Thrombosis

Contribution of Gene Sequence Variations of the Hepatic Cytochrome P450 3A4 Enzyme to Variability in Individual Responsiveness to Clopidogrel


Objectives—Metabolic activity of cytochrome P450 (CYP) 3A4 has been associated with clopidogrel response variability. Because metabolic activity of CYP3A4 is genetically regulated, we hypothesized that genetic variations of this enzyme may contribute to clopidogrel response variability.

Methods and Results—The CYP3A4*1B, CYP3A4*3, IVS7+258A>G, IVS7+894C>T, and IVS10+12G>A polymorphisms of the CYP3A4 gene were assessed in 82 patients in a steady phase of clopidogrel therapy. Glycoprotein (platelet glycoprotein (GP) IIb/IIIa receptor activation and platelet aggregation were assessed. A cohort of 45 clopidogrel-naïve patients was studied to determine the modulating effects of these polymorphisms after loading dose (300 mg) administration. Only the IVS7+258A>G, IVS7+894C>T, and IVS10+12G>A polymorphisms were sufficiently polymorphic. During the steady phase of clopidogrel treatment, IVS10+12A allele carriers had reduced GP IIb/IIIa activation (P=0.025) and better responsiveness (P=0.02); similarly, clopidogrel-naïve patients carriers of the IVS10+12A allele had reduced GP IIb/IIIa activation during the first 24 hours after a loading dose (P=0.025), increased platelet inhibition (P=0.006), and a more optimal drug response (P=0.003). This polymorphism did not influence platelet aggregation profiles. No association was observed between the other polymorphisms and clopidogrel responsiveness.

Conclusions—The IVS10+12G>A polymorphism of the CYP3A4 gene modulates platelet activation in patients treated with clopidogrel and may therefore contribute to clopidogrel response variability. (Arterioscler Thromb Vasc Biol. 2006;26:1895-1900.)

Key Words: clopidogrel • platelet • polymorphism

Treatment with clopidogrel is associated with a broad variability in antiplatelet effects.1–4 This may be partly attributed to the levels of clopidogrel’s active metabolite.5 Clopidogrel in fact is an inactive pro-drug that requires oxidation by the hepatic cytochrome P450 3A4 (CYP3A4) to generate an active metabolite.6–7 The active metabolite of clopidogrel inhibits platelet activation through an irreversible blockage of the platelet adenosine diphosphate (ADP) P2Y12 receptor. The P2Y12 receptor inhibits adenylyl cyclase and in turn decreases platelet cAMP (cAMP) levels and cAMP-mediated phosphorylation of the vasodilator-stimulated phosphoprotein, critical for inhibition of glycoprotein (GP) IIb/IIIa receptor activation.8

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Drugs that are substrates or inhibit CYP3A4 can potentially interfere with the conversion of clopidogrel into its active metabolite.9–11 Accordingly, the metabolic activity of the CYP3A4 enzyme, which varies considerably among individuals,12 has shown to influence platelet reactivity after a clopidogrel loading dose.13 Because genetic predisposition is the major determinant of heterogeneity in metabolic activity of the CYP3A4 enzyme,12,14–15 we hypothesized that single-nucleotide polymorphisms (SNPs) of CYP3A4 may account for interindividual variability of platelet reactivity in patients treated with clopidogrel. In the present study we examined the influence of SNPs of the CYP3A4 enzyme on platelet reactivity and responsiveness to clopidogrel in patients with coronary artery disease.

Materials and Methods

Detailed Materials and Methods are provided in the expanded methods, available online at http://atvb.ahajournals.org.

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Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

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Population

Eighty-two patients with stable coronary artery disease using combined aspirin and clopidogrel therapy were studied. Because ethnicity may contribute to variability in genotype distribution of specific SNPs, to avoid a patient selection bias only white patients homogeneous for ethnic background were included.12,14–15 All patients were from the central regions of Spain. In addition, because clopidogrel may take several days or weeks to achieve full antiplatelet effects, which play a role in individual response variability, only patients who were in a steady phase (≥1 month) of clopidogrel (75 mg/daily) treatment were included. All patients were clinically stable from the time that they initiated dual antiplatelet therapy and were on the same aspirin dose (100 mg/daily). Platelet reactivity was assessed 2 to 4 hours after drug intake. Because platelet reactivity may vary over time, platelet function measurements were repeated at a separate time point in patients who were still using dual antiplatelet therapy (70%) to sustain our study hypothesis. Compliance to the same aspirin dose (100 mg/daily). Platelet reactivity was assessed after clopidogrel (75 mg/daily) treatment was ensured by pill count and/or interview.

