High baseline serum total and LDL cholesterol levels are associated with \textit{MDR1} haplotypes in Brazilian hypercholesterolemic individuals of European descent

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Abstract

The \textit{MDR1} gene encodes the P-glycoprotein, an efflux transporter with broad substrate specificity. P-glycoprotein has raised great interest in pharmacogenetics because it transports a variety of structurally divergent drugs, including lipid-lowering drugs. The synonymous single-nucleotide polymorphism C3435T and the nonsynonymous single-nucleotide polymorphism G2677T/A in \textit{MDR1} have been indicated as potential determinants of variability in drug disposition and efficacy. In order to evaluate the effect of G2677T/A and C3435T \textit{MDR1} polymorphisms on serum levels of lipids before and after atorvastatin administration, 69 unrelated hypercholesterolemic individuals from São Paulo city, Brazil, were selected and treated with 10 mg atorvastatin orally once daily for four weeks. \textit{MDR1} polymorphisms were analyzed by PCR-RFLP. C3435T and G2677T/T polymorphisms were found to be linked. The allelic frequencies for C3435T polymorphism were 0.536 and 0.464 for the 3435C and 3435T alleles, respectively, while for G2677T/A polymorphism allele frequencies were 0.580 for the 2677G allele, 0.384 for the 2677T allele and 0.036 for the 2677A allele. There was no significant relation between atorvastatin response and \textit{MDR1} polymorphisms (repeated measures ANOVA; \(P > 0.05\)). However, haplotype analysis revealed an association between T/T carriers and higher basal serum total (TC) and LDL cholesterol levels (TC: 303 ± 56, LDL-C: 216 ± 57 mg/dl, respectively) compared with non-T/T carriers (TC: 278 ± 28, LDL-C: 189 ± 24 mg/dl; repeated measures ANOVA/Tukey test; \(P < 0.05\)). These data indicate that \textit{MDR1} polymorphism may have an important contribution to the control of basal serum cholesterol levels in Brazilian hypercholesterolemic individuals of European descent.
Introduction

Statins are 3-hydroxy-3-methylglutaryl co-enzyme A (CoA) reductase (HMGR) inhibitors that have an effective cholesterol lowering effect. Atorvastatin is an active hydroxy acid statin that reduces low-density lipoprotein (LDL) cholesterol by 40 to 60% at single daily doses of 10 to 80 mg (1). Atorvastatin undergoes varying degrees of metabolism in both animals and humans, catalyzed primarily by CYP3A4, an isoform of cytochrome P450, in the liver and gut (2). Other reported biotransformation pathways include lactonization of statin hydroxy acid and β-oxidation at the common dihydroxy heptanoic or heptanoic acid side chain (3). The statin acids are converted to the corresponding lactones by the acyl glucuronide intermediate, as demonstrated by Prueksaritanont et al. (4). Both acyl glucuronide and acyl CoA derivatives may revert to the statin acids by hydrolysis. Similar considerations apply to oxidative metabolites of the statins. The statin lactones are hydrolyzed to their open acids chemically or enzymatically by esterases or the recently identified paraoxonases. Atorvastatin and its metabolites are excreted primarily into the bile (1) by transporters, represented especially by the ATP-binding cassette (ABC) family (5).

ABC transporters are a superfamily of integral transmembrane proteins that uses energy of ATP hydrolysis to translocate a broad spectrum of molecules across the cell membrane. ABC proteins have transmembrane domains, intracellular ATP-binding domains, and a substrate-binding domain (6).

ABCB1 is a 170-kDa transporter protein named P-glycoprotein (P-gp) that has been associated with the transport of cellular lipids and drugs (6). P-gp is involved in the elimination of atorvastatin, which has been described as an inhibitor of P-gp (7). Constitutive expression of P-gp transporter in normal tissues has been shown to play an important role in drug disposition and response (reviewed in Ref. 8). The co-localization of P-gp with the drug-metabolizing enzyme CYP3A4 in the small intestine and liver suggests its role in the oral bioavailability, distribution, and excretion of drugs (9).

