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RESEARCH

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# Apolipoprotein E mRNA expression in mononuclear cells from normolipidemic and hypercholesterolemic individuals treated with atorvastatin

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## Abstract

**Background:** Apolipoprotein E (apoE) is a key component of the lipid metabolism. Polymorphisms at the apoE gene (*APOE*) have been associated with cardiovascular disease, lipid levels and lipid-lowering response to statins. We evaluated the effects on *APOE* expression of hypercholesterolemia, *APOE*  $\epsilon 2/\epsilon 3/\epsilon 4$  genotypes and atorvastatin treatment in Brazilian individuals. The relationship of *APOE* genotypes and plasma lipids and atorvastatin response was also tested in this population.

**Methods:** *APOE*  $\epsilon 2/\epsilon 3/\epsilon 4$  and plasma lipids were evaluated in 181 normolipidemic (NL) and 181 hypercholesterolemic (HC) subjects. HC individuals with indication for lowering-cholesterol treatment (n = 141) were treated with atorvastatin (10 mg/day/4-weeks). *APOE* genotypes and *APOE* mRNA in peripheral blood mononuclear cells (PBMC) were analyzed by TaqMan real time PCR.

**Results:** HC had lower *APOE* expression than NL group ( $p < 0.05$ ) and individuals with low *APOE* expression showed higher plasma total and LDL cholesterol and apoB, as well as higher apoAI ( $p < 0.05$ ). Individuals carrying  $\epsilon 2$  allele have reduced risk for hypercholesterolemia (OR: 0.27, 95% I.C.: 0.08-0.85,  $p < 0.05$ ) and NL  $\epsilon 2$  carriers had lower total and LDL cholesterol and apoB levels, and higher HDL cholesterol than non-carriers ( $p < 0.05$ ). *APOE* genotypes did not affect *APOE* expression and atorvastatin response. Atorvastatin treatment do not modify *APOE* expression, however those individuals without LDL cholesterol goal achievement after atorvastatin treatment according to the IV Brazilian Guidelines for Dyslipidemia and Atherosclerosis Prevention had lower *APOE* expression than patients with desirable response after the treatment ( $p < 0.05$ ).

**Conclusions:** *APOE* expression in PBMC is modulated by hypercholesterolemia and the *APOE* mRNA level regulates the plasma lipid profile. Moreover the expression profile is not modulated neither by atorvastatin nor *APOE* genotypes. In our population, *APOE*  $\epsilon 2$  allele confers protection against hypercholesterolemia and a less atherogenic lipid profile. Moreover, low *APOE* expression after treatment of patients with poor response suggests a possible role of *APOE* level in atorvastatin response.

**Keywords:** apolipoproteina E, hypercholesterolemia, single nucleotide polymorphism (SNP), *APOE* gene expression, atorvastatin

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## Background

Dyslipidemia is an important risk factor in the development of atherosclerosis and cardiovascular events. Research on cardiovascular diseases has led to a better knowledge of the molecular basis of atherosclerosis and the identification of a key role of apolipoprotein (apo) E in this process [1]. ApoE is a major constituent of triglyceride-rich chylomicrons, very low density lipoprotein (VLDL) and some subclasses of high density lipoprotein (HDL) particles, participating in the clearance of these particles from circulation by serving as a ligand for their catabolism via low density lipoprotein (LDL) and apoE receptors [2].

The gene encoding the apoE protein (*APOE*) is polymorphic resulting in three major isoforms ( $\epsilon 2$ ,  $\epsilon 3$  and  $\epsilon 4$ ) caused by two single nucleotide polymorphisms (SNPs) in the exon 4 of the *APOE*, resulting in cysteine-arginine interchanges at residuals 112 and 158 of the protein, which has been associated with a number of pathophysiological conditions, including cardiovascular and neurological diseases [3].

The inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), or statins, are among the most prescribed drugs worldwide and provide extensive benefits in the prevention of primary and secondary cardiovascular diseases [4]. However, there is considerable interindividual variation in the lipid-lowering response to statins, which is attributed to the interaction between multiple environmental factors and genetic determinants involved in the pharmacokinetic and pharmacodynamic pathways of these drugs [5].

The *APOE* gene is among the most extensively studied genes involved in the statins pharmacodynamic. The studies investigating the potential modifying role of *APOE* genotypes on lipid response to statin therapy have produced data frequently contradictory and so far inconclusive [6]. Evidence suggests that allele  $\epsilon 4$  carriers appear to have attenuated lipid-lowering response and  $\epsilon 2$  carriers have enhanced response [5], however differential interpretation of these results among investigators difficult the consensus [6]. Moreover genome-wide association studies (GWAS) have also shown an association of the *APOE* locus with hypercholesterolemia but they were unsuccessful in establishing its relationship with statin response when whole-genome platforms were used [7].

The regulation of *APOE* expression is very complex with participation of several factors controlling its transcription, including some factors that also regulate the expression of other proteins that control the lipid traffic [1]. Nevertheless, possible regulation of *APOE* transcription by polymorphisms and statins has been poorly studied.

The purpose of this study is to investigate the effects on *APOE* mRNA expression profile of hypercholesterolemia

and *APOE* genotypes using PBMC from normolipidemics and hypercholesterolemic individuals. Moreover, we also analyzed the influence of atorvastatin treatment on *APOE* expression in hypercholesterolemic individuals, as well as the relationship of *APOE* genotypes with plasma lipid and atorvastatin response in our population.

## Results

### Characteristics of study population

Main characteristics of normolipidemic and hypercholesterolemic individuals are shown in Table 1. Frequencies of ethnics, gender, family history of coronary artery disease (CAD), cigarette smoking and physical activity were similar between NL and HC groups. However, mean values of age and body mass index (BMI), as well as frequencies of menopause, hypertension and obesity were higher in HC than NL group ( $p < 0.05$ ). As expected, HC individuals had a more atherogenic lipid profile showing higher total, LDL and VLDL cholesterol, triglycerides and ApoB compared with NL subjects ( $p < 0.05$ ).