In addition to standard protocols.16 –17 Overall, 80% of patients were in a steady phase of clopidogrel (75 mg/daily) treatment (70%) to sustain our study hypothesis. Compliance to the same aspirin dose (100 mg/daily). Platelet reactivity was assessed after clopidogrel (75 mg/daily) treatment was ensured by pill count and/or interview. Patients on other antithrombotic drugs (oral anticoagulants, dipyridamole, cilostazol) or using clopidogrel for <1 month were not eligible for the study.

A separate cohort of 45 clopidogrel naïve patients scheduled to undergo elective coronary stenting receiving a 300-mg clopidogrel loading dose was also studied. All patients were on aspirin for at least 7 days and received clopidogrel at the time of coronary intervention. Platelet reactivity was assessed before clopidogrel administration (patients only on aspirin) and 4 and 24 hours after front loading. During intervention, unfractionated heparin (100 IU/kg) was administered according to standard practice. Only bare metal stents were used. Exclusion criteria were use of drugs or platelet GP IIb/IIIa blockers before, during, or after intervention; before use of thienopyridines; and/or aspirin intolerance/allergy.

This study was approved by the Ethical Committee of the San Carlos University Hospital and all patients gave informed consent.

Genotyping

Genomic DNA was extracted from whole blood samples according to standard protocols.16–17 Overall >30 SNPs of CYP3A4 have been described.12,14–15 These SNPs have been identified in different regions of the CYP3A4 gene. However, SNPs of the CYP3A4 gene are not homogeneously present and vary among different ethnicities. Therefore, the selection of SNPs for this study was based on the allele heterozygosity information for the CYP3A4 gene in the CEPH population derived from the HapMap Project Public Release #20.18 All the HapMap genotyped SNPs with a minor allele frequency >1% in the CEPH population were selected for the study: CYP3A4*1B, CYP3A4*3, IVS7+258A>G, IVS7+894C>T, and IVS10+12G>A. Genotyping of the CYP3A4*1B (c.–392A>G) SNP, located in the promoter region of the CYP3A4 gene, was performed using polymerase chain reaction (PCR) and amplification of the restriction endonuclease Rsal; 2 fragments of 240 and 100 bp for the G allele were obtained, and a single fragment of 340 bp was obtained for the A allele.

Although these SNPs have been previously reported, their prevalence in our population composed of patients with coronary artery disease is unknown. Therefore, a priori, these SNPs were considered for functional implication analysis only if sufficiently polymorphic and in Hardy-Weinberg equilibrium. Patients were predefined to be divided into 2 groups according to a dominant model, in which subjects homozygous for the wild-type allele are compared with subjects carrying ≥1 variant allele.17

GP IIb/IIIa Activation

Platelet reactivity was determined by assessing platelet surface expression of activated GP IIb/IIIa after 2 μmol/L ADP (ChronoLog Corp, Havertown, Pa) stimuli through flow cytometry, as previously described.1,2

Assessment of Platelet Aggregation

ADP-induced (6 μmol/L and 20 μmol/L) platelet aggregation was assessed in duplicate using platelet rich plasma (PRP) by the turbidimetric method in a 2-channel aggregometer (Chrono-Log 490 Model; Chrono-Log Corp), as previously described.1,2

Variability in Clopidogrel Responsiveness

In patients using sustained clopidogrel treatment, variability in clopidogrel induced antiplatelet effects was assessed by determining the distribution of platelet reactivity using a coefficient of variability (CV). A significant variability was defined when CV (CV = SD/mean) was >0.25 in continuous variables with a normal distribution.2 To assess the influence of SNPs of the CYP3A4 gene on variability of individual response to clopidogrel, this patient population was stratified into 3 groups on the basis of the tertiles of platelet reactivity (low, moderate, and high platelet reactivity). Patients in the lower tertile were considered as more optimal responders as opposed to those in the higher tertile, who were considered poor responders.