Expression of P-gp is regulated by the pregnane X receptor (PXR), a ligand-activated transcription factor that coordinately stimulates the expression of a large program of genes involved in the solubilization and excretion of xenobiotics from the body. Among them are those encoding P450 enzymes, UDP-glucuronosyltransferases, glutathione-S-transferase and various transporters, such as multidrug resistance-associated protein 2 and organic anion transporter peptide 2 (10). It has been reported that statins may be ligands for PXR since lovastatin was characterized as a PXR activator (11).

P-gp is encoded by a polymorphic gene named multidrug resistance 1 (MDR1) located on chromosome 7. The MDR1 gene has more than 20 polymorphisms, some of which have been associated with altered P-gp expression and activity in vivo (8).

The silent C3435T (exon 26) and the G2677T/A (exon 21), that lead to the amino acid exchanges G2677T (Ala893Ser) and G2677A (Ala893Thr), are common MDR1 polymorphisms. The frequencies of MDR1 3435T and 2677T alleles are influenced by ethnicity. Caucasian and Asian individuals have a higher frequency of the 3435T allele (37 to 66%) than African individuals (10 to 27%) (8,12,13). The frequency of the 2677T allele is also higher in Asians and Caucasians (38 to 62%) than in African Americans (15%) (8,14-16). On the other hand, the 2677A allele frequency is higher in Japanese (15 to 22%) (14,17) than in Caucasians (2 to 4%) (15,18,19).

It has been reported that C3435T and G2677T/A polymorphisms influence the duodenal expression of P-gp and alter the absorption and/or disposition of many drugs (20,21).

C3435T polymorphism was found to be
associated with variation in intestinal P-gp levels, influencing the uptake of orally administered P-gp substrates such as digoxin (22), fexofenadine (16) and cyclosporine (23). Subjects carrying the 3435TT genotype have remarkably lower duodenal P-gp expression and higher plasma digoxin levels in comparison to subjects with CC or CT genotypes (22,24,25). In contrast to these observations, lower plasma digoxin levels after orally administered digoxin and higher MDR1 expression in duodenal enterocytes were found in Japanese individuals carrying the 3435T allele (20,26).

The MDR1 G2677T/A polymorphism has also been associated with variations of P-gp activity. In vitro experiments have shown that cells expressing the 2677T variant have an enhanced efflux of digoxin when compared to those expressing the 2677G allele (16). Moreover, individuals carrying the 2677T allele had higher P-gp activity in vivo, measured by plasma fexofenadine levels, than those with the 2677G allele (16). MDR1 mRNA expression relative to villin mRNA in duodenal enterocytes was higher in subjects carrying the 2677T or 2677A polymorphic alleles (14).

Since it has been proposed that atorvastatin alters P-gp activity, it is possible that variations in MDR1 may also affect lipid response to atorvastatin. The present study was designed to evaluate the effects of human MDR1 polymorphisms, specifically G2677T/A and C3435T, on serum levels of lipids before and after atorvastatin administration to unrelated Brazilian individuals with hypercholesterolemia.

### Material and Methods

#### Subjects and study protocol

Sixty-nine unrelated individuals (28 men, 41 women, mean age: 59 years) were studied. They were admitted to the Dante Pazzanese Institute of Cardiology (São Paulo, SP, Brazil) from 2001 to 2003, with primary hypercholesterolemia, according to NCEP (27). Individuals with plasma triglycerides above 400 mg/dl, with hypothyroidism or diabetes mellitus or treated with oral contraceptives were not included in the study. The characteristics of the subjects are presented in Table 1.

Four weeks before the study all subjects met with a dietitian and were instructed to consume a low cholesterol diet. One week before the start of atorvastatin treatment, all subjects were evaluated for fasting serum levels of glucose, lipids and hepatic enzymes. Individuals with LDL cholesterol higher than 160 mg/dl were started on atorvastatin therapy, 10 mg orally once daily for four weeks. After treatment, subjects underwent another

<table>
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<th>Table 1. Characteristics of Brazilian hypercholesterolemic individuals of European descent.</th>
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<td>Range</td>
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<tr>
<td>Men ≥45 years</td>
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<td>Women ≥55 years</td>
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<td>Family history of coronary artery disease</td>
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<td>Concomitant medication</td>
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Data regarding sex, risk factors and concomitant medication are reported as percent with the number of individuals given in parentheses. *Arterial pressure ≥140/90 mmHg; $no$ hormonal replacement therapy; $first-degree relationship for men below 55 years and for women below 65 years; $angiotensin-converting enzyme inhibitors, beta blockers, thiazide diuretics, potassium sparing$ diuretics, and calcium channel blockers. Individuals whose clinical characteristics were not available were not included in the statistical analysis.
evaluation of fasting serum levels of glucose, lipids and hepatic enzymes. The study protocol was approved by Dante Pazzanese Institute of Cardiology Ethics Committee and informed consent was obtained from each patient.

