

# Influence of *CYP3A5* and *ABCB1* gene polymorphisms and other factors on tacrolimus dosing in Caucasian liver and kidney transplant patients

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**Abstract.** Tacrolimus is a substrate of cytochrome P4503A (*CYP3A*) enzymes as well as of the drug transporter *ABCB1*. We have investigated the possible influence of *CYP3A5* and *ABCB1* single nucleotide polymorphisms (SNPs) and other factors (e.g. albumin, hematocrit and steroids) on tacrolimus blood levels achieved in a population of Caucasian liver (n=51) and kidney (n=50) transplant recipients. At 1, 3 and 6 months after transplantation, tacrolimus doses (mg/kg/day) and trough blood levels ( $C_0$ ) were recorded and the weight-adjusted tacrolimus dosage (mg/kg/day) was calculated. Polymerase chain reaction followed by restriction fragment length polymorphism analysis was used for genotyping *CYP3A5*\*1 and \*3 [6986A>G] as well as *ABCB1* at exons 21 [2677G>T/A] and 26 [3435C>T] in both liver transplant donors and recipients and in kidney transplant recipients. Of the 152 subjects studied, 84.9% showed a *CYP3A5*\*3/\*3 genotype. The total frequency of the allelic variant \*3 was 93%. For the *G2677T/A* and *C3435T* polymorphisms the total frequencies of the allelic variants T/A and T were 44.7 and 46.7%, respectively. At 1, 3 and 6 months after transplantation the dose-adjusted  $C_0$  levels were significantly lower in patients with one copy of the \*1 allele compared to those homozygous for the \*3 allele. In the case of liver transplant patients the tacrolimus dose requirements were dominantly influenced by the polymorphisms of the *CYP3A5* gene in the donors. With regard to the *ABCB1* SNPs, in general they did not show any appreciable influence on tacrolimus dosing requirements; however, kidney transplant recipients

carrying the 2677T/A allele required significantly higher daily tacrolimus doses than subjects homozygous for the wild-type allele. Identification of *CYP3A5* single nucleotide polymorphisms prior to transplantation could contribute to evaluate the appropriate initial dosage of tacrolimus in the patients.

## Introduction

Tacrolimus is an immunosuppressive drug widely used and of great benefit in transplant patients. However, its narrow therapeutic index and elevated inter- and intra-individual pharmacokinetic variability require the close monitoring of its administration regimens (1). Achieving the desired target blood concentrations as soon as possible after transplantation is crucial to avoid rejection or excessive immunosuppression and to limit significant dose-related adverse reactions such as nephrotoxicity, neurotoxicity and hyperglycemia (1). To date, trough whole blood concentration ( $C_0$ ) represents the most common parameter used for the therapeutic monitoring of tacrolimus; it is measured 12 h after dose administration and correlates well with the area under the concentration-time curve (2,3).

Tacrolimus has poor bioavailability after oral administration (~25%; range, 4-93%) and is extensively metabolized by the cytochrome P4503A (*CYP3A*) oxidative enzymes *CYP3A4* and *CYP3A5* in the liver and small intestine (1,4). Furthermore, tacrolimus is also a substrate of P-glycoprotein (P-gp), a membrane drug efflux transporter encoded by the multi-drug resistance (*MDR1*) gene, also known as *ABCB1* (5). P-gp is present in several human tissues and organs and affects the disposition of several xenobiotics by limiting their absorption from the gut lumen and increasing their biliary and urinary excretion (6,7).

Several studies have suggested a link between the variability in the pharmacokinetics of tacrolimus and the polymorphisms of the *CYP3A5* and *ABCB1* genes (8,9). It is known that a single nucleotide polymorphism (SNP) *CYP3A5*\*3 in the homozygous state, correlates with reduced *CYP3A5* activity because of alternative splicing and formation of a truncated protein that is

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non-functional (10,11). Several studies have indeed indicated that this *CYP3A5* SNP can be associated with changes in tacrolimus dose requirements (9,12).

*ABCB1* also has a polymorphic expression, with at least 50 SNPs identified to date (13). It has been suggested that some of these SNPs, like 2677G>T/A at exon 21 and 3435C>T at exon 26, may alter the expression and function of P-gp (13). Although 3435C>T is a synonymous polymorphism, it may be in linkage disequilibrium with some non-synonymous SNP of *ABCB1*, including 2677G>T/A, which causes a serine-alanine substitution and results in a low expression of intestinal P-gp (14). In addition, recent data have suggested that 3435C>T may reduce *ABCB1* mRNA stability in the liver (15) or affect the insertion and folding of P-gp into the membrane resulting in an altered substrate specificity of the transporter (16).

Accordingly, at equivalent dose levels, subjects with the exon 21 and 26 polymorphisms in the *ABCB1* gene would experience greater tacrolimus bioavailability and level/dose ratios than those carrying the wild-type genotypes (13). However, the conclusions drawn so far on the actual influence of *ABCB1* SNPs on tacrolimus or also cyclosporine pharmacokinetics are largely controversial (12,14,17,18).

Importantly, the allele frequencies of the afore-mentioned *CYP3A5* and *ABCB1* SNPs can widely differ depending on ethnicities (19-21). Further, interactions have been described between tacrolimus and other drugs commonly used in transplantation, such as steroids. Steroids can induce both *CYP3A* and P-gp activity through pathways which involve different nuclear receptors and it is controversial whether their concomitant use with tacrolimus may change the pharmacokinetics of the latter agent with important clinical consequences (22). It has also been suggested that the *CYP3A5*\*1 non-carriers may be more susceptible to the inductive effects of steroids owing to their lower basal activity of *CYP3A* (23).