In patients treated with a 300-mg clopidogrel loading dose, clopidogrel responsiveness was defined according to the degree of inhibition of platelet reactivity 24 hours after clopidogrel administration compared with baseline values. Patients were classified as nonresponders, low responders, and responders when platelet inhibition was <10%, 10% to 29%, and ≥30%, respectively.2

Statistical Analysis

Continuous variables are expressed as mean±SD. Categorical variables are expressed as frequencies and percentages. The Kolmogorov-Smirnov test was performed to assess normal distribution. Comparisons between categorical variables were performed using 2-tailed Fisher exact test or the Pearson χ² test as appropriate. The Student t test was used for normally distributed continuous variables. Hardy-Weinberg equilibrium was estimated by χ² test for each SNP. Lewontin D’ was used as a measure of pairwise linkage disequilibrium.19 Haplotype analysis and linkage disequilibrium calculations were performed using R statistical package with haplostats and genetic libraries.20 A multivariate analysis of variance (MANOVA) was used to test intra-subject variance of platelet function parameters in patients receiving a 300-mg loading dose and their change over the study time period.2 P<0.05 was considered statistically significant. Statistical analysis was performed using SPSS®v11.0 software (SPSS Inc, Chicago, Ill).

Results

Genotyping

The variant allele of the CYP3A4*1B polymorphism was present only in 3% of the overall patient population. The variant allele of the CYP3A4*3 was not observed in any patient. Therefore, the extremely low prevalence of these
alleles did not allow them to be considered as genetic modulators of clopidogrel induced antiplatelet effects. The IVS7+258A>G, IVS7+894C>T, and IVS10+12G>A polymorphisms were in Hardy-Weinberg equilibrium and sufficiently polymorphic to be considered as potential modulators of platelet reactivity. Genetic distribution of the IVS7+258A>G, IVS7+894C>T, and IVS10+12G>A polymorphisms is described in the Table. Linkage disequilibrium was observed for some of the polymorphisms, particularly among the IVS7+894C>T, the IVS10+12G>A, and the CYP3A4*1B SNP (supplemental Table I, available online at http://atvb.ahajournals.org). Two common haplotypes constituted by 4 SNPs represent 58.3% and 28.1% of the studied population (supplemental Table II).

Platelet Reactivity in Patients Using Sustained Clopidogrel Therapy

Platelet reactivity in patients using sustained clopidogrel therapy was highly variable (CV = 0.65) and followed a normal distribution. The duration of clopidogrel treatment in these patients was 8.6 ± 4.9 months. Increased GP IIb/IIIa receptor activation was observed in noncarriers compared with carriers of the IVS10+12A allele (P = 0.025; Figure 1a). Repeated platelet function measurements were performed in 70% (n = 54) of this patient population observing similar variability profile (CV = 0.66), as well as differences in platelet activation measures in noncarriers compared with carriers of the IVS10+12A allele (30.6 ± 19.5% versus 17.4 ± 10.9%; P = 0.035). The distribution of IVS10+12A allele carrier patients related to their clinical characteristics was not significantly different (supplemental Table III). There were no differences between groups, including the use of CYP3A4 and non-CYP3A4 pathway metabolized statins.

After tertile distribution analysis of platelet reactivity profiles, carriers of the variant allele were more likely to have low platelet activation (P = 0.02; Figure 1b). Similar platelet activation distribution (lower, 0.7 to 16.7%; middle, 16.7 to 35.1%; upper, 35.1 to 74.7%) and allocation of carriers and noncarriers of the variant allele were observed in the subgroup of patients with repeated measurements (P = 0.03). A broad variability in platelet aggregation profiles was also observed after 6 μmol/L (CV = 0.44) and 20 μmol/L (CV = 0.32) ADP stimuli. However, noncarriers and carriers of the variant allele of the IVS10+12G>A polymorphism had similar degrees of platelet aggregation (31.7 ± 14.1% versus 32.9 ± 15.7%, P = 0.7 for 6 μmol/L ADP; and 45.4 ± 14.8% versus 46.5 ± 15.9%, P = 0.8 for 20 μmol/L ADP). Hence, their distribution according to platelet aggregation tertiles was also similar (P = 0.7 for 6 μmol/L ADP and P = 0.9 for 20 μmol/L ADP). Similar findings were observed in the subgroup of patients with repeated measurements (data not shown). No association was observed between platelet reactivity and the other genotyped SNPs in patients using sustained clopidogrel therapy. The analysis performed on the identified haplotypes showed no significant association.

