

Original Article

Impact of 3'UTR genetic variants in PCSK9 and LDLR genes on plasma lipid traits and response to atorvastatin in Brazilian subjects: a pilot study

Tomás Zambrano^{1,2}, Mario Hiroyuki Hirata¹, Álvaro Cerda^{1,2}, Egidio L Dorea³, Gelba A Pinto³, Maria C Gusumakuma³, Marcelo C Bertolami⁴, Luis A Salazar², Rosario Dominguez Crespo Hirata¹

¹School of Pharmaceutical Sciences, University of Sao Paulo, São Paulo, Brazil; ²Center of Molecular Biology and Pharmacogenetics, Scientific and Technological Bioresource Nucleus (BIOREN), Universidad de La Frontera, Temuco, Chile; ³University Hospital, University of Sao Paulo, São Paulo, Brazil

Received January 28, 2015; Accepted March 26, 2015; Epub April 15, 2015; Published April 30, 2015

Abstract: Background: Hypercholesterolemia is a complex trait, resulting from a genetic interaction with lifestyle habits. Polymorphisms are a major source of genetic heterogeneity, and variations in 2 key cholesterol homeostasis genes; low-density lipoprotein receptor (*LDLR*) and proprotein convertase subtilisin/kexin type-9 (*PCSK9*), lead to dyslipidemia. So, we investigated the relation of 2 variants located in the 3'-UTR (3'-untranslated region) of *LDLR* (rs14158, G>A) and *PCSK9* (rs17111557, C>T) with lipid profile and atorvastatin response. Methods: SNP influence on lipid profile was assessed in hypercholesterolemic patients (HC; n = 89) using atorvastatin (10 mg/day/4 weeks) and in normolipidemic subjects (NL; n = 171). Genotyping was completed through real-time PCR using TaqMan assays. Results: rs14158 G allele was higher in HC than in NL group (P = 0.043). NL subjects carrying the T allele of the *PCSK9* variant had lower high-density lipoprotein cholesterol (HDL-c) than C allele carriers (P = 0.009). There was no association between *LDLR* and *PCSK9* SNPs and atorvastatin response. Additionally, the *PCSK9* variant creates a microRNA interaction site, which could implicate an epigenetic mechanism in *PCSK9*-dependent HDL-C regulation. Conclusions: The rs14158 SNP contributes to hypercholesterolemia. Also, a putative microRNA regulation may influence HDL-C variability observed in rs17111557 carriers. Cholesterol-lowering response to atorvastatin is not influenced by *LDLR* and *PCSK9* variants.

Keywords: Atorvastatin, cholesterol, LDLR, PCSK9, polymorphism, microRNAs

Introduction

Cholesterol is an essential molecule. Besides giving fluidity to the plasma membrane, is the precursor for bile acids, steroid hormones, and vitamin D, among several other functions. Its homeostasis is a complex and finely controlled mechanism, produced by the interaction between genetic and environmental factors. Any impairment of these factors can lead to dyslipidemia, causing an elevation in plasma concentrations of low-density lipoprotein (LDL) cholesterol, a well-established risk factor for atherosclerosis and subsequent severe cardiovascular events, such as myocardial infarction, thrombosis, stroke and death [1-3].

LDL particles are formed as a catabolic product of triglyceride-rich lipoprotein metabolism.

They are removed from the bloodstream by hepatic LDL receptors (*LDLR*) -mediated endocytosis, previous interaction of the receptor with its ligand, apolipoprotein B-100 [4]. In addition, *LDLR* activity on the cell surface is negatively regulated at the post-translational level by binding of its epidermal growth factor-like repeat A (EGF-A) domain, to the proprotein convertase subtilisin/kexin type 9 (*PCSK9*) protease. This *LDLR*-*PCSK9* complex is subsequently internalized into the cell, leading to proteolytic degradation of the receptor in the acidic ambient of the lysosome, rather than recycling to the plasmatic membrane [5].

LDL cholesterol levels are largely determined by the activity of hepatic *LDLR* [6], and normal function of these receptors depends on interacting properly with apoB-100 and *PCSK9*.

Thus, genetic variations in these molecules become critical, and can establish a disorder called autosomal dominant hypercholesterolemia (ADH), also known as familial hypercholesterolemia (FH). The ADH phenotype is characterized by a dramatic elevation in LDL cholesterol levels, deposits of cholesterol in peripheral tissues, tendon xanthomas, enhanced atherosclerosis progression, and increased risk for premature coronary heart disease (CHD) [7, 8].

Variations identified in the LDLR make up a wide majority of ADH cases, with an estimated prevalence of 1:500 in its heterozygous form [9]. Additionally, findings in a Brazilian cohort documented seven previously undescribed *LDLR* mutations [10]. On the contrary, variations in *APOB* account for a small number of cases [6]. The highly polymorphic *PCSK9* gene is the third locus implicated in ADH [11]. Gain-of-function variations can reduce LDLR activity, causing elevated plasma LDL cholesterol levels. In contrast, loss-of-function mutations are associated with enhanced LDL cholesterol reduction and improved cardiovascular health [12].

Statins are potent competitive inhibitors of 3-hydroxymethyl-glutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme involved in the cholesterol biosynthetic pathway. HMGCR inhibitors have a wide range of beneficial biological activities, being commonly prescribed for the treatment of patients with hypercholesterolemia, inducing an effective and substantial decrease in LDL cholesterol and reducing significantly cardiovascular morbidity and mortality [13, 14]. In addition to the improvement in lipid profile, different studies also reported a number of pleiotropic effects, including anti-inflammatory, antioxidant and fibrinolytic effects [15-17].