### Association of hypercholesterolemia and plasma lipids with *APOE* gene expression

As represented in the Figure 1A, NL individuals showed higher levels of *APOE* mRNA expression in PBMC than HC individuals at baseline (NL: median  $11.7 \times 10^{-5}$  and inter-quartile (IQ) range:  $7.6 \times 10^{-5} - 20 \times 10^{-5}$ ; ATORVA baseline: median:  $8.1 \times 10^{-5}$  and IQ range:  $3.7 \times 10^{-5} - 17 \times 10^{-5}$ ;  $p = 0.004$ ).

Spearman's correlation test demonstrated that *APOE* mRNA expression in PBMC is negatively correlated with total cholesterol ( $r = -0.195$ ,  $p = 0.008$ ) and LDL cholesterol ( $r = -0.214$ ,  $p = 0.004$ ), and positively correlated with apoAI concentration ( $r = 0.202$ ,  $p = 0.007$ ) (data not shown).

Due to the weak lineal correlation and in order to corroborate the association of *APOE* mRNA expression levels with plasma lipid concentration and hypercholesterolemia, comparison analyses by grouping patients according to tercile of *APOE* mRNA values were performed (first tercile:  $2^{-\Delta C_t} < 7.7 \times 10^{-5}$ ; second tercile:  $2^{-\Delta C_t}$ :  $7.7 \times 10^{-5} - 17.1 \times 10^{-5}$ ; third tercile:  $2^{-\Delta C_t} > 17.1 \times 10^{-5}$ ). First, second and third tercile were considered as low, intermediate and high expression, respectively. As observed in Figure 2, individuals with low *APOE* mRNA expression had higher plasma concentration of total and LDL cholesterol and apoB and lower levels of apoAI ( $p < 0.05$ ). Further univariate logistic regression analysis demonstrated that individuals with mRNA values into the low expression group had increased risk to hypercholesterolemia than individuals with higher (intermediate and high expression) values of *APOE* expression (OR: 2.06, 95% C.I.: 1.10-3.87,  $p = 0.025$ ). Moreover, when compared with the individuals

**Table 1 Demographic and laboratory characteristics of study groups**

Parameter	Hypercholesterolemic (n = 181)	Normolipidemic (n = 181)	p-value
Age, years	56.1 ± 10.8	46.9 ± 6.8	< 0.001
Ethnics [European/African], %	62/38	65/35	0.581
Gender, % of women	67	73	0.205
Menopause, %	52	19	< 0.001
Family history of CAD, %	55	45	0.062
Hypertension, %	57	36	< 0.001
Obesity, %	29	15	0.002
Cigarette smoking, %	16	18	0.673
Physical activity, %	49	45	0.553
BMI, kg/m <sup>2</sup>	27.8 ± 4.1	26.2 ± 4.2	< 0.001
Total cholesterol, mg/dL	270 ± 41	173 ± 18	< 0.001
LDL cholesterol, mg/dL	183 ± 36	98 ± 18	< 0.001
HDL cholesterol, mg/dL	57 ± 14	58 ± 12	0.386
Triglycerides, mg/dL	150 ± 65	82 ± 28	< 0.001
VLDL cholesterol, mg/dL	30 ± 13	16 ± 6	< 0.001
ApoAI, mg/dL	136 ± 26	141 ± 26	0.101
ApoB, mg/dL	143 ± 27	85 ± 23	< 0.001

Non-continuous variables are compared by chi-square test. Continuous variables are presented as media ± SD and compared by *t* test. CAD, coronary artery disease; LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; ApoAI, apolipoprotein AI; ApoB, apolipoprotein B. Conversion factors to convert to Systeme International (SI) units are 0.02586 for cholesterol (mmol/l), 0.01129 for triglycerides (mmol/l) and 0.01 for apolipoproteins (g/l).

with the highest *APOE* mRNA levels, to belong to the low expression group represent an increased risk of 2.25 (OR: 2.25, 95% CI:1.08-4.67, *p*=0.030).

#### Relationship of *APOE* genotypes with basal lipids, atorvastatin response and *APOE* expression

Genotype and allele frequencies are presented in Table 2. Frequencies of SNP genotypes were as expected from a HWE (*p* > 0.05, data not shown) in both, NL and HC groups. The genotype distribution was different between NL and HC groups (*p* = 0.003) showing higher frequencies of  $\epsilon 2\epsilon 3$  and  $\epsilon 2\epsilon 4$  genotypes in NL individuals.  $\epsilon 2$  allele was more frequent in NL group (7.7%) than in HC (1.7%, *p* < 0.001) and logistic regression analysis showed that  $\epsilon 2$  carriers had decreased risk of hypercholesterolemia (OR: 0.27; C.I.: 0.08 - 0.85; *p* < 0.025), after adjustment for covariates gender, ethnics, history of CAD, age, hypertension, obesity, cigarette smoking, physical activity and alcohol consumption.

Individuals carrying the  $\epsilon 2$  allele showed lower total and LDL cholesterol and apoB, as well as higher HDL cholesterol (*p* < 0.05) compared with the  $\epsilon 3$  and  $\epsilon 4$  carriers in NL group (Table 3). No differences were observed on basal plasma lipids in HC group according to *APOE* genotypes.

