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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

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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

Table 1. Clinical and demographic characteristics of the study groups

Variables		NL (171)	HC (89)	*p-value*
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	*basal*	176.1 ± 20.7	283.8 ± 39.1	*< 0.0001*
	treatment	---	197.5 ± 30.6	*< 0.0001*
LDL cholesterol, mg/dL	*basal*	99.1 ± 18.1	195.7 ± 35.0	*< 0.0001*
	treatment	---	116.8 ± 26.7	*< 0.0001*
HDL cholesterol, mg/dL	*basal*	60.6 ± 13.3	56.7 ± 14.5	*0.030*
	treatment	---	54.66 ± 13.6	*0.002*
VLDL cholesterol, mg/dL	*basal*	16.2 ± 5.2	31.1 ± 13.5	< 0.0001
	treatment	---	25.9 ± 10.9	*< 0.0001*
Triglycerides, mg/dL	*basal*	80.9 ± 26.0	155.7 ± 67.8	*< 0.0001*
	treatment	---	129.9 ± 54.4	*< 0.0001*
Apo AI, mg/dL	*basal*	142.7 ± 27.4	134.2 ± 27.6	*0.023*
	treatment	---	136.5 ± 28.6	*0.231*
Apo B, mg/dL	*basal*	83.6 ± 22.5	143.5 ± 25.0	*< 0.0001*
	treatment	---	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 treatment. 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_34685359_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-

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

Model	Genotype %	HC (89)	NL (171)	OR [95% C.I.]	*p-value*
LDLR 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*
PCSK9 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 rs17111557 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

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

		Brazilian	HCB	CEU	JPT	YRI	ASW	MEX	LWK	TSI	Source
Gene	Polymorphism	NL									
LDLR	rs14158 G>A	26.3	41.9	25.7	46.5	16.4	12.2	26.0	7.8	17.0	HapMap
			P = 0.005	*P = 0.862*	P < 0.0001	*P = 0.005*	P = 0.003	P = 0.949	P < 0.0001	P = 0.017	
PCSK9	rs17111557 C>T	4.7	0	0.8	4.1	10.6	7.1	2.0	17.4	--	HapMap
			P = 0.037	P = 0.202	P = 0.075	P = 0.031	P = 0.396	P = 0.271	P = 0.0002		

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 Toscans 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	*LDLR* rs14158			*PCSK9* rs17111557		
	GG (91)	AG + AA (80)	*P*	CC (155)	CT + TT (16)	*P*
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-

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

Variables, mg/dL		*LDLR* rs14158			*PCSK9* rs17111557		
		GG (59)	AG + AA (30)	*P*	CC (79)	CT + TT (10)	*P*
Total cholesterol	*basal*	281.0 ± 37.6	289.1 ± 42.0	*0.358*	282.9 ± 35.1	279.1 ± 45.9	*0.754*
	treatment	195.1 ± 26.0	202.3 ± 38.3	*0.302*	196.8 ± 29.2	194.6 ± 34.8	*0.827*
	% change	-30.0 ± 8.6	-30.0 ± 8.5	*0.988*	-30.1 ± 8.8	-30.0 ± 7.1	*0.982*
LDL cholesterol	*basal*	190.4 ± 33.4	198.4 ± 38.4	*0.608*	193. 6 ± 33.2	194.7 ± 29.2	*0.924*
	treatment	116.1 ± 23.6	118.3 ± 32.3	*0.711*	115.6 ± 26.3	114.6 ± 22.4	*0.907*
	% change	-39.7 ± 10.9	40.4 ± 10.8	*0.773*	-39.9 ± 11.1	-40.7 ± 9.6	*0.827*
HDL cholesterol	*basal*	54.9 ± 14.1	60.2 ± 14.8	*0.104*	57.0 ± 14.5	59.2 ± 19.0	*0.673*
	treatment	53.3 ± 13.3	57.1 ± 14.1	*0.219*	54.8 ± 13.6	57.3 ± 18.3	*0.605*
	% change	-2.2 ± 9.6	-4.3 ± 10.8	*0.352*	-3.1 ± 10.5	-3.2 ± 5.0	*0.977*
VLDL cholesterol	*basal*	31.4 ± 13.0	30.5 ± 14.8	*0.754*	32.0 ± 13.9	25.2 ± 8.9	*0.134*
	treatment	25.6 ± 8.8	26.4 ± 14.2	*0.740*	26.2 ± 11.4	22.7 ± 5.3	*0.340*
	% change	-12.4 ± 26.7	-11.4 ± 27.0	*0.874*	-13.7 ± 27.7	-4.9 ± 20.5	*0.334*
Triglycerides	*basal*	157.3 ± 65.1	152.5 ± 73.8	*0.754*	160.3 ± 69.7	125.5 ± 44.3	*0.129*
	treatment	128.6 ± 44.2	132.6 ± 71.3	*0.746*	131.4 ± 57.0	113.1 ± 26.3	*0.322*
	% change	-12.4 ± 26.7	-11.4 ± 27.0	*0.874*	-13.7 ± 27.7	-4.9 ± 20.5	*0.334*
ApoAI	*basal*	130.5 ± 26.8	141.3 ± 28.1	*0.083*	135.8 ± 29.5	127.0 ± 26.3	*0.375*
	treatment	132.3 ± 27.8	144.4 ± 28.8	*0.061*	139.0 ± 28.9	132.3 ± 25.4	*0.490*
	% change	1.5 ± 12.0	2.7 ± 11.4	*0.675*	1.2 ± 15.1	3.8 ± 8.5	*0.587*
ApoB	*basal*	145.6 ± 24.6	139.3 ± 25.6	*0.276*	142.2 ± 26.7	140.6 ± 25.3	*0.859*
	treatment	98.0 ± 19.5	97.3 ± 22.4	*0.877*	98.3 ± 21.4	96.6 ± 20.3	*0.816*
	% change	-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; ApoAI, apolipoprotein AI; 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