Inhibition of cholesterol biosynthesis by statins comes along with an increase in hepatic LDLR, promoting uptake and clearance of LDL particles from plasma [18]. Paradoxically, these drugs also upregulate *PCSK9* mRNA [19], increasing serum PCSK9 protein levels and disturbing the lipid-lowering efficacy of statins in an dose-dependent manner [20].

Since cholesterol metabolism is usually disrupted in coronary artery disease, as well as the decisive functions that *PCSK9* and *LDLR*

have in cholesterol homeostasis, variations located in the regulatory regions of these candidate genes are potentially informative. So, this study aimed to investigate two previously unexplored single nucleotide polymorphisms (SNPs) in *PCSK9* and *LDLR* located in the 3'-untranslated region (3'UTR), and their association with lipid profile and lipid-lowering response to atorvastatin in a sample of the Brazilian population.

Materials and methods

Study population

Hypercholesterolemic patients (HC, n = 89) with LDL cholesterol over 160 mg/dL, and 171 normolipidemic subjects (NL) with LDL cholesterol lower than 130 mg/dL, triglycerides below 150 mg/dL, whose other lipid levels were normal according to American Heart Association (AHA) guidelines [21] and without any symptoms of an ongoing disease, were selected from the University Hospital of the University of Sao Paulo and the Institute Dante Pazzanese of Cardiology, Sao Paulo city, Brazil. Subjects with diabetes mellitus, hypertriglyceridemia, liver, renal or thyroid disease, pregnant women or those using oral contraceptives or under any other cause of secondary dyslipidemia were discarded from the study.

Patients ranged between 40 to 70 years old and were not under any medication known to affect lipoprotein metabolism (eg, cholestyramine, colestipol, niacin, clofibrate, gemfibrozil, probucol, hydroxymethylglutaryl-coenzyme A reductase inhibitors, β -blockers, thiazide diuretics, diphenylhydantoin, cis-retinoic acid, ascorbic acid, estrogens, progestins, anabolic steroids, hydrocortisone, fish oil capsules, or thyroxin) at the time of screening. Information on height, weight, hypertension, obesity, tobacco smoking, alcohol consumption, physical activity, menopause status, familial history of coronary artery disease (CAD) and medication was simultaneously obtained.

HC individuals went through a washout phase of 4 weeks, with indication of a low fat diet following the AHA recommendations [22]. After this phase, subjects were treated with atorvastatin (10 mg/day/4 weeks).

Serum lipids levels were obtained at baseline and after atorvastatin treatment. Also, alanine

3'UTR SNPs in PCSK9 and LDLR in Brazilians

Table 1. Clinical and demographic characteristics of the study groups

Variables	NL (171)	HC (89)	<i>p-value</i>
Age, years	47.3 ± 7.1	55.8 ± 10.6	< 0.0001
Women, %	78	67	0.054
Menopause, %	27	87	< 0.0001
Hypertension, %	37	52	0.018
Systolic pressure, mmHg	124.7 ± 18.4	130.7 ± 19.6	0.047
Diastolic pressure, mmHg	79.4 ± 13.6	84.7 ± 13.9	0.015
Obesity, %	17	31	0.011
Body mass index, kg/m ²	26.4 ± 4.3	27.9 ± 4.1	0.007
Family history of CAD, %	46	54%	0.214
Tobacco consumption, %	23	22	0.889
Physical activity, %	44	48	0.519
Total cholesterol, mg/dL	<i>basal</i> 176.1 ± 20.7	283.8 ± 39.1	< 0.0001
	<i>treatment</i> --	197.5 ± 30.6	< 0.0001
LDL cholesterol, mg/dL	<i>basal</i> 99.1 ± 18.1	195.7 ± 35.0	< 0.0001
	<i>treatment</i> --	116.8 ± 26.7	< 0.0001
HDL cholesterol, mg/dL	<i>basal</i> 60.6 ± 13.3	56.7 ± 14.5	0.030
	<i>treatment</i> --	54.66 ± 13.6	0.002
VLDL cholesterol, mg/dL	<i>basal</i> 16.2 ± 5.2	31.1 ± 13.5	< 0.0001
	<i>treatment</i> --	25.9 ± 10.9	< 0.0001
Triglycerides, mg/dL	<i>basal</i> 80.9 ± 26.0	155.7 ± 67.8	< 0.0001
	<i>treatment</i> --	129.9 ± 54.4	< 0.0001
Apo AI, mg/dL	<i>basal</i> 142.7 ± 27.4	134.2 ± 27.6	0.023
	<i>treatment</i> --	136.5 ± 28.6	0.231
Apo B, mg/dL	<i>basal</i> 83.6 ± 22.5	143.5 ± 25.0	< 0.0001
	<i>treatment</i> --	97.8 ± 20.4	< 0.0001

Number of subjects in parentheses. Continuous variables presented as mean ± SD and compared by *t*-test. HC, hypercholesterolemic; NL, normolipidemic; CAD, coronary artery disease; LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; ApoAI, apolipoprotein AI; apo B, apolipoprotein B.

aminotransferase (ALT) and creatine kinase (CK) enzymes were measured to evaluate possible liver and muscle adverse reactions to the treatment.

The study protocol was approved by the Ethics Committees of the University Hospital of University of Sao Paulo (Protocol # 811/08), School of Pharmaceutical Sciences of University of Sao Paulo (Protocol # 472), and Institute Dante Pazzanese of Cardiology (Protocol # 2077/2000). Each subject agreed to participate in the study by signing an informed consent.

Biochemical determinations

Blood samples were collected after an overnight fast of at least 12 hours. HC subjects had blood sampling at basal time and after treat-

ment. Plasma total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, ALT and CK were measured by routine laboratory methods. LDL cholesterol was calculated by Friedewald's formula [23].