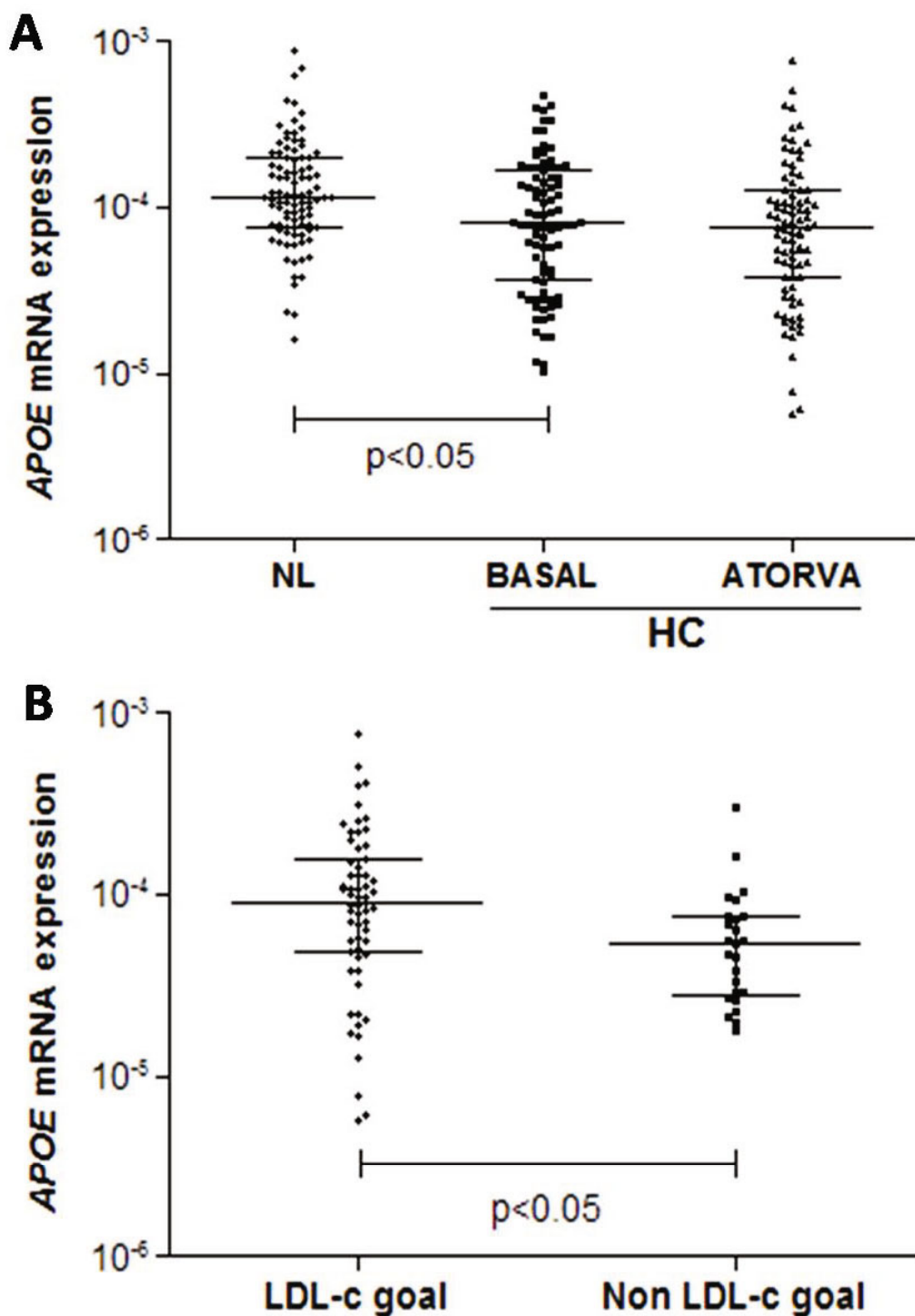
Association between *APOE* genotypes and basal plasma lipids in NL and HC individual was further explored in a multiple linear regression analysis adjusted for covariates gender, age, ethnics, hypertension, obesity, family history

of CAD, physical activity, cigarette smoking and alcohol consumption. In women group, menopause status did not modified *APOE* genotypes effects, therefore this variable was not included in the regression analysis. In NL group, the presence of  $\epsilon 2$  allele was associated with a significant decrease of 11.6, 18.1 and 13.8 mg/dL in values of total cholesterol, LDL cholesterol and apoB, respectively (Table 4). Moreover,  $\epsilon 2$  allele was also associated with an increment of 9 mg/dL of HDL cholesterol. On the other hand, presence of  $\epsilon 4$  allele was related to an increment of 6.7, 8.8 and 9.0 of total cholesterol, LDL cholesterol and apoB, respectively (Table 4). Confirming the previous results, we did not found any association between *APOE* genotypes and basal plasma lipids in HC individuals in the multiple linear regression model (data not shown).

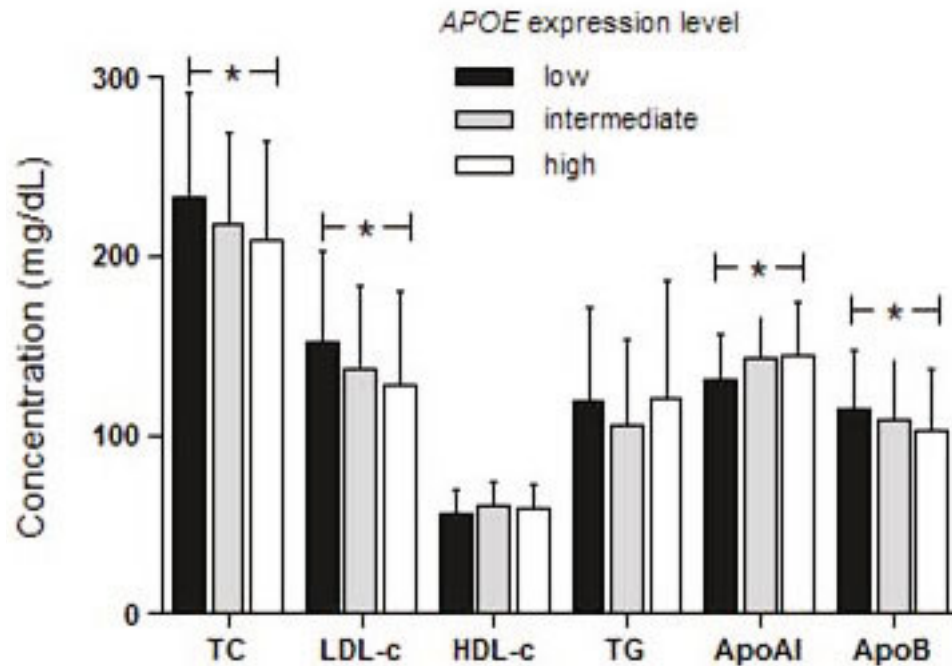
No differences were observed on *APOE* mRNA expression according to *APOE*  $\epsilon 2/\epsilon 3/\epsilon 4$  genotypes (*p* > 0.05, data not shown). Moreover the genotype distribution among *APOE* mRNA expression level groups was similar ( $\chi^2 = 0.695$ , 4 *df*, *p* = 0.952; data not shown).

