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Genotype-Phenotype Relationships for the Renin-Angiotensin-Aldosterone System in a Normal Population

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Abstract—The renin-angiotensin-aldosterone system plays an important role in blood pressure regulation by influencing salt-water homeostasis and vascular tone. The purpose of the present study was to search for associations of single nucleotide polymorphisms on 3 major candidate genes of this system with the plasma concentrations of the corresponding renin-angiotensin-aldosterone system components considered as quantitative phenotypes. Genotyping was performed in 114 normotensive subjects for different variants of the angiotensinogen (AGT) gene (C-532T, G-6A, M235T), the angiotensin I–converting enzyme (ACE) gene [4656(CT)2/3], the aldosterone synthase (CYP11B2), and the type I angiotensin II receptor (AT1R) gene (A1166C) by hybridization with allele-specific oligonucleotides (ASO) or enzymatic digestion of polymerase chain reaction products. Plasma levels of AGT, ACE, angiotensin II (Ang II), aldosterone, and immunoreactive active renin were measured according to standard techniques. Platelet binding sites for Ang II were analyzed by the binding of radioiodinated Ang II to purified platelets. Bmax and KD values of the Ang II binding sites on platelets of each individual were calculated to examine a possible relationship between these parameters and the AT1R genotype. A highly significant association of the ACE 4656(CT)2/3 variant with plasma ACE levels was observed (P<0.0001). ANOVA showed a significant effect of the AGT C-532T polymorphism on AGT plasma levels (P=0.017), but no significant effect was detectable with the other AGT polymorphisms tested, such as the G-6A or the M235T. A significant effect association was also found between the C-344T polymorphism of the CYP11B2 gene and plasma aldosterone levels, with the T allele associated with higher levels (P=0.02). No genotype effect of the AT1R A1166C polymorphism was detected either on the Bmax or the KD value of the Ang II receptors on platelets.

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Key Words: angiotensinogen • angiotensin-converting enzyme • angiotensin II • aldosterone
• polymorphism • blood pressure

Single nucleotide biallelic polymorphisms are powerful tools when used to search for a linkage disequilibrium between a marker genotype and a disease, mainly of multifactorial origin. With these markers, candidate genes of the renin-angiotensin-aldosterone system (RAAS), including those of angiotensinogen (AGT), the angiotensin I–converting enzyme (ACE), and type I angiotensin (Ang) II receptor (AT1R), have been investigated in association studies with cardiovascular diseases, such as hypertension or myocardial infarction. As examples of these associations, the ACE insertion/deletion (I/D) polymorphism was found associated with myocardial infarction, cardiac hypertrophy after physical exercise, and Alzheimer disease,1,2 and the AGT or AT1R genes were found to be associated with hypertension.3,4

Therefore, it is of interest to establish relationships between these marker genotypes and intermediate phenotypes related to the disease and to the gene tested to understand the physiopathology of these associations, thus helping to clarify inconsistent results issued from some association studies. Among these observations, the strong effect of the ACE I/D genotype on plasma ACE levels has been reproduced in several studies. Significant associations have also been found between AGT markers and plasma AGT in 2 independent studies.3,5

In this study, we analyzed the relationships between several marker genotypes of the ACE, AGT, aldosterone synthase (CYP11B2), and AT1R genes and biochemical parameters related to these genes in a group of normal individuals. We confirmed previously reported effects of ACE and AGT genes on their gene products in plasma and identified a polymorphism of the 5′ region of AGT as a candidate functional variant, because it showed the most pronounced
association with AGT plasma levels. Our results indicate that the AT1R sites on platelets are of limited density and that there is no effect of the genotype on receptor number or affinity. A significant increase of plasma aldosterone (pAldo) levels was associated with the T allele of the C-344T polymorphism of the CYP11B2 gene.

**Methods**

**Subjects**

The study was approved by the review committee of Hôpital Saint-Antoine, and all subjects gave informed consent. One hundred fourteen white adults (87 men, 27 women) were selected from the Center for Preventive Medicine and the staff of the laboratory. Subjects with acute or chronic disease, a body mass index >28 kg/m², antihypertensive drug therapy, or an alcohol intake >50 g/24 h were excluded. After 10 minutes of rest in the sitting position, subjects had their blood pressure measured with a sphygmomanometer.