Platelet Reactivity After a 300-mg Clopidogrel Loading Dose

GP IIb/IIIa receptor activation was similar in noncarriers and carriers of the variant allele of the IVS10+12G>A poly-

![Image](http://atvb.ahajournals.org/)

Figure 1. A, ADP-induced (2 μmol/L) glycoprotein IIb/IIIa receptor activation in patients carriers (gray) and noncarriers (white) of the variant allele of the IVS10+12G>A polymorphism on sustained clopidogrel therapy. Values are expressed as percentage of positive platelets (mean ± SD) and compared using Student t test. B, ADP-induced (2 μmol/L) glycoprotein IIb/IIIa receptor activation in patients carriers (gray) and noncarriers (white) of the variant allele of the IVS10+12G>A polymorphism on sustained clopidogrel therapy according to tertile distribution. Probability value was calculated using χ² test.
morphism at baseline, before clopidogrel front-loading. No significant difference was observed in the IVS10+12G>A genotype distribution regarding to their clinical characteristics (supplemental Table III). After loading dose administration, a lower reduction of GP IIb/IIIa receptor activation was observed in noncarriers of the variant allele of this polymorphism at 4 and 24 hours, with platelet activation significantly higher in these patients during the overall study time course \((P=0.025)\) (Figure 2). At 24 hours the degree of platelet inhibition was significantly higher in carriers of the variant allele \((P=0.006)\;\text{Figure 3a})). Noncarriers of the variant allele had a poorer response to clopidogrel as compared with carriers (non responders, 36% versus 11%; low responders, 36% versus 0%; responders, 28% versus 89%; \(P=0.003)\;\text{Figure 3b})). ADP-induced platelet aggregation significantly reduced after clopidogrel administration; however, it was similar in both groups (data not shown). No association was observed between platelet reactivity and the other genotyped SNPs in patients on 300 mg clopidogrel loading dose. The analysis performed on the identified haplotypes showed no significant association.

**Discussion**

The present study demonstrates the role of gene sequence variations of CYP3A4 in modulating platelet activation in patients treated with clopidogrel. In particular, carriers of the IVS10+12A allele had reduced GP IIb/IIIa activation and a better response to clopidogrel compared with noncarriers of the variant allele. The hypothesis of the modulating effect of this polymorphism on platelet activation in patients treated with clopidogrel is supported not only by the differences in platelet activation observed in patients already in a steady phase of clopidogrel treatment, but also in the acute phase of treatment. Although platelet reactivity may vary over time, analogous findings persisted in patients undergoing repeated measurements in the sustained phase of clopidogrel treatment, further sustaining our study hypothesis. The modulating effects of the IVS10+12G>A polymorphism as assessed by the degree of inhibition of platelet activation is noteworthy in clopidogrel naïve patients carriers of the A allele who, except for 1 patient, were all clopidogrel responders. However, this polymorphism was not associated with a modulating effect on platelet aggregation. These discrepancies with platelet activation may be attributed to the mechanism of action of clopidogrel. In fact, because clopidogrel is per se an inhibitor of platelet activation, this may explain why GP IIb/IIIa receptor activation and not platelet aggregation resulted modulated by the IVS10+12G>A polymorphism of the CYP3A4 gene. In patients treated with clopidogrel, inhibition of platelet aggregation represents a downstream effect after inhibition of platelet activation. In addition, the resulting degree of platelet aggregation is influenced by numerous stimuli, which may affect to a lesser extent platelet activation.2 Because clopidogrel mediates its effects through vasodilator-stimulated phosphoprotein phosphorylation, which has a pivotal role for GP IIb/IIIa receptor inhibition, assessment of GP IIb/IIIa receptor activation may be a more sensitive platelet biomarker to assess the modulating effects of CYP3A4 gene polymorphisms.

