr, Orecchini G, Bertolini S. Variation in the low density lipoprotein receptor gene is associated with differences in plasma low density lipoprotein cholesterol levels in young and old normal individuals from Italy. Arterioscler Thromb 1991;11:509–516.
20. Humphries SE, Kessling AM, Horsthemke B, et al. A common DNA polymorphism of the low-density lipoprotein (LDL) receptor gene and its use in diagnosis. Lancet 1985;1:1003–1005.
21. Humphries SE, King-Underwood L, Gudnason V, et al. Six DNA polymorphisms in the low density lipoprotein receptor gene: their genetic relationship and an example of their use for identifying affected relatives of patients witch familial hypercholesterolemia. J Med Genet 1993;30:273–279.
22. Hobbs H, Brown M, Goldstein J. Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. Hum Mutat 1992; 1:445–466.
23. Pereira E, Ferreira R, Hermelin B, et al. Recurrent and novel LDL receptor gene mutations causing heterozygous familial hypercholesterolemia in La Habana. Hum Genet 1995;96:319–322.
24. Hobbs H, Leitersdorf E, Goldstein J, Brown M, Russell D. Multiple crm- mutations in familial hypercholesterolemia. evidence for 13 alleles, including four deletions. J Clin Invest 1988;81:909–917.
25. Levy E, Minnich A, Cacan, SL, et al. Association of an Exon 3 mutation (Trp66-Gly) of the LDL receptor with variable expression of familial hypercholesterolemia in a French Canadian family. Biochem Mol Med 1997;60:59–69.
26. Bertolini S, Coviello D, Masturzo P, et al. RFLPs of the LDL-Receptor gene: their use in the diagnosis of FH and evaluation of different levels of gene expression on normal subjects. Eur J Epidemiol 1992;8:18–25.
27. Pedersen JC, Berg K. Normal DNA polymorphism at the low density lipoprotein receptor (LDLR) locus associated with serum cholesterol level. Clin Genet 1988;34:302–312.
28. Berkman N, Weir BS, Schwartz SP, Reshef A, Leitersdorf E. Haplotype analysis at the low density lipoprotein receptor locus: application to the study of familial hypercholesterolemia in Israel. Hum Genet 1992;88:405–410.
29. Ahn YI, Kamboh IM, Aston CE, Ferrell RE, Hamman RF. Role of common genetic polymorphisms in the LDL receptor gene in affecting plasma cholesterol levels in the general population. Arterioscler Thromb Vasc Biol 1994;14:663–670.
30. Wiseman SA, Powell JT, Humphries SE, Press M. The magnitude of the hypercholesterolemia of hypothyroidism is associated with variation in the low density lipoprotein receptor gene. J Clin Endocrinol Metab 1993;77:108–112.
31. Myant NB, Gallagher JJ, Knight BL, et al. Clinical signs of familial hypercholesterolemia in patients with familial defective apolipoprotein B-100 and normal low density lipoprotein receptor function. Arterioscler Thromb 1991;11:691–703.
32. Summary of the second report of the National Cholesterol Education Program-NCEP expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel II). J Am Med Assoc 1993;269:3015–3023.
33. Fossati P, Medicci R. Abstract book. International symposium on cholesterol control and cardiovascular diseases: prevention and therapy. Milan, Italy, 1987. Tarrytown, NY: Apud, Bayer Corporation, Diagnostic Division, cholesterol-fast color.
34. Fossati P, Principe L. Serum triglycerides determined colorymetrically with an enzyme of low-density lipoprotein cholesterol in plasma without use of the preparative ultracentrifuge. Clin Chem 1982;28:2077–2080.
35. Burstein M, Scholnick HR, Morfin R. Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. J Lipid Res 1970;11:583–595.
36. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972;18:499–502.
37. Salazar LA, Hirata MH, Cavalli SA, Machado MO, Hirata RDC. Optimized procedure for DNA isolation from fresh and cryopreserved clotted human blood useful in clinical molecular testing. Clin Chem 1998; 44:1748–1750.
38. Rosner B. Fundamentals of biostatistics, 2nd ed. Boston: PWS; 1986.
39. Miserez AR, Schuster H, Chidetti N, Keller U. Polymorphic haplotypes and recombination rates at the LDL receptor gene locus in subjects with and without familial hypercholesterolemia who are from different populations. Am J Hum Genet 1993;52:808–826.
40. Chaves FJ, Puig O, Garcia-Sogo M, et al. Seven DNA polymorphism in the LDL receptor gene: application to the study of familial hypercholesterolemia in Spain. Clin Genet 1996;50:28–35.
41. Puig O, Chaves FJ, Garcia-Sogo M, et al. A three-allelic plymorphic system in exon 12 of the LDL receptor gene is highly informative for segregation analysis of familial hypercholesterolemia in the Spanish population. Clin Genet 1996;50:50–53.
42. Taylor R, Jeenah M, Seed M, Humphries S. Four DNA polymorphisms in the LDL receptor gene: their genetic relationship and use in the study of variation at the LDL receptor locus. J Med Genet 1988;25:653–259.
43. Gudnason V, Zhou T, Thormar K, et al. Detection of the low density lipoprotein receptor gene Pvu II intron 15 polymorphism using the polymerase chain reaction: association with plasma lipid traits in healthy men and women. Dis Markers 1998;13:209–220.
44. Russell D, Schneider W, Yamamoto T, Luskey K, Brown M, Goldstein

J. Domain map of the LDL receptor: sequence homology with epidermal growth factor precursor. Cell 1984;37:577–585.
45. Reshef A, Meiner V, Dann EJ, Granat M, Leitersdorf E. Prenatal diagnosis of familial hypercholesterolemia caused by the "Lebanese" mutation at the low density lipoprotein receptor locus. Hum Genet 1992;89:237–239.
46. Schuster H, Humphries S, Rauh G, et al. Association of DNA-haplotypes in the human LDL-receptor gene with normal serum cholesterol levels. Clin Genet 1990;38:401–409.
47. Poledne R, Pisa Z, Berg K. Normal genetic variation in the low density lipoprotein receptor (LDLR) locus influences cholesterol levels in children. Clin Genet 1993;43:122–126.