**Biochemical Measurements**

Blood was collected into tubes that contained heparin for determination of plasma concentrations of proteins, creatinine, sodium, and potassium. These determinations were performed immediately by the usual techniques as used for blood samples.

**Measurement of Plasma RAAS Parameters**

**Analytical Determinations**

Blood was collected into cold tubes that contained potassium EDTA for determinations of the various parameters of the RAAS and were also supplemented with 2% ethanol, 2 mg/mL neomycin, and 25 μmol/L 1,10-phenanthroline for Ang II determination. Plasma was stored at −20°C. pAldo was measured by radioimmunoassay (RIA) according to a previously reported technique. Ang II was measured according to the method of Nussberger et al. with an Ang II monoclonal antibody, which was obtained in the laboratory, that did not cross-react with Ang I. Active renin was measured with a commercial kit (Sigma Diagnostics) on the basis of the hydrolysis of creatinine, sodium, and potassium. These determinations were performed immediately by the usual techniques adapted to a RAXT Technicon autoanalyzer. Untimed urine specimens were obtained by voiding for measurement of creatinine, sodium, and potassium concentrations with the same techniques as used for blood samples.

**Preparation of Platelets**

Forty to 50 mL of blood was drawn from a forearm vein. The first milliliter of blood was discarded, the rest was transferred into a glass tube in ice, and sodium citrate was added as an anticoagulant. Platelets were separated according to Mann et al. with minor modifications. Platelet-rich plasma was obtained by centrifugation at 100g for 15 minutes at room temperature. It was then washed with 20 volumes of cold Tris-buffered saline (Tris-HCl 50 mmol/L, NaCl 154 mmol/L, Na₂EDTA 5 mmol/L, pH 7.35) that contained 0.05% BSA and centrifuged at 1000g for 10 minutes at room temperature. Washings and centrifugations were repeated once more, and the platelets were then resuspended in medium 199 buffer (Gibco Life Technologies) that contained 5 mmol/L EDTA, 0.2% BSA, and 1 mg/mL bacitracin (pH 7.4 at 22°C) called an assay buffer. Platelet number in the final suspension was determined automatically in a Coulter counter.

**Binding Studies**

Incubations were performed in a total volume of 100 μL that included 80 μL of platelet suspension. Saturation equilibrium binding studies were performed in the presence of increasing concentrations of 125I-labeled Ang II (0.037 to 0.450 nmol/L). Nonspecific binding was ascertained in the presence of 1 μmol/L of unlabeled Ang II. Incubation was performed for 120 minutes at 37°C in a shaking water bath. At the end of the incubation period, 2 mL of ice-cold assay buffer was added to each tube. The tubes were centrifuged at 2000g for 5 minutes at 4°C. The supernatants were discarded, and the pellets were resuspended again in 2 mL of ice-cold assay buffer. This procedure was repeated twice. Radioactivity of the final pellet was measured in a LKB-Wallac gamma counter with 60% efficiency. The results of the saturation binding experiments were analyzed by Scatchard transformation of the data with the “Ligand” program to calculate the apparent Kd and the number of sites (Bmax).

**DNA Extraction**

Genomic DNAs from subjects were extracted by standard techniques.

**Genotyping for ACE, AGT, AT1R, and CYP11B2 Polymorphisms**

For the A1166C polymorphism of AT1R, a polymerase chain reaction (PCR) products were digested overnight by the addition of 5 U of DdeI restriction enzyme. In the presence of the 1166C allele, the PCR product (404 bp) was cut into 2 fragments of 118 bp and 286 bp in length and visualized on ethidium bromide-stained 1.5% agarose gels.