Atorvastatin treatment reduced the plasma lipids with exception of apoAI in ATORVA group (Additional file 1). Results of CK and ALT demonstrated that there were no muscle or liver adverse reaction cases during the four-week atorvastatin treatment (data not shown).

*APOE* genotypes did not influence basal and post-treatment plasma lipids in individuals treated with atorvastatin (Table 5). Further multiple linear regression



**Figure 1** PBMC APOE mRNA expression in normolipidemic (NL) and hipercolesterolemic individuals before (HC - BASAL) and after treatment with 10 mg/day of atorvastatin (HC - ATORVA) [A] and according to LDL cholesterol goal achievement in response to atorvastatin [B]. Values are presented as dispersion graph with bars indicating median and interquartile range and compared by Mann-Whitney U test or Wilcoxon signed test.



**Figure 2 Plasma lipid profile according to APOE mRNA expression levels.** Patients were grouped in levels of APOE mRNA expression according to tercile values (first tercile [low]:  $2^{-\Delta Ct} < 7.7 \times 10^{-5}$ ; second tercile [intermediate]:  $2^{-\Delta Ct}: 7.7 \times 10^{-5} - 17.1 \times 10^{-5}$ ; third tercile [high]:  $2^{-\Delta Ct} > 17.1 \times 10^{-5}$ ) and plasma lipids were compared among the groups by one way ANOVA and Tukey test. TC, total cholesterol; LDL-c, low density lipoprotein cholesterol; HDL-c, high density lipoprotein cholesterol; TG, triglycerides; apoAI, apolipoprotein AI; apoB, apolipoprotein B; (\*),  $p < 0.05$ . Conversion factors to convert to Systeme International (SI) units are 0.02586 for cholesterol (mmol/l), 0.01129 for triglycerides (mmol/l) and 0.01 for apolipoproteins (g/l).

analysis confirmed these results (data not shown). Moreover, 10 mg/day atorvastatin treatment did not modify the APOE mRNA expression ( $p > 0.05$ ; Figure 1A). Despite the absence of atorvastatin effects on APOE mRNA expression, those individuals without LDL

cholesterol goal achievement according to the IV Brazilian Guidelines for Dyslipidemia and Atherosclerosis Prevention [8] had lower expression ( $p = 0.009$ ; Figure 1B) than patients with desirable response after the treatment.

**Table 2 Genotype and allele frequencies of APOE polymorphisms in hypercholesterolemic and normolipidemic individuals**

Genotypes	$\epsilon 2\epsilon 2$	$\epsilon 2\epsilon 3$	$\epsilon 2\epsilon 4$	$\epsilon 3\epsilon 3$	$\epsilon 3\epsilon 4$	$\epsilon 4\epsilon 4$
HC (n = 181)	0.0% (0)	2.8% (5)	0.6% (1)	66.3% (120)	26.5%(48)	3.8% (7)
NL (n = 181)	0.6% (1)	9.4% (17)	5.0% (9)	61.3% (111)	22.7%(41)	1.1% (2)
$\chi^2 = 17.624; 5 \text{ df}; p = 0.003$						
Alleles	$\epsilon 2$	$\epsilon 3$	$\epsilon 4$			
HC	1.7%	80.9%	17.3%			
NL	7.7%	77.4%	14.9%			
$\chi^2 = 15.223; 2 \text{ df}; p < 0.001$						
Allele	O.R.	95%C.I.		p-value		
$\epsilon 3$	1.00	-		-		
$\epsilon 2$	0.27	0.08-0.85		0.025		
$\epsilon 4$	1.31	0.76-2.26		0.331		

Number of individuals is in parenthesis. HC, hypercholesterolemic; NL, normolipidemic; df, degrees of freedom; O.R., odds ratio; C.I. confidence interval. O.R. values and 95% C.I. were obtained from logistic regression analysis using hypercholesterolemia as dependent variable with gender, ethnics, history of coronary artery disease, age, hypertension, obesity, cigarette smoking, physical activity and alcohol consumption as covariates for the model. Influence of  $\epsilon 2$  and  $\epsilon 4$  alleles on risk for hypercholesterolemia was assessed comparing with the reference allele  $\epsilon 3$ .



**Table 3 Influence of APOE polymorphisms on basal serum lipids in normolipidemic and hipercholesterolemic individuals**

Parameter	ε2	ε3	ε4	p-value
<b>Normolipidemics</b>	(18)	(111)	(43)	–
Total cholesterol, mg/dL	160 ± 23 <sup>a</sup>	173 ± 18 <sup>b</sup>	177 ± 15 <sup>b</sup>	0.003
LDL cholesterol, mg/dL	81 ± 18 <sup>a</sup>	99 ± 18 <sup>b</sup>	105 ± 16 <sup>b</sup>	< 0.001
HDL cholesterol, mg/dL	65 ± 11 <sup>a</sup>	57 ± 12 <sup>b</sup>	56 ± 12 <sup>b</sup>	0.023
VLDL cholesterol, mg/dL	14 ± 5	17 ± 6	16 ± 5	0.179
Triglycerides, mg/dL	72 ± 25	84 ± 28	80 ± 26	0.179
ApoAI, mg/dL	150 ± 20	140 ± 25	137 ± 31	0.205
ApoB, mg/dL	71 ± 19 <sup>a</sup>	86 ± 23 <sup>b</sup>	90 ± 22 <sup>b</sup>	0.011
<b>Hypercholesterolemic</b>	(5)	(120)	(55)	–
Total cholesterol, mg/dL	253 ± 25	271 ± 43	267 ± 31	0.668
LDL cholesterol, mg/dL	166 ± 22	183 ± 39	181 ± 25	0.622
HDL cholesterol, mg/dL	62 ± 18	57 ± 14	56 ± 13	0.679
Triglycerides, mg/dL	133 ± 42	151 ± 71	148 ± 52	0.713
VLDL cholesterol, mg/dL	27 ± 8	30 ± 14	30 ± 10	0.713
ApoAI, mg/dL	140 ± 34	138 ± 29	132 ± 19	0.418
ApoB, mg/dL	128 ± 29	143 ± 28	143 ± 25	0.450