PCSK9 and LDLR genotyping

DNA was obtained from blood samples anticoagulated with EDTA using a salting-out procedure described elsewhere [24]. PCSK9 rs17111557 C>T and LDLR rs14158 G>A polymorphisms were analyzed by allelic discrimination using the TaqMan real-time PCR system. Validated SNP genotyping assays (ID C_34685-359_10 for rs17111557 and C_8726960_10 for rs14158) were acquired from Applied Biosystems (Applied Biosystems, CA, USA). The reaction was optimized in a total volume of 10 µl using 20 ng of DNA. A 7500 Fast

Real-Time PCR system (Applied Biosystems, CA, USA) was used to detect the fluorescence of DNA amplification. A 20 percent of the samples were randomly selected and reanalyzed as quality control criteria.

Statistical analysis

Data were analyzed using Sigma Stat software v. 3.5 (SPSS Inc., Chicago IL). Allelic frequencies and genotype distribution were estimated by gene counting. Hardy-Weinberg equilibrium (HWE) was also evaluated. Four association inheritance models (codominant, dominant, recessive and overdominant) were explored, which correspond to different parameterizations or grouping of the genotypes. Differences in non-continuous variables, genotype and allelic distributions were compared by chi-square test. Normality distribution for all con-

3'UTR SNPs in PCSK9 and LDLR in Brazilians

Table 2. Inheritance models for *LDLR* and *PCSK9* polymorphisms and relationship with hypercholesterolemia

Model	Genotype %	HC (89)	NL (171)	OR [95% C.I.]	<i>p</i> -value
<i>LDLR</i> rs14158					
Codominant	G/G	66.3 (59)	53.2 (91)	1.00	
	A/G	30.3 (27)	40.9 (70)	0.59 [0.34-1.03]	0.064
	A/A	3.4 (3)	5.8 (10)	0.46 [0.12-1.75]	0.246
Dominant	G/G	66.3 (59)	53.2 (91)	1.00	
	A/G + A/A	33.7 (30)	46.8 (80)	0.58 [0.34-0.99]	0.043
Recessive	G/G + A/G	96.6 (86)	94.2 (161)	1.00	
	A/A	3.4 (3)	5.8 (10)	0.56 [0.15-2.09]	0.390
Overdominant	G/G + A/A	69.7 (62)	59.1 (101)	1.00	
	A/G	30.3 (27)	40.9 (70)	0.62 [0.36-1.08]	0.095
<i>PCSK9</i> rs17111557					
Codominant	C/C	88.8 (79)	90.6 (155)	1.00	
	C/T	10.1 (9)	9.4 (16)	0.91 [0.38-2.14]	0.822
	T/T	1.1 (1)	0 (0)	0.17 [0.006-4.23]	0.170
Dominant	C/C	88.8 (79)	90.6 (155)	1.00	
	C/T + T/T	11.2 (10)	9.4 (16)	1.22 [0.53-2.83]	0.630
Recessive	C/ + C/T	98.9 (88)	100 (171)	1.00	
	T/T	1.1 (1)	0 (0)	5.81 [0.23-144.2]	0.282
Overdominant	C/C + T/T	89.9 (80)	90.6 (155)	1.00	
	C/T	10.1 (9)	9.4 (16)	1.09 [0.46-2.57]	0.844

Number of subjects in parentheses. HC, hypercholesterolemic; NL, normolipidemic.

tinuous variables was tested by Kolmogorov-Smirnov test for statistical analysis. Differences in mean values were evaluated by *t*-test. Paired *t*-test was used to analyze the effects of atorvastatin treatment. Statistical significance was set at $P < 0.05$. Additionally, regulatory microRNAs involved in the modulation of the rs17-111557 SNP located in the 3'-UTR of *PCSK9* were selected from computational identification using a combination of the MicroSNiPer and miRNASNP algorithms. The regulatory network was generated using the Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City) software.

Results

Subjects demographics and clinical profiles

Demographic, clinical and laboratory data of NL and HC subjects was registered in **Table 1**. The frequency of tobacco consumption, family history of CAD and physical activity was similar between NL and HC groups. On the other hand, the mean value of age and body mass index (BMI), along with the frequency of menopause, hypertension and obesity were higher in the HC

group ($P < 0.05$). As expected, this subjects presented an atherogenic lipid profile, showing higher concentrations of total cholesterol, LDL cholesterol, VLDL cholesterol, and triglycerides than the NL group ($P < 0.05$). In addition, HDL-cholesterol values were significantly lower in HC subjects ($P = 0.030$). No cases of adverse drug reactions were observed.

LDLR and PCSK9 single nucleotide polymorphisms

The *LDLR* rs14158 G>A and *PCSK9* rs17111557 C>T polymorphisms were in Hardy-Weinberg equilibrium in NL and HC groups ($P > 0.05$), corroborating the unbiased selection of the subjects under study.

Minor allele frequency (MAF) of the *LDLR* SNP was lower in HC than in NL group ($P = 0.043$, **Table 2**). Hence, the *LDLR* G allele is associated to hypercholesterolemia in Brazilian subjects. On the other hand, genotypes and MAF of the *PCSK9* variant were similar between the HC and NL group ($P > 0.05$).

Also, MAF of major populations listed in HAPMAP, YRI (Yoruba tribe of Ibadan), ASW

3'UTR SNPs in PCSK9 and LDLR in Brazilians

Table 3. Minor allele frequencies for *LDLR* and *PCSK9* polymorphisms in different populations

Gene	Polymorphism	Brazilian	HCB	CEU	JPT	YRI	ASW	MEX	LWK	TSI	Source
<i>LDLR</i>	rs14158 G>A	26.3	41.9	25.7	46.5	16.4	12.2	26.0	7.8	17.0	HapMap
<i>PCSK9</i>	rs17111557 C>T	4.7	0	0.8	4.1	10.6	7.1	2.0	17.4	-	HapMap

HCB-Han Chinese in Beijing, China; CEU-Utah residents with Northern and Western European ancestry from the CEPH collection; JPT-Japanese in Tokyo, Japan; YRI-Yoruba in Ibadan, Nigeria; ASW African ancestry in Southwest USA; MEX Mexican ancestry in Los Angeles, California; LWK-Luhya in Webuye, Kenya; TSI Tuscans in Italy; NL, normolipidemic. *P*-values correspond to chi-square analyses.