**Lipid and lipoprotein measurements**

Blood samples were collected from the individuals after an overnight fast, before and after atorvastatin administration. Serum total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglyceride concentrations were measured using standard enzymatic methods with an automated analyzer (Hitachi 912, Hitachi Ltd., Tokyo, Japan). HDL cholesterol was measured after phosphotungstic acid and magnesium precipitation. LDL and very low-density lipoprotein (VLDL) cholesterol concentrations were calculated using the Friedwald formula (28).

**Genomic DNA analysis**

Genomic DNA was extracted from EDTA-anticoagulated blood by a salting-out procedure optimized in our laboratory (29). G2677T, G2677A, and C3435T MDRI polymorphic regions were amplified by the polymerase chain reaction (PCR). PCR assays were performed with 50 ng genomic DNA, amplification buffer (50 mM KCl, 20 mM (NH₄)₂SO₄, 2 mM MgCl₂, 75 mM Tris-HCl, pH 9.0), 200 µM primers, and 0.5 U DNA polymerase (Biotools B&M Labs., S.A., Madrid, Spain). For both G2677T and G2677A genotyping, the forward primer 5'-TTGTTTTGCAGGCTATAGGTTCC-3' and the reverse were used as described previously (15). For C3435T polymorphism, we designed the forward and reverse primers 5'-TCCTTAATCTCAGTAACCTGGCA-3' and 5'-ATGAAAGCGATTTAGTTGGCC-3', respectively. The thermal cycler protocol consisted of initial denaturation at 98°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min and extension at 72°C for 2 min. Amplification was carried out in a thermal cycler, PTC-200 (MJ Research Inc., Waltham, MA, USA). PCR products were analyzed by 1.5% agarose gel electrophoresis after ethidium bromide staining.

G2677T, G2677A and C3435T polymorphisms were detected by digestion of PCR-amplified products using the restriction enzymes BshNI, Rsfl and MboI, respectively. Enzymatic digestions were performed at 37°C for 1 h in a total volume of 10 µl using 1 U restriction endonuclease and 1x restriction buffer (33 mM Tris-acetate, 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA, pH 7.9). Restriction fragments were identified by 8% polyacrylamide gel electrophoresis after silver staining.

**Statistical analysis**

Genotype distribution and allelic frequency for the polymorphisms were estimated by gene counting. Differences in genotype frequency distribution from that expected from Hardy-Weinberg equilibrium were determined by the chi-square test. Haplotype frequencies were estimated on the basis of the Expectation-Maximization algorithm (30) using the population genetics data analysis program Arlequin (31). The computer package SAS System 6.12 for Windows (SAS Institute Inc., Cary, NC, USA) was used to analyze data. All the continuous variables are presented as means ± SD. In order to improve statistical power, the genotypes for C3435T (CT and TT) and G2677T/A (GT, GA, TT, TA) polymorphisms were combined into a single “T” allele carrier for C3435T and a single “T/A” allele carrier for G2677T/A polymorphism. Relationships between the genotypes and categorical variables (cigarette smoking, age, sex, menopause, hypertension) were evaluated by the chi-square test. The effect of each polymorphism on lipid and lipoprotein
levels before and after atorvastatin administration (10 mg/day) was evaluated by repeated measures ANOVA followed by the Tukey test. Serum lipid and lipoprotein data before and after treatment were compared using the profile test by contrast. All variables were log transformed (log10) for analysis due to lack of normal distribution. Significance was defined as P < 0.05.