Number of individuals is in parenthesis. Values are expressed as media ± SD and compared by one way ANOVA. HC, hypercholesterolemic; NL, normolipidemic; LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; ApoAI, apolipoprotein AI; ApoB, apolipoprotein B. ε2 represent ε2ε2 and ε2ε3 carriers; ε3 symbolize ε3ε3 genotype and ε4 represent ε3ε4 and ε4ε4 individuals. <sup>a, b</sup>, different letters at the same line represent statistical significance. Conversion factors to convert to Systeme Internacional (SI) units are 0.02586 for cholesterol (mmol/l), 0.01129 for triglycerides (mmol/l) and 0.01 for apolipoproteins (g/l).

## Discussion

Diverse studies have proposed an important role of the *APOE* in hypercholesterolemia and statin response based in this association with *APOE* ε2/ε3/ε4 genotypes, but little information is known about the relationship of these variables with the expression status of *APOE*. Here, we describe the mRNA expression profile in PBMC from NL individuals and HC patients treated with atorvastatin.

Absence or structural mutations of *APOE* cause significant disorders in lipid metabolism and cardiovascular diseases. Deficiency of apoE results in massive accumulation of remnant lipoproteins, leading to severe hypercholesterolemia and atherosclerosis in human and apoE

knockout mice [1]. Here, we reported that HC individuals have lower *APOE* mRNA expression than NL individuals, which is concordant with the previous information related to apoE deficiency.

The *APOE* mRNA expression is extremely complex with regulation in a tissue-specific manner and in response to cellular changes and extra and intra-cellular factors [1]. The expression of cholesterol acceptors in the efflux process such as apoAI, as well as transporter proteins involved in this process have been described to activate *APOE* transcription in human adipocytes and macrophages [9,10]. Accordingly, we observed that individuals with low expression of *APOE* present a decreased apoAI plasma concentration. Moreover, we also reported

**Table 4 Influence of APOE genotypes on basal serum lipids in normolipidemic individuals by multiple linear regression analysis**

Variable	ε2	p-value	ε3	p-value	ε4	p-value
	B (SE)		B (SE)		B (SE)	
Total cholesterol	-11.6 (5.3)	0.030	-1.0 (3.3)	0.753	6.7 (3.3)	0.047
LDL cholesterol	-18.1 (5.2)	0.001	-0.7 (3.4)	0.847	8.8 (3.4)	0.010
HDL cholesterol	9.0 (3.4)	0.009	-1.2 (2.2)	0.568	-1.8 (2.2)	0.399
VLDL cholesterol	-1.8 (1.5)	0.236	0.8 (0.9)	0.352	-0.3 (0.9)	0.799
ApoB	-13.8 (6.9)	0.046	-2.6 (4.2)	0.534	9.0 (4.5)	0.047
ApoAI	11.3 (7.6)	0.138	0.7 (4.5)	0.879	-5.4 (4.9)	0.269

B and (SE) represent regression coefficient and standard error and are expressed in mg/dL. B values were obtained from multiple linear regression analysis introducing gender, ethnics, family history of coronary artery disease, age, hypertension, obesity, cigarette smoking, physical activity and alcohol consumption as covariates. HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; apoB, apolipoprotein B; apoAI, apolipoprotein AI. Conversion factors to convert to Systeme Internacional (SI) units are 0.02586 for cholesterol (mmol/l), 0.01129 for triglycerides (mmol/l) and 0.01 for apolipoproteins (g/l).

**Table 5 Influence of APOE genotypes on serum lipids in hypercholesterolemic individuals treated with atorvastatin (10 mg/day/4-weeks)**

Parameter		ε2 (5)	ε3 (92)	ε4 (44)	p-value
Total cholesterol, mg/dL	Basal	253 ± 25	284 ± 39	275 ± 27	0.100
	Treatment	174 ± 28	198 ± 31	198 ± 27	0.285
	Change, %	-32 ± 6	-30 ± 10	-28 ± 8	0.332
LDL cholesterol, mg/dL	Basal	166 ± 22	194 ± 37	188 ± 21	0.166
	Treatment	96 ± 20	116 ± 28	119 ± 22	0.262
	Change, %	-42 ± 9	-40 ± 13	-37 ± 10	0.408
HDL cholesterol, mg/dL	Basal	62 ± 18	57 ± 15	55 ± 11	0.534
	Treatment	55 ± 14	55 ± 14	53 ± 10	0.745
	Change, %	-11 ± 11	-3 ± 11	-3 ± 9	0.270
VLDL cholesterol, mg/dL	Basal	27 ± 8	32 ± 14	31 ± 11	0.672
	Treatment	24 ± 12	26 ± 12	26 ± 9	0.880
	Change, %	-10 ± 38	-14 ± 30	-12 ± 25	0.902
Triglycerides, mg/dL	Basal	133 ± 42	159 ± 72	155 ± 53	0.672
	Treatment	118 ± 58	130 ± 58	131 ± 43	0.880
	Change, %	-10 ± 38	-14 ± 30	-12 ± 25	0.902
ApoA1, mg/dL	Basal	140 ± 34	138 ± 29	132 ± 19	0.418
	Treatment	151 ± 54	140 ± 28	133 ± 24	0.259
	Change, %	+6 ± 16	+2 ± 11	+1 ± 13	0.650
ApoB, mg/dL	Basal	128 ± 29	143 ± 28	143 ± 25	0.532
	Treatment	97 ± 23	99 ± 21	97 ± 19	0.984
	Change, %	-24 ± 7	-30 ± 13	-31 ± 11	0.494