Genotyping of the different variants of AGT (C-532T, G-6A, M235T) and ACE [4656(CT)₆] was performed by hybridization with an allele-specific oligonucleotide. DNA fragments that contained the polymorphisms were amplified by PCR with forward and reverse primers as indicated in Table 1. The PCR products were

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**TABLE 1. Primers Used for Amplification of the Regions Containing the Analyzed Polymorphisms**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>Primer</th>
<th>Product Size, bp</th>
<th>Annealing Temperature, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGT</td>
<td>5’ region</td>
<td>5’TTC CAG AAG GCA CTT TTC AC-3’</td>
<td>594</td>
<td>56</td>
</tr>
<tr>
<td>Exon 1</td>
<td>5’TAG TAC CCA GAA CAA CGG CA-3’</td>
<td>354</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>AGT</td>
<td>Exon 2</td>
<td>5’-GAT GGG CAC AAG GTC CTG TC-3’</td>
<td>177</td>
<td>67</td>
</tr>
<tr>
<td>Intron 2</td>
<td>5’-GCCAGC AGA GAG GGT TTG CT-3’</td>
<td>404</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>ACE</td>
<td>U6</td>
<td>5’-AGA AGC CTG CAT GTT TTG AG-3’</td>
<td>5’-GCT ACA CTC CAG CGT CTG AGG-3’</td>
<td>9</td>
</tr>
<tr>
<td>L6</td>
<td>5’-CCT GGT GCT CCT CTA ACG ATT TA-3’</td>
<td>9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**References**

1. Hydrolysis of creatinine, sodium, and potassium.
3. Analytical Determinations.
5. DNA Extraction.
7. **Downloaded from hyper.ahajournals.org by on May 21, 2008**
denatured in 150 μL of 0.5 mol/L NaOH and 1.5 mol/L NaCl and spotted onto nylon membranes (N+I, ICN). Membranes were neutralized in 2× SSC and cross-linked with UV light (DNA Transilluminator, Fotodyne). Each membrane was hybridized in 7% polyethylen glycol/10% sodium dodecyl sulfate at specific temperatures (Tm-5°C) for 4 hours, with 100 pmol of either of the 2 oligonucleotides (Table 2) that were end-labeled with [γ-32P]dATP with T4 polynucleotide kinase. The membranes were washed twice at room temperature in 1× SSC for 5 minutes and then incubated for 5 minutes in 0.5× SSC at specific temperatures (Tm-3°C) before autoradiography. Genotype determination of the C-344T polymorphism of CYP11B2 was performed by allele-specific oligonucleotide hybridization as described previously.17

Statistical Analysis
Results are expressed as mean±SD.

Analysis of Variance
The association of the different polymorphisms with their corresponding gene product plasma levels or blood pressure levels was tested by 1-way ANOVA. Blood pressure values were adjusted for age and gender. Effects of the combination of the G-6A and C-532T polymorphisms of AGT were tested by 2-way ANOVA. Values are considered significantly different at P<0.05. Biochemical values were log-transformed before analysis.

Multiple Regression Analysis
Multiple regression analyses were performed with the aim of evaluating the effects of the different polymorphisms on pACE, pAGT, pAldo, and on the KD and B max of platelet AT1R sites after adjustment for covariates [gender, systolic blood pressure (SBP), urinary Na+ excretion].

Results

Anthropometric Variables and Biochemical Measurements
The main anthropometric characteristics and biochemical measurements are shown in Table 3. The mean age of the subjects was 49.5±5.4 years. The number of subjects available for each measurement is given because in some cases, measurements failed for technical reasons. Significant positive correlations were found between plasma immunoreactive active renin (pIr-AR) and plasma Ang II (pAng II; r=0.69, P<0.0001), pIr-AR and pAldo (r=0.31, P<0.0008), and pAng II and pAldo (r=0.23, P<0.02).

Platelet Binding Sites for Ang II
Competitive displacement experiments with unlabeled Ang II, [125I]-Ang II, and nonpeptide AT1 (losartan) and AT2 (PD123177) receptor antagonists demonstrated that only AT1 receptors were present at the platelet surface (data not shown). Saturation binding studies allowed the binding parameters to be characterized. Scatchard transformation was performed with the data from each saturation binding curve to calculate the number of sites (B max) and the apparent KD. The corresponding values (mean±SEM) were 5.3±0.54 fmol/8×108 platelets and 0.47±0.04 nmol/L. This KD value is similar to that observed for AT1 receptors in another study on platelets.18 The receptor number corresponds to 4 to 5 sites per platelet. This limited density is also in accordance with previously published studies.12,19 A significant positive correlation was found between the KD and the B max values (r=0.35; P=0.009) and confirmed the general finding that the affinity of a ligand for its receptor is negatively correlated with the receptor density. No correlation was found to be significant with the other biochemical parameters.