Results

MDR1 polymorphisms

The allelic frequencies and genotype distribution of the MDR1 polymorphisms for the Brazilian individuals of European descent studied here are summarized in Table 2. The genotype frequencies of the MDR1 polymorphisms were in Hardy-Weinberg equilibrium. The relative frequency of the MDR1 2677T allele was 0.384, while the frequency of the 2677A allele was much lower (0.036). For C3435T polymorphism, the relative frequency of the 3435T allele was 0.464. A strong linkage disequilibrium was observed between the G2677T/A and C3435T polymorphisms (χ² = 34.88, P < 0.0001, 2 d.f.). Association between genotypes from the MDR1 polymorphisms resulted in 18 possible haplotypes, 10 of which were found in the Brazilian subjects of European descent. The most frequent haplotype was 2677GT/3435CT (36.2%), followed by GG/CC (15.9%), GG/CT (11.5%) and TT/TT (10.1%), whereas the frequency of the GT/TT (7.3%), GT/CC (7.2%), GA/CC (4.4%), GG/TT and TA/CT (2.9%), and TT/CT (1.5%) haplotypes was lower.

Effects of MDR1 polymorphisms

The relationships between genotypes and categorical variables (cigarette smoking, age, sex, menopause, hypertension) were evaluated by the chi-square test. There was no significant influence of the variables on genotype distribution (data not shown).

The results of repeated measures ANOVA regarding the effect of MDR1 C3435T and G2677T/A polymorphisms on serum lipid and lipoprotein concentrations before and after treatment with atorvastatin are presented in Table 3. The serum lipid levels of individuals carrying the 2677G allele (GG genotype) were similar to those found in GG non-carriers (GT, GA, TT, AA, TA genotypes) after atorvastatin treatment (P > 0.05). For C3435T polymorphism, 3435C carriers (CC genotype) had a lipid profile similar to that of CC non-carriers (CT and TT genotypes; P > 0.05).

No significant interaction between G2677T/A and C3435T MDR1 polymorphisms and atorvastatin treatment was observed regarding treatment-induced changes...
We also evaluated the effect of *MDR1* haplotypes between baseline and atorvastatin treatment on serum concentrations of lipids and lipoproteins. The individuals were grouped as T/T haplotype carriers (GT/CT, GT/TT, TT/CT, TT/TT, and TA/CT haplotypes) and non-T/T haplotype carriers (GG/CC, GG/CT, GT/CC, GG/TT, and GA/CC haplotypes). As shown in Figure 1, baseline serum levels of total (Figure 1A) and LDL (Figure 1B) cholesterol were significantly higher in Brazilian individuals of European descent carrying the T/T haplotype (total cholesterol: 303 ± 56, LDL: 216 ± 57 mg/dl) than in those carrying the non-T/T haplotype (total cholesterol: 278 ± 28, LDL: 189 ± 24 mg/dl) (Tukey test: P < 0.05). However, after atorvastatin treatment, serum lipid and lipoprotein levels were similar for TT haplotype carriers and non-carriers.

As shown in Figure 2, there was no significant interaction between T/T and non-T/T haplotype carriers and atorvastatin treatment-induced changes in total and LDL cholesterol in Brazilian subjects of European descent.

**Discussion**

Significant ethnic differences in allele frequency and genotype distribution of the *MDR1* C3435T and G2677T/A polymorphisms have been shown (8). In the population of Brazilian subjects of European descent studied here, the relative frequency of the common 3435C allele (0.558) was simi-
lar to that found in other Caucasian populations (8,12,15,16,22,32).

Analysis of the \textit{MDR1} G2677T/A polymorphism showed that the relative frequencies of the 2677G (0.580), 2677T (0.384) and G2677A (0.036) alleles for Brazilians of European descent did not differ from those observed in other Caucasian populations (8,15,16,18).

In our population we observed linkage disequilibrium between C3435T and G2677T/A polymorphisms, as demonstrated for European American and Asian populations (16, 26,30,33).

High baseline levels of total and LDL cholesterol were found in Brazilian individuals of European descent carrying the 3435T allele compared to those with the 3435CC genotype, but the results failed to reach statistical significance. Kajinami et al. (34) have reported that hypercholesterolemic white women carrying at least one 2677nonG (2677T or 2677A) allele showed significantly higher LDL cholesterol levels than non-carriers, but this was not seen in men.