Number of individuals is in parenthesis. +/- symbols in change indicate reduction or increment of each parameter. Values are presented as media ± SD and compared by one way ANOVA. LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein. ε2 : ε2ε2 and ε2ε3 genotypes; ε3: ε3ε3 genotype; ε4: ε3ε4 and ε4ε4 genotypes. Conversion factors to convert to Systeme Internacional (SI) units are 0.02586 for cholesterol (mmol/l), 0.01129 for triglycerides (mmol/l) and 0.01 for apolipoproteins (g/l).

that these individuals have increased concentrations of total and LDL cholesterol and apoB. The relation of plasma levels of LDL cholesterol with APOE expression in PBMC was previously reported in children with obesity [11]. The increased concentrations of particles which depend on the LDL receptor (LDLR) for their clearance from plasma is consistent with the key role of apoE as a high affinity ligand for the LDLR in the cholesterol homeostasis [12].

APOE allele frequencies have demonstrated to be heterogeneous among different populations, but the ε3 allele is almost invariably the most common and ε2 the rarest allele [13]. In this study, the frequencies of APOE alleles observed in the overall population (ε2: 5%, ε3: 79% and ε4:16%) were similar to earlier studies in European descendant population [13,14], African American [15] and Brazilian populations [16].

Several genetic factors have been related to hypercholesterolemia, however in most of the cases the contribution of these genetic factors to the risk for hypercholesterolemia depends on environmental factors. Our results showed differences in genotype and allele frequencies between normolipidemic and hypercholesterolemic groups, suggesting that APOE ε2 allele confers protection against hypercholesterolemia. This characteristic persists

even after adjustment for covariates that have been largely associated with hypercholesterolemia, such as gender, ethnics, history of CAD, age, hypertension, obesity, cigarette smoking and physical activity, suggesting that APOE ε2 could be considered an independent factor that protect against hypercholesterolemia in our sample population.

Ferreira and co-workers [16] did not found differences in APOE ε2/ε3/ε4 genotypes between normolipidemic and dyslipidemic Brazilian individuals. On the other hand, and in line with our results, it has been reported higher frequency of ε2 allele in normolipidemic than hypercholesterolemic individuals from South America [17]. Moreover, a previous study in the Brazilian population reported that, compared with the ε2 allele, the presence of ε3 allele increases more than two times the risk for dyslipidemia (OR: 2.31, CI:1.06-5.06) [18], which is in agreement with our results.

The effects of APOE polymorphisms on plasma lipids have been described by several studies and the evidence suggests that APOE ε2 is associated with lower, whereas ε4 with higher, concentrations of plasma total cholesterol, LDL cholesterol and apoB in comparison with the ε3 allele [19]. We reported a less atherogenic lipid profile of ε2 allele, as well as a contribution of ε4 allele for higher total and LDL cholesterol and apoB in normolipidemic



individuals. However, the association between *APOE* polymorphisms and plasma lipids were detected exclusively in the normolipidemic group. In agreement with this characteristic, an association of *APOE* genotypes with basal plasma lipids in normolipidemic individuals, but not in dyslipidemic patients, was previously reported in our population [16]. The authors described that  $\epsilon 2$  allele carriers had significantly lower total, LDL and non-HDL cholesterol compared to  $\epsilon 3$  and  $\epsilon 4$  allele carriers only in normolipidemic individuals. Moreover, other studies were not able to demonstrate any association between *APOE*  $\epsilon 2/\epsilon 3/\epsilon 4$  genotypes and total and LDL cholesterol in patients with familial hypercholesterolemia [20] and polygenic dyslipidemia [21,22]. Nevertheless, the lack of association of *APOE* polymorphisms with plasma lipids in hypercholesterolemic patients in our sample seems to be attributable to the small number of individuals carrying the  $\epsilon 2$  allele that could be considered an important limitation of our study.

The variation on plasma lipids according to *APOE*  $\epsilon 2/\epsilon 3/\epsilon 4$  genotypes are believed to stem mainly from structural and biophysical properties of apoE isoforms [3]. ApoE4-containing lipoproteins exhibit a high binding ability to their receptors that cause a more efficient catabolism and an accelerated clearance of chylomicrons and VLDL-remnants, leading to down regulation of LDLR and HMGCR and to increased LDL cholesterol levels in plasma. On the contrary, lipoproteins containing the apoE2 isoform present lower affinity compared to apoE4 and apoE3 isoforms that result in decreased cholesterol levels.

In the present study, no differences were observed in the change of lipid levels in response to atorvastatin treatment according to *APOE* genotypes. Although many studies have evaluated the influence of *APOE* polymorphism on statin response, some of these studies had controversial results. Whereas there is a strong line of evidence linking *APOE*  $\epsilon 2/\epsilon 3/\epsilon 4$  genotypes with the efficacy of statin treatment [23-26], other studies did not reveal any association between *APOE* genotypes and response to treatments with various statins [27-29]. Commonly, evidence supports that *APOE*  $\epsilon 3$  allele is associated with better response than  $\epsilon 4$  allele in term of LDL cholesterol decrease and, in addition, individuals carrying the  $\epsilon 2$  allele have greater reduction of LDL cholesterol than  $\epsilon 3$  homozygotes [30]. These differences result from the improved activity of HMGCR in  $\epsilon 2$  compared to  $\epsilon 3$  allele carriers due to the modulation of intracellular cholesterol by the upregulation of hepatic LDLR, which has lower affinity for the apoE2 isoform that results in an improved response of  $\epsilon 2$  allele carriers to the inhibition of HMGCR by statins. On the other hand, the LDLR presents higher affinity for apoE4 isoform and the effect of statin therapy is diminished in  $\epsilon 4$  allele carriers when compared to  $\epsilon 2$  or  $\epsilon 3$  [3].