Association Between Gene Polymorphisms and Their Respective Intermediate Phenotypes
For all polymorphisms determined, there was no significant deviation from Hardy-Weinberg equilibrium in this group of subjects. Biochemical measurements of the RAAS (pIr-AR, pAGT, pACE, pAng II, pAldo) were analyzed according to the genotypes of the subjects by ANOVA and in multivariate analyses (Table 4).

ACE Genotype
A marked difference in the pACE level was found between genotype groups of the ACE gene, with the 4656(CT)2/3 allele-specific oligonucleotide hybridization.
repeat of the 3' region of the ACE gene. There was a nearly 5-fold lower level of mean plasma ACE in subjects homozygous for the 4656(CT)\textsubscript{3} versus subjects homozygous for the (CT)\textsubscript{2} genotype. No other relationships were found for this genotype, in particular with pAng II and pAldo.

**AGT Genotype**

Three different polymorphisms of the AGT gene were tested for their effect on pAGT levels. Higher levels of pAGT were found with the less frequent alleles of the M235T and of the G-6A polymorphisms (235T and -6A alleles, respectively), but none of these variations reached statistical significance. Only the C-532T polymorphism was associated with a significant difference in pAGT levels. Higher levels of pAGT were associated with the less frequent allele (C-532T allele) of the polymorphism. By multiple regression analysis that included SBP, age, and all the polymorphisms tested, C-532T was the single polymorphism that significantly explained the AGT variance ($P<0.02$; $n=87$).

An analysis of the effect of the combination of the G-6A and C-532T polymorphisms on pAGT was also performed. These 2 polymorphisms are in complete linkage disequilibrium, as previously observed\textsuperscript{20}; the T allele of the C-532T polymorphism is always associated with the A allele of the G-6A polymorphism. As shown on Table 5, there was no significant difference in pAGT between the 3 genotype classes for the G-6A polymorphism among the CC subjects for the C-532T polymorphism. Inversely, pAGT was significantly increased in subjects with the CT or TT genotype versus those with the CC genotype of the C-532T polymorphism, in the 2 genotype classes of the G-6A polymorphism (GA or AA) in which these genotypes can be observed, as a consequence of the complete linkage disequilibrium.

There was also a weakly significant effect of the C-532T polymorphism on the pACE level, and lower plasma values were observed with the T allele (Table 4). There was no effect of the genotype on pAng II or pAldo levels.

\textbf{A1166C Genotype of the ATIR Gene}

No relationship was found between this polymorphism and any of the biochemical measurements performed. In particular, the ATIR binding sites ($K_o$ and $B_{max}$) were not affected by this genotype.

\textbf{C-344T Polymorphism of the CYP11B2 Gene}

pAldo levels were significantly different in the 3 genotype groups ($P=0.02$). Heterozygous subjects exhibited the highest values, and TT homozygotes exhibited intermediate levels.

**Association Between Gene Polymorphisms and Blood Pressure**

No significant association between the polymorphisms of ACE, AGT, and CYP11B2 was found with either SBP or diastolic (DBP), age-, and gender-adjusted blood pressure. For the A1166C polymorphism of ATIR, a borderline association was found with DBP but not with SBP when subjects with the AC or CC genotype were compared with those with the AA genotype (79.8 versus 75.7 mm Hg; $P=0.06$).

**Discussion**

The aim of this study was to assess the effect of polymorphisms of the RAAS genes on biochemical components
directly related to these genes, such as pACE, pAldo, and pAGT, but also including other parameters that have not been studied previously in relation to these genotypes, such as platelet Ang II receptors and pAng II levels. This study was performed in a series of untreated subjects without primary selection for hypertension, in whom sodium excretion was assessed, and who did not receive any treatment that could alter the biochemical variables studied.

**Association of AGT Polymorphisms With Plasma AGT Levels**

Different studies showed that the 235T allele of the M235T polymorphism was associated with higher pAGT levels versus noncarriers.\(^3\)\(^,\)\(^5\) However, these results were found in a single series of hypertensive subjects and in children. Thus, our study is the first to be performed on untreated normotensive adults with 3 biallelic polymorphisms of the AGT gene, from the 5′ region (C-532T and G-6A) and from exon 2 (M235T) of the gene. Although trends were observed for higher levels of pAGT in subjects with the 235T allele and with the -6A allele, these associations were not significant.