Table 4. Influence of *LDLR* and *PCSK9* polymorphisms on lipid profile in normolipidemic subjects

Variables, mg/dL	<i>LDLR</i> rs14158			<i>PCSK9</i> rs17111557		
	GG (91)	AG + AA (80)	<i>P</i>	CC (155)	CT + TT (16)	<i>P</i>
Total cholesterol	173.3 ± 20.7	179.2 ± 20.3	0.062	176.3 ± 21.6	177.9 ± 16.1	0.773
LDL cholesterol	96.8 ± 18.4	101.8 ± 17.4	0.073	98.3 ± 18.2	107.8 ± 14.0	0.059
HDL cholesterol	60.5 ± 12.5	60.6 ± 14.2	0.942	61.6 ± 13.4	52.6 ± 6.8	0.009
VLDL cholesterol	15.6 ± 4.8	16.8 ± 5.4	0.139	16.1 ± 5.2	17.5 ± 5.5	0.341
Triglycerides	78.2 ± 24.4	84.1 ± 27.4	0.139	80.9 ± 26.0	87.5 ± 27.5	0.720
ApoAI	141.4 ± 28.7	144.2 ± 25.9	0.543	144.0 ± 27.6	135.9 ± 25.9	0.268
ApoB	81.5 ± 21.2	86.1 ± 24.0	0.219	82.2 ± 22.0	93.0 ± 25.0	0.071

Number of subjects in parentheses. Values are expressed as mean ± SD and compared by *t*-test. LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; ApoAI, apolipoprotein AI; apo B, apolipoprotein B.

(African ancestry in southwest USA), LWK (Luhya in Webuye, Kenya), TSI (Tuscans of Italy) and JPT (Japanese of Tokyo area) were listed and compared against our data (Table 3).

LDLR and *PCSK9* SNPs, lipid profile and response to atorvastatin

As shown in Table 4, in the NL group, the *LDLR* variant did not have an influence on the serum lipid profile. Interestingly, *PCSK9* rs17111557 T allele carriers showed lower HDL cholesterol than C allele carriers (*P* = 0.009), when using the dominant inheritance model.

Secondly, in the HC group we did not observed a relationship between lipid levels and genetic variations in the two loci studied. When evaluating atorvastatin response, no significant association of the *PCSK9* and *LDLR* polymorphisms was observed after completion of the treatment (Table 5).

MicroRNA regulation of *PCSK9*

Figure 1 shows the computational prediction of miRNA binding sites for the *PCSK9* rs17111557 SNP, detailing the (A) sequence, (B) structure and (C) interaction occurring when the G>A

change is present, along with its (D) regulatory pathway, using the hsa-miR-548t as an example.

Discussion

In this work, we have investigated two SNPs located in the 3'-UTR of *LDLR* and *PCSK9* genes and their relationship with plasma lipids and lipid-lowering response to atorvastatin in Brazilian population. Currently, Caucasians make up a wide majority of the subjects involved in most of *LDLR* and *PCSK9* genotype-phenotype correlation studies, narrowing the findings to this specific cohort. In this way, factors influencing the association of the allele with disease, either positively or negatively, such as ethnicity and gender, could not be determined without population based allele frequencies [25] and non-Caucasian population attributable risk would remain unknown. However, frequencies of risk alleles defining susceptibility to hypercholesterolemia differ considerably from one population to another, where natural selection and genetic drift account for the two usual factors responsible [26]. Additionally, genetic variation can also be influenced by geography [27]. So, MAFs deter-

3'UTR SNPs in PCSK9 and LDLR in Brazilians

Table 5. Influence of *LDLR* and *PCSK9* polymorphisms on lipid profile in HC patients treated with atorvastatin