In the last years, GWAS have provided new perspectives and a more comprehensive approach for identifying genetic loci associated to statin response. Thompson *et al.* (2009), using a platform of 291, 988 SNPs did not observe any association between genotypes and atorvastatin response at beginning, when 1, 984 individuals were analyzed, however further analysis in 5745 individuals from the Treating to New Target (TNT) trial using a candidate gene approach reported a strong association between *APOE*  $\epsilon 2/\epsilon 3/\epsilon 4$  genotypes and LDL cholesterol statin response [31]. Furthermore, a recent study has evaluated the response to diverse statins using a GWAS approach involving nearly 4, 000 individuals from three different trials of statin efficacy [Cholesterol and Pharmacogenetics (simvastatin), Pravastatin/Inflammation CRP evaluation (pravastatin) and TNT (atorvastatin)] [32]. The authors did not found any association between *APOE* SNPs and statin response, however the SNP rs4429638, located in the *APOC1* gene and near *APOE*, was associated with change LDL cholesterol suggesting a possible involvement of *APOE* locus in statin efficacy.

Despite the number of studies investigating the response to statins according to *APOE* genotypes, the effect of HMGCR inhibitors on apoE protein and mRNA expression has been poorly studied, particularly using *in vivo* models. Atorvastatin and cerivastatin demonstrated to reduce apoE protein secretion and *APOE* mRNA expression in THP-1 derived macrophages after 24h of treatment in a dose dependent manner [33]. Conversely, in cultured human monocyte-derived macrophages, lovastatin increased *APOE* mRNA levels but decreased apoE secretion [34], phenomena that the authors attributed to the increase of apoE not destined for secretion. On the other hand, regarding *in vivo* studies, Guan *et al.* reported that *APOE* mRNA levels in mononuclear cells of hyperlipidemic diabetic patients taking simvastatin (5-10 mg/day) did not differ from those without statin treatment [35]. These contradictory results from *in vitro* experiments and the data reported for Guan and co-workers and our observations in PBMC from hypercholesterolemic individuals (not change after 10 mg/day atorvastatin treatment) could be explained by the differences in the cellular models used by the authors. Moreover, we observed that patients without LDL cholesterol goal achievement had lower *APOE* mRNA expression that could suggest a possible involvement of the modulation of this gene in the statin response.

## Conclusions

Hypercholesterolemic patients had lower *APOE* mRNA levels than normolipidemic individuals and the *APOE* expression levels were associated with differences in the plasma lipid profile, corroborating the key role of the *APOE* in the cholesterol metabolism and suggesting that

*APOE* mRNA expression may be a good marker for hypercholesterolemia in our sample population. Moreover, although there was no effect of  $\epsilon 2/\epsilon 3/\epsilon 4$  polymorphisms on *APOE* gene expression, the presence of  $\epsilon 2$  allele confers protection against hypercholesterolemia in Brazilian subjects. Moreover, there was no evidence of an involvement of *APOE* genotypes with atorvastatin lipid-lowering response and the atorvastatin treatment does not modify the mRNA expression in hypercholesterolemic subjects, however the low *APOE* mRNA expression after treatment showed by individuals with poor cholesterol lowering-response suggests a possible involvement of *APOE* level in atorvastatin response.

## Methods

### Study population and therapeutic protocol

Characteristic of study population and therapeutic protocol were previously described [36]. Three-hundred-sixty-two (255 women and 107 men, aged 29 to 81 y) individuals were randomly selected at the University Hospital of University of Sao Paulo and the Institute Dante Pazzanese of Cardiology, Sao Paulo city, Brazil. One-hundred-eighty-one subjects were classified as hypercholesterolemic (HC) according to the IV Brazilian Guidelines for Dyslipidemia and Atherosclerosis Prevention [8] and 181 were considered normolipidemics (NL) LDL cholesterol < 3.36 mmol/L (130 mg/dL). Individuals with diabetes mellitus; hypertriglyceridemia [triglycerides > 4.42 mmol/L (350 mg/dL)]; liver, renal or thyroid disease; pregnant women or under treatment of oral contraceptives; and other causes of secondary dyslipidemia were not included in the study.

HC individuals went through a four-week washout having a low fat diet according to the American Heart Association recommendation [37]. One-hundred-forty-one individuals from HC group had indication of lowering-cholesterol drug therapy in order to reach the LDL cholesterol goal according to the IV Brazilian Guidelines for Dyslipidemia and Atherosclerosis Prevention [8]. Afterwards, these individuals were treated with 10 mg/day of atorvastatin during 4 weeks (ATORVA group). Serum lipids were measured to evaluate atorvastatin cholesterol-lowering response. The study protocol was approved by the ethics committees of the Institute Dante Pazzanese of Cardiology, University Hospital and the School of Pharmaceutical Sciences of the University of the Sao Paulo. Each individual agreed to participate in the study by signing an informed consent.

### Biochemical measurements

Blood samples were collected after an overnight (12 h) fast. ATORVA patients had blood drawn previous and after the 4-week atorvastatin treatment. Plasma total

cholesterol, HDL cholesterol and triglycerides were measured by routine enzymatic colorimetric methods. Plasma apo AI and apo B were measured by nephelometry. LDL and VLDL cholesterol were estimated by Friedewald formula [38]. Serum ALT and CK concentrations were determined by kinetic methods to evaluate atorvastatin effects on liver and muscle tissues.

### *APOE* genotyping

Genomic DNA was extracted from EDTA-treated blood samples using salting out procedure [39]. SNPs rs7412 and rs429358, that determinate the *APOE*  $\epsilon 2$ ,  $\epsilon 3$  and  $\epsilon 4$  alleles were analyzed by allelic discrimination using TaqMan real time PCR system. Validated SNP genotyping assays (ID number C\_904973\_1 for rs429358 and C\_904973\_1 for rs7412) were purchased from Applied Biosystems (Applied Biosystems, CA, USA). Both SNP genotyping reactions were optimized in a total volume of 8  $\mu$ l using 20 ng of DNA and fluorescence was detected in a 7500 Fast Real-Time PCR system (Applied Biosystems, CA, USA). Control samples with known *APOE* genotypes were included in each PCR run, which were analyzed by the alternative PCR-RFLP method [40].