Clopidogrel response variability has been primarily assessed during the acute phases of treatment during which clopidogrel’s pharmacokinetic and pharmacodynamic profiles vary considerably.5,21 One may argue that such variability depends on the fact that drug-induced metabolic activity of CYP3A4 requires several days or weeks of treatment before this reaches a plateau and thus a more uniform response. This is supported by the fact that clopidogrel responsiveness ameliorates with length of treatment.5 However, a broad variability in clopidogrel induced effects continued to be observed even in patients in a steady phase of treatment.

Several factors may contribute to clopidogrel response variability.22 SNPs of targets within the clopidogrel pathway, namely CYP3A4 enzyme, P2Y12 receptor, and GP IIb/IIIa
receptor, have been suggested to contribute to individual response variability to clopidogrel. A minor haplotype of the P2Y12 receptor was found to be associated with increased platelet reactivity in nonmedicated healthy volunteers. However, these findings could not be duplicated by several authors studying patients with coronary artery disease treated with clopidogrel. In addition to P2Y12 SNP analysis, Lev et al failed to observe any impact of SNPs of the P2Y12, and GPIIIa receptors on responsiveness to acute front-loading of clopidogrel. We recently demonstrated the lack of association between SNPs of the GP IIIa and P2Y12 receptors and platelet reactivity in patients on chronic clopidogrel therapy. These findings are likely related to the fact that an active metabolite and not clopidogrel per se, is responsible for inhibition of the P2Y12 receptor, therefore suggesting that an upstream target within clopidogrel’s metabolic pathway to have a more important modulating role of its downstream antiplatelet effects. One may argue that CYP3A5 has overlapping substrate specificity with CYP3A4 and may contribute to clopidogrel’s metabolism and be a determinant of drug response as well. However, gene sequence variations of the CYP3A5 enzyme in patients with coronary artery disease have not been associated with variability in clopidogrel responsiveness. This latter observation may be explained by the fact that CYP3A4 is the dominant CYP3A enzyme among whites and therefore may be more important in clopidogrel responsiveness. This latter observation may be explained by the fact that CYP3A4 is the dominant CYP3A enzyme among whites and therefore may be more important in clopidogrel metabolism. Drug–drug interactions and clinical status are also responsible for variability in platelet reactivity and clopidogrel responsiveness. However, our patient population was very homogenous not only for ethnicity but also for clinical status and treatment.

In the present study, the overall prevalence of clopidogrel nonresponders compares favorably with that previously described. Notably, noncarriers of the IVS10+12A allele were predominant. This may explain the high prevalence of clopidogrel nonresponders using standard clopidogrel dosing. Recent reports have shown that higher doses of clopidogrel improve responsiveness. However, our study did not assess if SNPs of the CYP3A4 gene continue to contribute to variability in platelet reactivity when higher clopidogrel doses are used. Nevertheless, it is noteworthy to emphasize that although increasing clopidogrel doses improves responsiveness, a broad variability in antiplatelet effects persist independent of the dose used. Platelet reactivity in fact may be within higher or lower ranges according to the dose used but still remain highly variable. This is related to the fact that clopidogrel’s antiplatelet effects primarily depend on the metabolic activity of CYP3A4 which is per se heterogeneous. On the contrary, direct P2Y12 antagonists such as AZD6140 or cangrelor, which do not require hepatic metabolism to be active, do not present such heterogeneous effects.

Although this study was not aimed to assess clinical events, it is important to underscore that increased post-treatment platelet reactivity and suboptimal response to antiplatelet drugs, including clopidogrel, which were both observed in this study, are associated with increased atherothrombotic risk. Larger population studies are warranted to assess if the functional findings from this study have clinical sequelae and the assessment of candidate genetic traits appears as a promising arena of research to identify such high-risk individuals. Ultimately, the identification of relevant genetic markers may guide a more rationale and individualized use of drugs and dosages, thus optimizing therapeutic regimens which are essential for the prevention of atherothrombotic events.