Variables, mg/dL	<i>LDLR</i> rs14158			<i>PCSK9</i> rs17111557			
	GG (59)	AG + AA (30)	<i>P</i>	CC (79)	CT + TT (10)	<i>P</i>	
Total cholesterol	<i>basal</i>	281.0 ± 37.6	289.1 ± 42.0	0.358	282.9 ± 35.1	279.1 ± 45.9	0.754
	<i>treatment</i>	195.1 ± 26.0	202.3 ± 38.3	0.302	196.8 ± 29.2	194.6 ± 34.8	0.827
	<i>% change</i>	-30.0 ± 8.6	-30.0 ± 8.5	0.988	-30.1 ± 8.8	-30.0 ± 7.1	0.982
LDL cholesterol	<i>basal</i>	190.4 ± 33.4	198.4 ± 38.4	0.608	193.6 ± 33.2	194.7 ± 29.2	0.924
	<i>treatment</i>	116.1 ± 23.6	118.3 ± 32.3	0.711	115.6 ± 26.3	114.6 ± 22.4	0.907
	<i>% change</i>	-39.7 ± 10.9	40.4 ± 10.8	0.773	-39.9 ± 11.1	-40.7 ± 9.6	0.827
HDL cholesterol	<i>basal</i>	54.9 ± 14.1	60.2 ± 14.8	0.104	57.0 ± 14.5	59.2 ± 19.0	0.673
	<i>treatment</i>	53.3 ± 13.3	57.1 ± 14.1	0.219	54.8 ± 13.6	57.3 ± 18.3	0.605
	<i>% change</i>	-2.2 ± 9.6	-4.3 ± 10.8	0.352	-3.1 ± 10.5	-3.2 ± 5.0	0.977
VLDL cholesterol	<i>basal</i>	31.4 ± 13.0	30.5 ± 14.8	0.754	32.0 ± 13.9	25.2 ± 8.9	0.134
	<i>treatment</i>	25.6 ± 8.8	26.4 ± 14.2	0.740	26.2 ± 11.4	22.7 ± 5.3	0.340
	<i>% change</i>	-12.4 ± 26.7	-11.4 ± 27.0	0.874	-13.7 ± 27.7	-4.9 ± 20.5	0.334
Triglycerides	<i>basal</i>	157.3 ± 65.1	152.5 ± 73.8	0.754	160.3 ± 69.7	125.5 ± 44.3	0.129
	<i>treatment</i>	128.6 ± 44.2	132.6 ± 71.3	0.746	131.4 ± 57.0	113.1 ± 26.3	0.322
	<i>% change</i>	-12.4 ± 26.7	-11.4 ± 27.0	0.874	-13.7 ± 27.7	-4.9 ± 20.5	0.334
ApoA1	<i>basal</i>	130.5 ± 26.8	141.3 ± 28.1	0.083	135.8 ± 29.5	127.0 ± 26.3	0.375
	<i>treatment</i>	132.3 ± 27.8	144.4 ± 28.8	0.061	139.0 ± 28.9	132.3 ± 25.4	0.490
	<i>% change</i>	1.5 ± 12.0	2.7 ± 11.4	0.675	1.2 ± 15.1	3.8 ± 8.5	0.587
ApoB	<i>basal</i>	145.6 ± 24.6	139.3 ± 25.6	0.276	142.2 ± 26.7	140.6 ± 25.3	0.859
	<i>treatment</i>	98.0 ± 19.5	97.3 ± 22.4	0.877	98.3 ± 21.4	96.6 ± 20.3	0.816
	<i>% change</i>	-32.8 ± 11.1	-29.7 ± 11.9	0.247	-48.8 ± 31.0	-46.1 ± 17.1	0.796

Number of subjects in parentheses. Values are expressed as mean ± SD and compared by t-test. LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; ApoA1, apolipoprotein A1; apo B, apolipoprotein B.

mined in this study were contrasted against information of polymorphisms from other populations (<http://www.ncbi.nlm.nih.gov/projects/SNP>). As expected, MAF comparison showed wide allelic variation among the different cohorts listed (Table 3). This finding is concordant with the complex cohort studied [28]. Specifically, as the result of long-lasting admixture between inter-ethnic individuals from three continents; Portuguese colonizers, African slaves and autochthonous inhabitants, Brazilians are known to be one of the most heterogeneous populations worldwide, with a strong European, African and Brazilian Native American ancestry in their mosaic genome according to DNA analyses [29, 30]. Also, in addition to the highly complex ancestry of Brazilian inhabitants, large intraethnic variability could also exist [31].

LDLR 3'UTR variant and relationship with atorvastatin response

This study shows that the *LDLR* rs14158 SNP did not influence the lipid profile in HC and in non-dyslipidemic individuals. However, the

wild-type allele was related to hypercholesterolemia in Brazilians. Few studies have evaluated the participation of this particular SNP in cholesterol homeostasis. Chen et al. reported a case control study comparing coronary heart disease (CHD) against healthy individuals, finding no differences in genotype and allelic frequencies for the *LDLR* rs14158 variant between these 2 groups, suggesting that this SNP do not cause a predisposition to CHD and does not influence lipid-lowering treatment [32]. Conversely, Van Zyl et al. uncovered a significant association between this common variant and increased levels of LDL cholesterol in black South African population [33], but without exploring lipid-lowering response. Although Southern Africa inhabitants are recognized to have a rich and significant genetic diversity [34], this population differs largely from the Brazilian genomic ancestry, determined by a strong European influence [35]. Thus, this could be a factor that could partially explain the diverging results from our study, as discussed earlier. To our knowledge, this is the first study showing that the rs14158 SNP is not related to

cholesterol concentrations for the first seven days of therapy [38]. Recently, the molecular mechanism by which this protease controls HDL cholesterol was suggested to be through specific PCSK9-mediated regulation of apolipoprotein E-containing HDL, showing that PCSK9 inactivation is vital to decrease HDL cholesterol concentrations [39]. However, different mechanisms could be involved. As widely recognized, 3'-UTRs contain important regulatory sequences controlling gene expression at the posttranscriptional level. More precisely, this region is also regulated by small non-coding ribonucleic acids (miRNAs), which control major cellular processes [40], regulating gene expression at the posttranscriptional level through complementary base-pairing within the 3'-UTR of the target mRNA, inducing translational repression or mRNA degradation [41]. According to diverse bioinformatic target-prediction tools, the rs17111557 T allele studied creates a miRNA interaction site absent in C allele carriers, and several miRNAs are predicted to bind the 3'-UTR of *PCSK9* in presence of this C>T change. As an example, among the various miRNAs described (Figure 1), the 7bp seed length of hsa-miR-548t interacts with the *PCSK9* SNP in position 614 of its 3'-UTR, allowing complementary base-pairing. MIR-related SNPs, including SNPs in target sites may function as regulatory SNPs through modifying miRNA regulation to affect the phenotypes and disease susceptibility [42], and when accurate target prediction is met, a correlation with post-translational protein repression levels exist [43]. Hence, microRNAs could be contributing to a possible epigenetic inhibition of the protease through specific binding in presence of the rs17111557 SNP, shaping a different mechanism by which to reach PCSK9 inactivation and subsequent lower HDL-C concentrations observed in our cohort. Nonetheless, further analyses are needed to support this regulatory pathway and experimental microRNA-SNP regulation remains to be determined. A plausible explanation for not finding this SNP-associated HDL modulation in the HC cohort is due to its hypercholesterolemic nature, reducing the power to detect basal associations with lipids. Moreover, it can also be attributed to statin medication can be attributed to statin medication. As indicated earlier, atorvastatin upregulates *PCSK9* mRNA and can significantly increase PCSK9 circulating protein levels by up to 34% [44], reducing also the desired lipid-