### *APOE* mRNA quantification in PBMC

*APOE* mRNA expression was measured in individuals from NL (n = 88) and ATORVA (n = 94) groups. EDTA-anticoagulated blood samples were used to obtain peripheral blood mononuclear cells (PBMC) as previously described [41] and immediately used for RNA extraction. Total RNA was extracted from PMBC using TRIzol<sup>®</sup> Reagent (Invitrogen-Life Technologies, CA, USA) following the manufacturer's suggested protocol. RNA was dissolved in DEPC-treated water and the concentration was measured by spectrophotometry using the NanoDrop<sup>®</sup> (NanoDrop Technologies INC., DE, USA). RNA integrity was evaluated using the bioanalyzer<sup>®</sup>2100 (Agilent technologies, CA, USA). Samples with RNA integrity number (RIN) lower than 5 were not used for mRNA experiments. cDNA was produced from 1  $\mu$ g of total RNA by Superscript<sup>™</sup> II Reverse Transcriptase (Invitrogen-Life Technologies, CA, USA).

*APOE* mRNA expression was measured by quantitative TaqMan real-time PCR (qPCR). The assay ID Hs00171168\_m1 was used to access the *APOE* mRNA detection. Genorm software <http://medgen.ugent.be/genorm> was used to select the most stable among six endogenous reference genes [ubiquitin C (*UBC*), glyceraldehyde-3-phosphate dehydrogenase (*GAPD*), beta-2-microglobulin (*B2M*), Hypoxanthine phosphoribosyl-transferase I (*HPRTI*), succinate dehydrogenase complex, subunit A (*SDHA*) and hydroxymethyl-bilane synthase (*HMBS*)], and the most stable in the

experimental conditions was *UBC*. The sequence of primers and probes used for *UBC* are described as follows: Forward, 5'-ATTTGGGTCGCGTTCTTG-3'; reverse, 5'-TGCCTTGACATTCTCGAT GGT-3'; and probe, VIC -TCGTCACCTTGACAATGC- MGB/NFQ. The qPCR assays were carried out in 96 well plates using a 7500 Fast Real-Time PCR system (Applied Biosystems, CA, USA). The relative quantification of *APOE* mRNA was calculated by the comparative Ct method using the formula  $2^{-\Delta C_t}$  [42].

### Statistical Analysis

Statistical analyses were performed using SPSS v.15 for windows (SPSS Inc., Madrid, Spain) and Minitab v.15 statistical software (Minitab Inc. State College, PA). The allele frequencies and genotype distribution were estimated by gene counting. SNPAnalyzer software v. 1.0 [43] was used to verify whether the genotypes distribution were in Hardy-Weinberg equilibrium (HWE) using the expectation-maximization algorithm. Differences in non-continuous variables, genotype and allelic distributions were compared by chi-square test. Normality distribution for all continuous variables was tested by Kolmogorov-Smirnov test and skewed variables were log transformed to improve normality for statistical analysis. Differences in mean values were evaluated by *t*-test or one-way ANOVA using Tukey post-hoc test. Paired *t*-test was used to analyze the effects of treatment in ATORVA group. In order to evaluate effects of *APOE* genotypes on the different variables, individuals were classified into three phenotypes: 1) E2 group, carrying the  $\epsilon 2\epsilon 2$  and  $\epsilon 2\epsilon 3$  genotypes; 2) E3 group, carrying the  $\epsilon 3\epsilon 3$  genotypes; and 3) E4 group carrying either  $\epsilon 3\epsilon 4$  or  $\epsilon 4\epsilon 4$  genotypes. Individuals with the  $\epsilon 2\epsilon 4$  were not assigned to any group and they were therefore excluded for the analysis. Mann Whitney U test or Kruskal-Wallis (two or three independent samples) or Wilcoxon signed rank test (paired samples) were used to evaluate differences in *APOE* mRNA gene expression. Correlations of *APOE* expression with other continuous variables were assessed by Spearman correlation test. Individuals were grouped in three groups (terciles) according to *APOE* expression values and plasma lipid concentrations were then compared among the groups using one-way ANOVA and Tukey post-hoc test. Multiple logistic regression analysis was used to examine the association of *APOE* genotypes with hypercholesterolemia including adjustment for relevant covariates. Multiple linear regression analysis was performed introducing variables of interest as dependent variables and *APOE* genotypes together to relevant covariates as independent variables, which regression coefficient were obtained for each independent variable. Statistical significance was set for  $p < 0.05$ .

### Additional material

**Additional file 1: Supplementary table.** Influence of 10 mg/day/4-weeks atorvastatin treatment on plasma lipids in ATORVA group (n = 141). Lipid plasma concentrations in individuals of the ATORVA group at baseline and after 10 mg/day/4-weeks atorvastatin treatment.

### List of abbreviations

ApoE: apolipoprotein E; *APOE*: apolipoprotein E gene; HDL: high density lipoprotein; LDL: low density lipoprotein; VLDL: very low density lipoprotein; LDLR, low density lipoprotein receptor; HMGCR: 3-hydroxy-3-methylglutaryl coenzyme A reductase; HC: hypercholesterolemic; NL: normolipidemic; ATORVA: group of patients treated with atorvastatin; PBMC; peripheral blood mononuclear cells; CAD: coronary artery disease; GWAS: genome-wide association studies; SNP: single nucleotide polymorphism.

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### Authors' contributions

AC carried out the experimental procedures, patients' selection, analysis of data and drafted the manuscript. FDVG, MAWW and SSA contribute to patients' selection and experimental procedures. MMSB, ELD, MCB and AAF contribute to patients' selection and monitoring patients under study protocol. MHH and RDCH participated in the design of the study, interpretation of data and elaboration of the manuscript. All authors have read and approved the final manuscript.

### Competing interests

The authors declare that they have no competing interests.

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