Only the C-532T polymorphism was significantly associated with pAGT concentration, both by 1-way ANOVA and by multivariate analysis. The M235T polymorphism is not considered to be functional, but the G-6A polymorphism was considered to be functional, at least in an in vitro assay with the AGT promoter fused to a reporter gene.\(^2\)\(^1\) In another study of 130 normal nuclear families, the C-532T polymorphism was the one best supported by statistics (ANOVA and combined segregation-linkage analysis) as the functional variant among all tested polymorphisms of the AGT gene (E.B., N. Chatelain, F.P., L. Tiret, S. Visvikis, M. Lathrop, F.S., F. Demenais, unpublished data, 1999).

When the effect of the C-532T on plasma AGT is analyzed in combination with the G-6A polymorphism, there is no detectable effect of the G-6A polymorphism on plasma AGT across the subjects according to this genotype in CC subjects. An effect on plasma AGT is detectable only according to the C-532T genotype, although the complete linkage disequilibrium between the 2 polymorphisms does not allow this effect to be fully analyzed. No functional study has yet been performed on the C-532T polymorphism. Because the C-532T polymorphism is located within a consensus sequence to the transcription factor AP-2, it might modulate AGT gene transcription.

We searched for an influence of the AGT genotypes on pAng II and pAldo levels through their effect on the AGT level, because we detected positive and significant correlations between plr-AR and pAng II, plr-AR and pAldo, as well as pAng II and pAldo. In this study, we were unable to detect an effect of the AGT genotype either on pAng II or on the pAldo concentration. This can be due to variations of these 2 parameters, with a variety of environmental factors that are difficult to standardize in a clinical study in humans. Alternatively, the differences in the pAGT levels may be too weak to produce a detectable effect or are counterbalanced by physiological retrocontrols. We observed a weakly significant (\(P=0.046\)) association between the AGT C-532T polymorphism and pACE level, with an apparently codominant effect and lower ACE values in subjects with the T allele of the C-532T polymorphism, which is associated with the highest levels of pAGT. If not spurious, this association might be due to a negative feedback of Ang II on ACE, as suggested in a previous study,\(^2\)\(^3\) although no effect in pAng II could be detected in our study.

**AT1R Polymorphism**

Because a polymorphism located in the 3′ untranslated region of this gene (A1166C) was shown to be associated with hypertension or increased pulse wave velocity, we searched for an association of this polymorphism with different parameters of the RAAS.\(^2\)\(^3\) The biological phenotypes that most directly related to the AT1R gene, and the most accessible, are the parameters of the Ang II binding sites that belong to the AT1R subtype and present on circulating cells. We confirmed that platelet Ang II binding sites are of the type I subclass by ligand binding specificity. The \(K_0\) value of Ang II binding in platelets was similar to that found in renal and vascular preparations,\(^2\)\(^4\) whereas the \(B_{\text{max}}\) value corresponded to a much lower number of receptor sites, which was in agreement with previously published reports.\(^2\)\(^1\)\(^-\)\(^2\)\(^3\)\(^,\)\(^19\) Our data are consistent with those already reported by Duggan et al.\(^2\)\(^5\) We did not detect any significant association between the genotypes for the A1166C polymorphism and the parameters of AT1R binding sites. Because of their low expression, one can hypothesize that the AT1R in platelets are not or are weakly submitted to transcriptional regulatory mechanisms that act in the other target tissues of Ang II. Our results indicate that this measurement cannot be used as an intermediate phenotype for the AT1R gene. No other biological variable was significantly associated with the AT1R polymorphism.