lowering effect of the drug. In this sense, PCSK9 inhibition is suggested to enhance effectiveness of statins and substantially lower the risk for CHD [45]. As conclusive evidence shows that HDL cholesterol levels are an important CHD risk factor, demonstrated by an inverse relationship with myocardial infarction in a follow-up of the Framingham Heart study [46], additional comprehension regarding epigenetic mechanisms impairing PCSK9 function is of great interest, holding promising clinical and therapeutic implications.

To our knowledge, this is the first report documenting the relationship of the *PCSK9* rs17111557 variant and lipid-lowering treatment to atorvastatin. Previously, Anderson et al. explored 2 different *PCSK9* variants in a Brazilian cohort, successfully linking the E670G variant with high basal LDL-C levels in hypercholesterolemic subjects, but without showing any influence of the polymorphisms on atorvastatin treatment [47]. In the same manner, this study did not reveal an association between atorvastatin therapy and the SNPs described. However, we must take into account that this lack of relationship is a common feature of pharmacogenetic studies, and several mechanisms such as age, gender and racial ancestry can underlie these differences [48].

A possible limitation in this study is the number of individuals enrolled, that could be influencing the different trends observed into not overcoming statistical significance, suggesting that a wider cohort could help clarify such tendencies. Besides, this study did not address the possible posttranslational regulation of the *PCSK9* variant with experimental identification of the microRNAs involved. This fact, along with determination of PCSK9 levels, could clarify the putative epigenetic mechanism implicated in lower HDL-cholesterol levels.

In summary, we have shown that in a Brazilian sample population, the common rs14158 SNP in the regulatory 3'UTR region of *LDLR* is related to hypercholesterolemia. Furthermore, the *PCSK9* rs17111557 variant affects HDL cholesterol in normolipidemic individuals, contributing to plasma HDL cholesterol variability possibly through an interesting putative epigenetic mechanism. However, neither of the polymorphisms was related to lipid-lowering therapy with atorvastatin.

Acknowledgements

This study was supported by grants from FAPESP-Brazil (2011/21697-1) and FONDECYT-Chile (1130675). T.Z. and A.C. were recipients of fellowships from CONICYT-Chile. M. H.H. and R.D.C.H. are recipients of fellowships from CNPq-Brazil. We thank the volunteers for their participation in this study.

Disclosure of conflict of interest

None.

Address correspondence to: Luis A Salazar, Centro de Biología Molecular & Farmacogenética, Facultad de Medicina, Universidad de La Frontera, Av. Francisco Salazar 01145, Casilla 54-D, Temuco, Chile. Tel: +56 45 2592895; Fax: +56 45 2592832; E-mail: luis.salazar@ufrontera.cl

References

- [1] Welch CL, Xia YR, Shechter I, Farese R, Mehrabian M, Mehdizadeh S, Warden CH and Lusis AJ. Genetic regulation of cholesterol homeostasis: chromosomal organization of candidate genes. *J Lipid Res* 1996; 37: 1406-1421.
- [2] Lacoste L, Lam JY, Hung J, Letchacovski G, Solymoss CB and Waters D. Hyperlipidemia and coronary disease. Correction of the increased thrombogenic potential with cholesterol reduction. *Circulation* 1995; 92: 3172-3177.
- [3] National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation* 2002; 106: 3143-3421.
- [4] Blasiolo DA, Oler AT and Attie AD. Regulation of ApoB secretion by the low density lipoprotein receptor requires exit from the endoplasmic reticulum and interaction with ApoE or ApoB. *J Biol Chem* 2008; 283: 11374-11381.
- [5] Kwon HJ, Lagace TA, McNutt MC, Horton JD and Deisenhofer J. Molecular basis for LDL receptor recognition by PCSK9. *Proc Natl Acad Sci U S A* 2008; 105: 1820-1825.
- [6] Pullinger CR, Hennessy LK, Chatterton JE, Liu W, Love JA, Mendel CM, Frost PH, Malloy MJ, Schumaker VN and Kane JP. Familial ligand-defective apolipoprotein B. Identification of a new mutation that decreases LDL receptor binding affinity. *J Clin Invest* 1995; 95: 1225-1234.
- [7] van der Graaf A, Avis HJ, Kusters DM, Vissers MN, Hutten BA, Defesche JC, Huijgen R, Fouchier SW, Wijburg FA, Kastelein JJ and Wiegman A. Molecular basis of autosomal dominant hypercholesterolemia: assessment in a large cohort of hypercholesterolemic children. *Circulation* 2011; 123: 1167-1173.
- [8] Twisk J, Gillian-Daniel DL, Tebon A, Wang L, Barrett PH and Attie AD. The role of the LDL receptor in apolipoprotein B secretion. *J Clin Invest* 2000; 105: 521-532.
- [9] Yang KC, Su YN, Shew JY, Yang KY, Tseng WK, Wu CC and Lee YT. LDLR and ApoB are major genetic causes of autosomal dominant hypercholesterolemia in a Taiwanese population. *J Formos Med Assoc* 2007; 106: 799-807.
- [10] Salazar LA, Hirata MH, Cavalli SA, Nakandakare ER, Forti N, Diament J, Giannini SD, Bertolami MC and Hirata RD. Molecular basis of familial hypercholesterolemia in Brazil: Identification of seven novel LDLR gene mutations. *Hum Mutat* 2002; 19: 462-463.
- [11] Abifadel M, Varret M, Rabes JP, Allard D, Ouaguerram K, Devillers M, Cruaud C, Benjannet S, Wickham L, Erlich D, Derre A, Villegier L, Farnier M, Beucier I, Bruckert E, Chambaz J, Chanut B, Lecerf JM, Luc G, Moulin P, Weissenbach J, Prat A, Krempf M, Junien C, Seidah NG and Boileau C. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nat Genet* 2003; 34: 154-156.
- [12] Abifadel M, Rabes JP, Devillers M, Munnich A, Erlich D, Junien C, Varret M and Boileau C. Mutations and polymorphisms in the proprotein convertase subtilisin kexin 9 (PCSK9) gene in cholesterol metabolism and disease. *Hum Mutat* 2009; 30: 520-529.
- [13] Law MR, Wald NJ and Rudnicka AR. Quantifying effect of statins on low density lipoprotein cholesterol, ischaemic heart disease, and stroke: systematic review and meta-analysis. *BMJ* 2003; 326: 1423.
- [14] Guo S, Wang R, Yang Z, Li K and Wang Q. Effects of atorvastatin on serum lipids, serum inflammation and plaque morphology in patients with stable atherosclerotic plaques. *Exp Ther Med* 2012; 4: 1069-1074.
- [15] Albert MA, Staggars J, Chew P and Ridker PM. The pravastatin inflammation CRP evaluation (PRINCE): rationale and design. *Am Heart J* 2001; 141: 893-898.
- [16] Liao JK. Isoprenoids as mediators of the biological effects of statins. *J Clin Invest* 2002; 110: 285-288.
- [17] Takemoto M and Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. *Arterioscler Thromb Vasc Biol* 2001; 21: 1712-1719.
- [18] Pocathikorn A, Taylor RR and Mamotte CD. Atorvastatin increases expression of low-den-