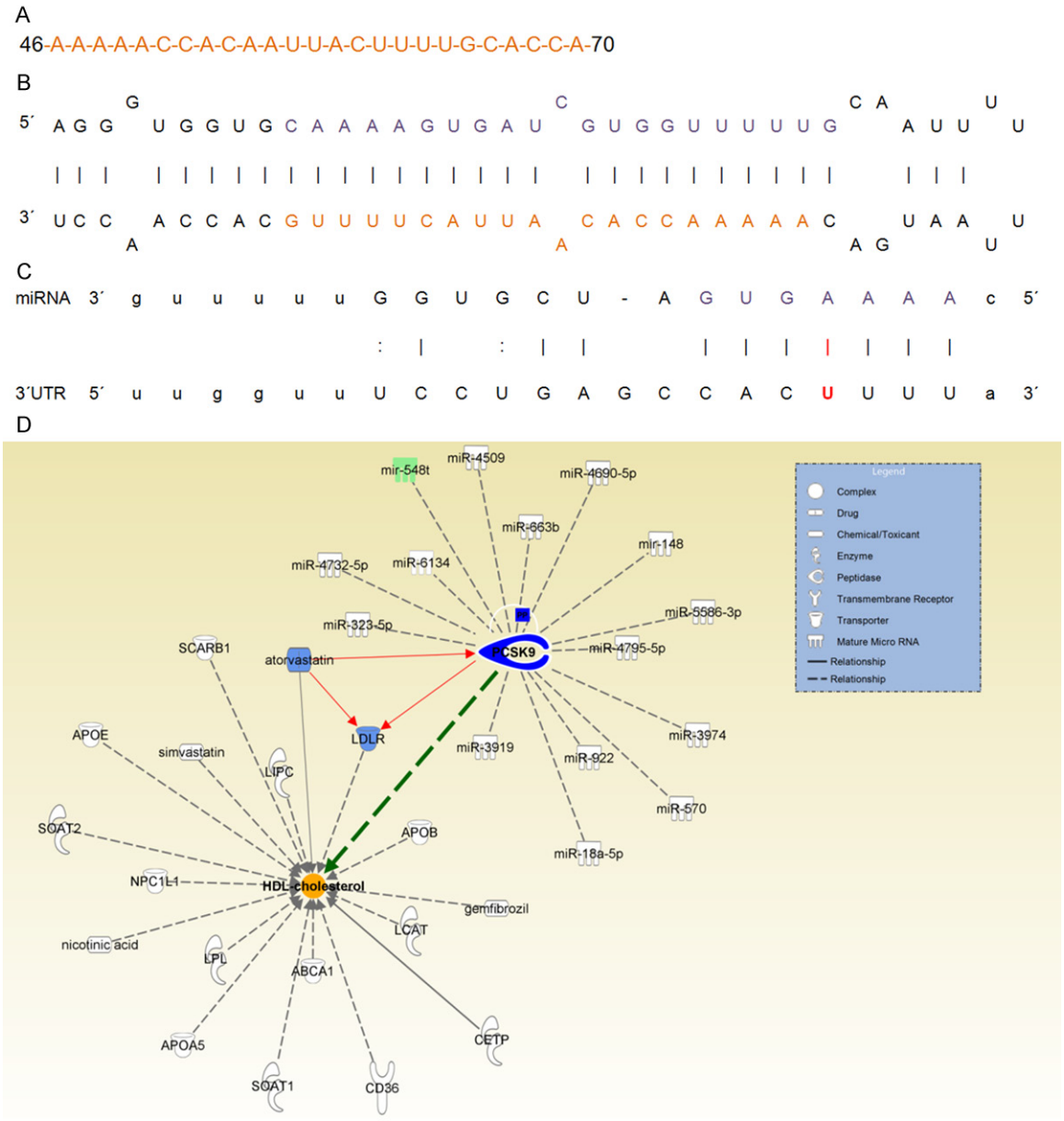

Figure 1. Putative microRNAs involved in decreased HDL-c through PCSK9 modulation. (A) hsa-miR-548t sequence, (B) stem-loop structure, (C) hsa-miR-548t interaction in position 614 with rs17111557 SNP, seed length: 7 bp, and (D) multiple microRNA-predicted regulation of the *PCSK9* rs17111557 C>T polymorphism.

lipid-lowering response with atorvastatin treatment in Brazilian subjects.

PCSK9 3’UTR variant and putative MIR regulation

The rs17111557 polymorphism in *PCSK9* did not impact the lipid concentration in hypercholesterolemic subjects. Also, it failed to demonstrate an association with atorvastatin treatment. Nonetheless, the T allele of this SNP revealed a significant association with reduced HDL cholesterol concentrations in NL subjects. As aforementioned, the key function of PCSK9 is LDLR proteolysis. However, several studies have shown an involvement of PCSK9 in HDL metabolism. In 2005, Rashid et al. demonstrated that mice lacking PCSK9 exhibited a 30% reduction in HDL cholesterol concentration [36]. In the same model, targeting of PCSK9 with an antisense oligonucleotide inhibitor showed a 54% reduction in HDL cholesterol [37], and in non-human primates, treatment with antibodies against PCSK9 reduced HDL

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 posttranslational 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.

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Disclosure of conflict of interest

None.

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