The AT1R A1166C polymorphism is the single polymorphism for which we found a nonsignificant but borderline association with blood pressure values. The subjects bearing the C allele have a slightly higher DBP than those who do not bear this allele (\(P=0.06\)). This result is consistent with the higher prevalence of this allele that we previously observed in hypertensive subjects.\(^4\)

**Association of ACE Polymorphisms With Plasma ACE Levels**

The 4656(CT)\(_{2/3}\) polymorphism in the 3′-untranslated region of the ACE gene consists of a repetition of 2 or 3 CT dinucleotides (CT\(_{2/3}\)) and is in complete linkage disequilibrium with the ACE I/D variant.\(^15\) We observed a highly significant association of the ACE 4656(CT)\(_{2/3}\) variant with pACE levels (\(P<0.0001\)). No effect on other variables was detected; in particular, no effect on pAGT, pAng II, or pAldo.

**Association of CYP11B2 Polymorphism With pAldo Levels**

The association we found between CYP11B2 polymorphisms and pAldo levels corroborate those of 2 independent studies that showed a similar relationship. In a series of 92 normal men, aged 30 to 55 years, Hautanen et al\(^2\)\(^6\) found a higher rate of urinary Aldo secretion in TT carriers than in CC carriers.
(P=0.016) of the C-344T polymorphism. Similarly, in a series of 486 normal subjects, Davies et al.\(^2\) found a higher excretion of tetrahydroaldosterone in T allele–bearing subjects than in those not bearing the T allele (P=0.024). As in our study for pAldo, the urinary levels of tetrahydroaldosterone were higher in heterozygotes bearing the T allele. Contrasting results were reported by Pojoga et al.\(^2\) who found a linear relationship between pAldo and the C-344T allele in a series of hypertensive subjects, but in this case, higher levels were found in C-bearing subjects.

In view of physiopathological hypotheses that proposed an inappropriate secretion of aldosterone in hypertension, the present results must be examined in the context of other results that associate the CYP11B2 polymorphism with hypertension in other case-control studies. Indeed, the T allele of the -344 genotype that we found associated with higher pAldo levels was also found significantly more frequently (P=0.010) in hypertensive patients (0.561) than in normotensive controls (0.488) by Brand et al.\(^1\) A similar frequency of the T allele in white hypertensives (0.56) was also found in the study by Pojoga et al.\(^2\) Davies et al also found a significant increase of the T allele in essential hypertensives from Scotland (P=0.009).\(^2\) Thus, it could be proposed that the T allele is more frequent in hypertensives and is associated with a higher activity of the CYP11B2 gene, which leads to higher aldosterone secretion at a given level of sodium intake. This would necessitate higher blood pressure to allow for the sodium excretion rate required to maintain sodium homeostasis. This hypothesis needs to be supported by further studies specifically designed for this purpose.

The potential functional role of the C-344T polymorphism was analyzed in few studies. By gel shift assay, White and Slutsker\(^2\) found that the -344C allele of the CYP11B2 promoter binds the steroidogenic transcription factor 1 (SF-1) 4 times more than the -344T allele on a molar basis. In contrast, Clyne et al.\(^3\) reported that the -344 surrounding sequence binds SF-1 but without functional consequences. Two different elements at positions -71/-64 and -129/-114 that consist of SF-1 and COUP-1 binding sites, respectively, are required for both basal and Ang II– or K\(^+\)-stimulated CYP11B2 transcription. Because of its reduced affinity to SF-1, the T allele might bind a smaller amount of the SF-1 factor, thus allowing more transcription factor to be bound on the functional site of the promoter.

**Conclusions**

This study shows that the C-352T polymorphism of the 5′ region of the AGT gene is most significantly associated with pA GT levels and that this effect can be detected in a relatively small sample of subjects (n=87). This result suggests that this polymorphism should be used preferentially in association studies with the AGT gene. We also detected a significant increase of pAldo levels associated with the T allele of the C-344T polymorphism of CYP11B2.

Although these results, together with those from the literature, suggest that the polymorphisms studied might be implicated in the predisposition to hypertension, we were unable to detect any significant relationship between the polymorphisms and blood pressure in our group of normal subjects.

We have been unable to detect relationships between the AT1R polymorphism and any biochemical parameters, including the number of Ang II binding sites of the AT1 type on platelets. It is clear that the tissue concentration of the different components and the kinetics of the system in the tissue microenvironment are not accessible in clinical studies. Therefore, pharmacological responses or physiological responses that reflect these kinetics should be also studied in relation with polymorphisms, in addition to biochemical parameters, to elucidate the associations found between marker genotypes and diseases.

**Acknowledgments**

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