3'UTR SNPs in PCSK9 and LDLR in Brazilians

- sity lipoprotein receptor mRNA in human circulating mononuclear cells. *Clin Exp Pharmacol Physiol* 2010; 37: 471-476.
- [19] Dubuc G, Chamberland A, Wassef H, Davignon J, Seidah NG, Bernier L and Prat A. Statins up-regulate PCSK9, the gene encoding the pro-protein convertase neural apoptosis-regulated convertase-1 implicated in familial hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 2004; 24: 1454-1459.
- [20] Welder G, Zineh I, Pacanowski MA, Troutt JS, Cao G and Konrad RJ. High-dose atorvastatin causes a rapid sustained increase in human serum PCSK9 and disrupts its correlation with LDL cholesterol. *J Lipid Res* 2010; 51: 2714-2721.
- [21] Stone NJ, Robinson JG, Lichtenstein AH, Bairey Merz CN, Blum CB, Eckel RH, Goldberg AC, Gordon D, Levy D, Lloyd-Jones DM, McBride P, Schwartz JS, Shero ST, Smith SC Jr, Watson K, Wilson PW, Eddleman KM, Jarrett NM, LaBresh K, Nevo L, Wnek J, Anderson JL, Halperin JL, Albert NM, Bozkurt B, Brindis RG, Curtis LH, DeMets D, Hochman JS, Kovacs RJ, Ohman EM, Pressler SJ, Sellke FW, Shen WK and Tomaselli GF. 2013 ACC/AHA guideline on the treatment of blood cholesterol to reduce atherosclerotic cardiovascular risk in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *Circulation* 2014; 129: S1-45.
- [22] Chahoud G, Aude YW and Mehta JL. Dietary recommendations in the prevention and treatment of coronary heart disease: do we have the ideal diet yet? *Am J Cardiol* 2004; 94: 1260-1267.
- [23] Friedewald WT, Levy RI and 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.
- [24] Salazar LA, Hirata MH, Cavalli SA, Machado MO and Hirata RD. Optimized procedure for DNA isolation from fresh and cryopreserved clotted human blood useful in clinical molecular testing. *Clin Chem* 1998; 44: 1748-1750.
- [25] Cross DS, Ivacic LC, Stefanski EL and McCarty CA. Population based allele frequencies of disease associated polymorphisms in the Personalized Medicine Research Project. *BMC Genet* 2010; 11: 51.
- [26] Alex L, Chahil JK, Lye SH, Bagali P and Ler LW. Differences in allele frequencies of autosomal dominant hypercholesterolemia SNPs in the Malaysian population. *J Hum Genet* 2012; 57: 358-362.
- [27] Handley LJ, Manica A, Goudet J and Balloux F. Going the distance: human population genetics in a clinal world. *Trends Genet* 2007; 23: 432-439.
- [28] Silbiger VN, Hirata MH, Luchessi AD, Genvigir FD, Cerda A, Rodrigues AC, Willrich MA, Arazi SS, Dorea EL, Bernik MM, Faludi AA, Bertolami MC, Santos C, Carracedo A, Salas A, Freire A, Lareu MV, Phillips C, Porras-Hurtado L, Fond-evila M and Hirata RD. Differentiation of African components of ancestry to stratify groups in a case-control study of a Brazilian urban population. *Genet Test Mol Biomarkers* 2012; 16: 524-530.
- [29] Alves-Silva J, da Silva Santos M, Guimaraes PE, Ferreira AC, Bandelt HJ, Pena SD and Prado VF. The ancestry of Brazilian mtDNA lineages. *Am J Hum Genet* 2000; 67: 444-461.
- [30] Pena SD, Bastos-Rodrigues L, Pimenta JR and Bydlowski SP. DNA tests probe the genomic ancestry of Brazilians. *Braz J Med Biol Res* 2009; 42: 870-876.
- [31] Garcia-Martin E, Martinez C, Ladero JM and Agundez JA. Interethnic and intraethnic variability of CYP2C8 and CYP2C9 polymorphisms in healthy individuals. *Mol Diagn Ther* 2006; 10: 29-40.
- [32] Chen W, Wang S, Ma Y, Zhou Y, Liu H, Strnad P, Kraemer FB, Krauss RM and Liu J. Analysis of polymorphisms in the 3' untranslated region of the LDL receptor gene and their effect on plasma cholesterol levels and drug response. *Int J Mol Med* 2008; 21: 345-353.
- [33] van Zyl T, Jerling JC, Conradie KR and Feskens EJ. Common and rare single nucleotide polymorphisms in the LDLR gene are present in a black South African population and associate with low-density lipoprotein cholesterol levels. *J Hum Genet* 2014; 59: 88-94.
- [34] Petersen DC, Libiger O, Tindall EA, Hardie RA, Hannick LI, Glashoff RH, Mukerji M, Fernandez P, Haacke W, Schork NJ and Hayes VM. Complex patterns of genomic admixture within southern Africa. *PLoS Genet* 2013; 9: e1003309.
- [35] Pena SD, Di Pietro G, Fuchshuber-Moraes M, Genro JP, Hutz MH, Kehdy Fde S, Kohlrausch F, Magno LA, Montenegro RC, Moraes MO, de Moraes ME, de Moraes MR, Ojopi EB, Perini JA, Racciopi C, Ribeiro-Dos-Santos AK, Rios-Santos F, Romano-Silva MA, Sortica VA and Suarez-Kurtz G. The genomic ancestry of individuals from different geographical regions of Brazil is more uniform than expected. *PLoS One* 2011; 6: e17063.
- [36] Rashid S, Curtis DE, Garuti R, Anderson NN, Bashmakov Y, Ho YK, Hammer RE, Moon YA and Horton JD. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. *Proc Natl Acad Sci U S A* 2005; 102: 5374-5379.
- [37] Graham MJ, Lemonidis KM, Whipple CP, Subramaniam A, Monia BP, Crooke ST and Crooke RM. Antisense inhibition of proprotein convertase subtilisin/kexin type 9 reduces serum