Haplotype analysis revealed that the Brazilians of European descent carrying the T/T haplotype had higher levels of total and LDL cholesterol. This observation suggests that the functional effects of P-gp may be haplotype-dependent and do not necessary need to be defined by a single polymorphism.

Increased \textit{MDR1} mRNA and protein expression has been demonstrated (16,20,26) by \textit{in vitro} and \textit{in vivo} studies. Nakamura et al. (20) described higher mRNA expression levels in duodenal enterocytes of healthy Japanese volunteers with the 3435TT genotype. Therefore, it is reasonable that the high levels of serum total and LDL cholesterol found in T/T haplotype carriers may be due to an increased \textit{MDR1} mRNA and protein P-gp expression.

P-gp is involved in the transport of free cholesterol from the plasma membrane to the endoplasmic reticulum (ER), the site of cholesterol esterification by acyl-CoA: cholesteryl acyltransferase (ACAT) (35). In addition, the positive correlation between \textit{MDR1} and ACAT mRNA expression levels found in atherosclerotic lesions suggests that P-gp may be involved in the accumulation of intracellular cholesterol ester and in the acceleration of cell proliferation rate in vessel sites prone to atherosclerosis (36).

The increased P-gp activity associated with the \textit{MDR1} variants may result in increased intracellular content of cholesterol esters that induces a reduction of cholesterol synthesis and LDL uptake mediated by HMGR and LDL receptor, respectively. Therefore, the lower number of LDL receptors on the cell membrane reduces the rate of removal of LDL particles, enhancing the cholesterolemia. Although this mechanism

![Figure 2. Serum concentration of total (A) and low-density lipoprotein (LDL) cholesterol (B) in Brazilian hypercholesterolemic individuals of European descent according to \textit{MDR1} haplotype, before (baseline) and after atorvastatin therapy (10 mg/day). Non-T/T carriers: GG/CC, GG/CT, GG/TT, GT/CC, GA/CC haplotypes; T/T carriers: GT/CT, GT/TT, TT/CT, TT/TT, TA/CT haplotypes. Repeated measures ANOVA: P > 0.05 for differences between non-TT and TT carriers.](image)
would explain the higher levels of total and LDL cholesterol found in Brazilian individuals of European descent who are T/T haplotype carriers, other molecules that regulate the intracellular cholesterol trafficking may also be involved.

Recently, it has been shown that caveolin-1, a component of caveolae, mediates the efflux of intracellular free cholesterol by transporting cholesterol from the ER to the plasma membrane (37). Therefore, the availability of a cholesterol substrate in the ER, which is the major determinant of ACAT activity, may be regulated by both P-gp and caveolin-1 (37). These findings would explain the lack of correlation between P-gp activity and intracellular cholesterol esterification found in HepG2 cells (38).

The C3435T and G2677T/A polymorphisms were associated with drug responses in patients treated with atorvastatin (34), nelfinavir (39), digoxin (22), and tacrolimus (40). We did not find a significant effect of these polymorphisms on the response to atorvastatin in hypercholesterolemic patients. Recently, Kajinami et al. (34) detected an association between 3435CC genotype and smaller reductions in LDL cholesterol, but larger increases in HDL cholesterol, relative to variant allele carriers among hypercholesterolemic white women after treatment with atorvastatin (10 mg/day). This effect was not found in men, suggesting a gender-specific effect. Also, haplotype analysis showed that women carrying the homozygous GC haplotype (2677GG and 3435CC genotypes) have a smaller response regarding reduction of LDL cholesterol than women with the non-GC haplotype.

The lack of association between lipid response to atorvastatin and MDR1 genotypes and haplotypes may be due to the size of our sample since we could not stratify our population by gender. In addition, this caused the statistical power of the test performed to be below the desired level. Therefore, other studies on P-gp expression and MDR1 polymorphisms involving larger samples (at least two to three times larger) are necessary to determine whether MDR1 variants in fact influence P-gp expression and consequently atorvastatin disposition. In addition, the positive association between our T/T haplotype carriers and higher basal total and LDL cholesterol would be greatly strengthened if the sample were larger.

We conclude that MDR1 polymorphism may have an important contribution to basal total and LDL cholesterol serum levels in Brazilian individuals of European descent with primary hypercholesterolemia.

References