**Study Limitations**

Measurement of CYP3A4’s metabolic activity was not performed in this study. However, a correlation between CYP3A4’s metabolic activity and clopidogrel response has been described and the functional findings of our study support the hypothesis that individuals genetic predisposition, a major determinant of CYP3A4’s metabolic activity, contributes to this phenomenon. Although all the described SNPs with minor allele frequency >1% have been genotyped in this study, we cannot exclude the contributing role of other SNPs in modulating clopidogrel response. IVS10+12G>A polymorphism of the CYP3A4 gene, which is in linkage disequilibrium with some other SNPs, may be a marker for an haplotype which regulates the metabolic activity of CYP3A4s by either modifying catalytic activity and/or enzyme expression, possibly through other, as yet undetected, SNPs.

**Acknowledgments**

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**Disclosures**

Dominick J. Angiolillo is on the speaker bureau and is a consultant for Sanofi-Aventis and Bristol Myers Squibb. The remaining authors report no conflicts.

**References**


Contribution of Gene Sequence Variations of the Hepatic Cytochrome P450 3A4 Enzyme to Variability in Individual Responsiveness to Clopidogrel


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Materials and Methods

*GP IIb/IIIa activation*

Platelet reactivity was determined by assessing platelet surface expression of activated GP IIb/IIIa following 2 μM ADP (ChronoLog Corp, Havertown, PA) stimuli through flow cytometry, as previously described (1,2). GP IIb/IIIa activation was assessed using a polyclonal fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human fibrinogen antibody (800nM, DAKO Diagnostics, Glostrup, Denmark), which detects fibrinogen binding to the activated GPIIb/IIIa receptor. An EPICS-XL PROFILE II Coulter flow cytometer (Coulter Corp. Miami, FL) was used for the assessment. After discard of the initial first millilitres of blood to avoid spontaneous platelet activation, whole blood was drawn into sterile tubes containing 3.8% trisodium citrate and then diluted with HEPES-tyrodes buffer (5mM HEPES hydroxyethylpiperazineethanesulfonic acid-, 137mM NaCl, 2,7mM NaHCO₃, 0,36mM NaH₂PO₄, 2mM CaCl₂, 5mM glucose, 0,2% BSA) to a final volume of 1:8:1 (blood:HEPES-Tyrodes:citrate) resulting in a 1/10 dilution of whole blood during sampling. Previously HEPES-Tyrodes buffer were filtered through 0.22μm sterile filters to avoid interference from particles. Then, 50 μL of diluted whole blood was stimulated in vitro with 2μM ADP before
immunolabelling. The corresponding antibody was then added and incubated for 20 min in dark at room temperature. After incubation, 300μl of 0.5% PBS-buffered paraformaldehyde was added for fixation. Samples were analyzed within 2 hours by flow cytometry and platelets were identified based on particle size (forward scatter) and complexity (side scatter). Light scatter and fluorescence data from 10000 platelet events were collected with all detectors in logarithmic mode. Acquisition and processing data were analyzed with XL2 software (Coulter Corp. Miami, FL). Platelet activation was expressed as the percentage of platelets positive for antibody binding.

Assessment of platelet aggregation

Blood was collected in tubes containing 3.8% trisodium citrate. Platelet aggregation was assessed in duplicate using platelet rich plasma (PRP) by the turbidimetric method in a 2-channel aggregometer (Chrono-Log 490 Model, Chrono-Log Corp., Havertown, PA), as previously described (1, 2, 17). Platelet agonists included 6 μM ADP and 20 μM ADP (Chrono-Log Corp., Havertown, PA) (1, 2, 17). PRP was obtained as a supernatant after centrifugation of citrated blood at 800 rpm for 10 minutes. Platelet poor plasma (PPP) was obtained by a second centrifugation of the blood fraction at 2500 rpm for 10 minutes. The platelet count in PRP was adjusted to the range of 250.000/μL by dilution with autologous plasma when platelet count was out of range. Light transmission was adjusted to 0% with PRP and to 100% for PPP for each measurement. Platelet aggregation was assessed within 2 hours from blood sampling. PRP was kept at 22º C prior to use and at 37ºC one minute before running the aggregatory test. Aggregation was assessed in siliconized tubes at 37ºC in constant
stirring conditions and curves were recorded for 5 minutes. Platelet aggregation was
determined as the maximal percent change in light transmittance from baseline using PPP
as reference.
**Online Table I.** Linkage disequilibrium observed between the studied SNPs. D’ with p value in brackets are reported for each SNP pair comparison.