3'UTR SNPs in PCSK9 and LDLR in Brazilians

- LDL in hyperlipidemic mice. *J Lipid Res* 2007; 48: 763-767.
- [38] Chan JC, Piper DE, Cao Q, Liu D, King C, Wang W, Tang J, Liu Q, Higbee J, Xia Z, Di Y, Shetterly S, Arimura Z, Salomonis H, Romanow WG, Thibault ST, Zhang R, Cao P, Yang XP, Yu T, Lu M, Retter MW, Kwon G, Henne K, Pan O, Tsai MM, Fuchslocher B, Yang E, Zhou L, Lee KJ, Daris M, Sheng J, Wang Y, Shen WD, Yeh WC, Emery M, Walker NP, Shan B, Schwarz M and Jackson SM. A proprotein convertase subtilisin/kexin type 9 neutralizing antibody reduces serum cholesterol in mice and nonhuman primates. *Proc Natl Acad Sci U S A* 2009; 106: 9820-9825.
- [39] Choi S, Aljakna A, Srivastava U, Peterson BR, Deng B, Prat A and Korstanje R. Decreased APOE-containing HDL subfractions and cholesterol efflux capacity of serum in mice lacking Pcsk9. *Lipids Health Dis* 2013; 12: 112.
- [40] Bushati N and Cohen SM. microRNA functions. *Annu Rev Cell Dev Biol* 2007; 23: 175-205.
- [41] Doench JG and Sharp PA. Specificity of microRNA target selection in translational repression. *Genes Dev* 2004; 18: 504-511.
- [42] Ryan BM, Robles AI and Harris CC. Genetic variation in microRNA networks: the implications for cancer research. *Nat Rev Cancer* 2010; 10: 389-402.
- [43] Maragkakis M, Alexiou P, Papadopoulos GL, Reczko M, Dalamagas T, Giannopoulos G, Goumas G, Koukis E, Kourtis K, Simossis VA, Sethupathy P, Vergoulis T, Koziris N, Sellis T, Tsanakas P and Hatzigeorgiou AG. Accurate microRNA target prediction correlates with protein repression levels. *BMC Bioinformatics* 2009; 10: 295.
- [44] Careskey HE, Davis RA, Alborn WE, Troutt JS, Cao G and Konrad RJ. Atorvastatin increases human serum levels of proprotein convertase subtilisin/kexin type 9. *J Lipid Res* 2008; 49: 394-398.
- [45] Brown MS and Goldstein JL. *Biomedicine*. Lowering LDL—not only how low, but how long? *Science* 2006; 311: 1721-1723.
- [46] Wilson PW. High-density lipoprotein, low-density lipoprotein and coronary artery disease. *Am J Cardiol* 1990; 66: 7A-10A.
- [47] Anderson JM, Cerda A, Hirata MH, Rodrigues AC, Dorea EL, Bernik MM, Bertolami MC, Faludi AA and Hirata RD. Influence of PCSK9 polymorphisms on plasma lipids and response to atorvastatin treatment in Brazilian subjects. *J Clin Lipidol* 2014; 8: 256-264.
- [48] Simon JA, Lin F, Hulley SB, Blanche PJ, Waters D, Shiboski S, Rotter JI, Nickerson DA, Yang H, Saad M and Krauss RM. Phenotypic predictors of response to simvastatin therapy among African-Americans and Caucasians: the Cholesterol and Pharmacogenetics (CAP) Study. *Am J Cardiol* 2006; 97: 843-850.