<table>
<thead>
<tr>
<th></th>
<th>IVS7+258A&gt;G</th>
<th>IVS7+894C&gt;T</th>
<th>IVS10+12G&gt;A</th>
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<tbody>
<tr>
<td>CYP 3A4*1B</td>
<td>0.141 (0.5585)</td>
<td>0.685 (1.19e-08)</td>
<td>0.757 (1.28e-12)</td>
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<tr>
<td>IVS7+258</td>
<td>-</td>
<td>0.200 (0.1052)</td>
<td>0.264 (0.0375)</td>
</tr>
<tr>
<td>IVS7+894</td>
<td>-</td>
<td>-</td>
<td>0.845 (&lt;2e-16)</td>
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</table>

Lewontin's D' was used as a measure of pairwise linkage disequilibrium (reference 19).
Online Table II. Frequency of the haplotypes of the CYP3A4 gene estimated in the study population.

<table>
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<tr>
<th>CYP 3A4*1B (c.-392A&gt;G)</th>
<th>CYP3A4*3 (p.M445T)</th>
<th>IVS7+258A&gt;G (c.670+258A&gt;G)</th>
<th>IVS7+894C&gt;T (c.670+894C&gt;T)</th>
<th>IVS10+12G&gt;A (c.1026+12G&gt;A)</th>
<th>Haplotype frequency</th>
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<td>A M A C G</td>
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<tr>
<td>A M G C G</td>
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<td></td>
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<tr>
<td>A M A T A</td>
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<tr>
<td>A M G T A</td>
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<tr>
<td>G M A T A</td>
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<td>A M A T G</td>
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<td>A M G C A</td>
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<tr>
<td>G M G T A</td>
<td>0.00076</td>
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</table>

Haplotype analyses were performed using R statistical package with haplo.stats and genetic libraries (http://www.R-project.org), (reference 20)
**Online Table III.** Demographics of the study population according to a dominant model distribution of the IVS10+12G>A polymorphism of the CYP3A4 gene

<table>
<thead>
<tr>
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<th>Long-term phase (n=82)</th>
<th>Acute-phase (n=45)</th>
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<tbody>
<tr>
<td></td>
<td>Non-carriers n=68</td>
<td>Carriers n=14</td>
</tr>
<tr>
<td></td>
<td>(83%)</td>
<td>(17%)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>63 ±11</td>
<td>61±11</td>
</tr>
<tr>
<td>Males</td>
<td>51 (75%)</td>
<td>11 (79 %)</td>
</tr>
<tr>
<td>Risk factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>31 (47%)</td>
<td>3 (21%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>36 (53%)</td>
<td>5 (36%)</td>
</tr>
<tr>
<td>Hyperlipemia</td>
<td>36 (53%)</td>
<td>8 (57%)</td>
</tr>
<tr>
<td>Current smokers</td>
<td>6 (9%)</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-Blockers</td>
<td>43 (63%)</td>
<td>9 (64%)</td>
</tr>
<tr>
<td>Nitrates</td>
<td>36 (53%)</td>
<td>9 (54%)</td>
</tr>
<tr>
<td>ACE-Inhibitors</td>
<td>29 (43%)</td>
<td>3 (21%)</td>
</tr>
<tr>
<td>Statins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3A4 pathway metabolized</td>
<td>37 (54%)</td>
<td>7 (50%)</td>
</tr>
<tr>
<td>non-3A4 pathway metabolized</td>
<td>4 (6%)</td>
<td>1 (7%)</td>
</tr>
</tbody>
</table>

Data are presented as the mean value ± SD or number (%).