The principal objective of this study was to analyze the possible effect of the *CYP3A5* and *ABCB1* SNPs, also in conjunction with steroids use, on tacrolimus dose requirements in a Caucasian, mainly Sicilian, population of liver and kidney transplant patients. We examined also the possible influences of age, gender and plasma protein and hematocrit values on tacrolimus clearance.

## Materials and methods

**Subjects.** A total of 101 subjects who underwent liver (n=39 male, 12 female) and kidney (26 male, 24 female) transplantation at the Mediterranean Institute for Transplantation and Advanced Specialized Therapies (ISMETT) were consecutively included in this study. For the liver transplant patients we took into account also their co-respective donors (n=51). The average age of the liver transplant recipients was 54±12.30 (range, 15-68) years; all were Caucasians and 48 (94%) were Sicilians. The average age of the transplant donors was 42.80±20.30 (range, 15-82) years; 25 were males and 26 were females and all were Caucasians (70.6% Sicilians). Regarding the kidney transplant patients, the average age was 42.94±13.55 (range, 17-62) years; all were Caucasians and 46 (92%) were Sicilians.

After transplantation, the recipients were treated with tacrolimus, alone or in combination with steroids (mostly

during the first month) and/or mycophenolate mofetil. The patients were checked to make sure that they were not taking any drug known to interact with tacrolimus. The average initial tacrolimus dose for liver and kidney transplant recipients was 0.017 and 0.05 mg/kg every 12 h, respectively. The dose was subsequently adjusted according to the whole blood trough ( $C_0$ ) levels. The target  $C_0$  level was set between 5 and 12 ng/ml for liver transplant patients and between 8 and 12 ng/ml for kidney transplant patients.

Body weight, laboratory data (albumin, serum creatinine and liver function tests), tacrolimus dosage (mg/kg/day) and  $C_0$  levels were recorded at 1, 3 and 6 months after transplantation. Blood samples for tacrolimus  $C_0$  levels determinations were drawn just prior to the morning dose. Whole blood tacrolimus concentrations were measured by EMIT 2000 immunoassay (DADE Behring, Hilden, Germany). The dose-adjusted  $C_0$  level (L/D) was calculated by dividing the tacrolimus  $C_0$  level by the corresponding 24 h dose. The information thus obtained was the tacrolimus dose needed to obtain a given  $C_0$  level. The protocol was approved by the Institutional Review Board and Ethic Committee of ISMETT and informed consent was obtained from all patients.

**Genotype identification.** Recipient and donor genotypes were determined by analyzing 200  $\mu$ l of EDTA-anticoagulated blood. Genomic DNA was extracted using the QIAmp DNA mini kit (Qiagen, Crawley, UK). The PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) assay was then applied to identify the *CYP3A5*\*3, 2677G>T/A and 3435C>T genetic polymorphisms. We used 150 ng of genomic DNA for PCR amplification. The forward primer for *CYP3A5*\*3 was 5'-CAT CAG TTA GTA GAC AGA TGA-3' and the reverse primer was 5'-GGT CCA AAC AGG GAA GAA ATA-3'; the forward primer for 2677G>T/A was 5'-TAC CCA TCA TTG CAA TAG CAG-3' and the reverse primer was 5'-TTT AGT TTG ACT CAC CTT TCT AG-3'; the forward primer for 3435C>T was 5'-CAT GCT CCC AGG CTG TTT AT-3' and the reverse primer was 5'-GTA ACT TGG CAG TTT CAG TG-3'. PCR was performed in a total volume of 50  $\mu$ l with 40 pmoles of each primer, 0.2 mM dNTP, 1X High Fidelity PCR buffer (600 mM Tris-SO<sub>4</sub> pH 8.9 and 180 mM NH<sub>4</sub>SO<sub>4</sub>), 2 mM MgSO<sub>4</sub> and 1.5 U of TaqDNA polymerase (Platinum Taq High Fidelity, Invitrogen, Carlsbad, CA, USA). PCR process included initial denaturation at 94°C for 7 min, followed by 35 cycles of denaturation for 1 min at 94°C, 35 cycles of annealing for 1 min at 55°C, and 35 cycles of synthesis for 1 min at 72°C. The final extension was carried out for 7 min at 72°C. The PCR products (10  $\mu$ l for each sample) were analyzed by electrophoresis on 1% agarose gel. The enzymatic digestion was performed for 2 h at 37°C using specific restriction enzymes. For *CYP3A5*\*3 we used 15  $\mu$ l of PCR product and 10 units of *SspI* plus 1X buffer (50 mM NaCl, 100 mM Tris-HCl, 10 mM MgCl<sub>2</sub> and 0.025% Triton X-100) in a total volume of 20  $\mu$ l; for 2677G>T/A, we used 15  $\mu$ l of PCR product and 20 units of *XbaI* plus 1X buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl<sub>2</sub> and 1 mM DTT) in a total volume of 20  $\mu$ l; and for 3435C>T we used 10  $\mu$ l of PCR product and 10 units of *DpnII* plus 1X buffer (100 mM NaCl, 50 mM Bis Tris-HCl, 10 mM MgCl<sub>2</sub> and 1 mM dithiothreitol) in a total volume of 15  $\mu$ l. The digestion products were then

Table I. Distribution of *CYP3A5* and *ABCB1* genotypes in liver transplant donors and recipients and in kidney transplant recipients.

Genotype	Allelic status	Liver transplant patients		Kidney transplant recipients (n=50) n (%)	Total expression % (n/152)
		Donors (n=51) n (%)	Recipients (n=51) n (%)		
<i>CYP3A5</i> *3	*3/*3	40 (78.4)	44 (86.3)	45 (90)	84.9 (129/152)
	*1/*3	10 (19.6)	6 (11.7)	5 (10)	13.8 (21/152)
	*1/*1	1 (2)	1 (2)	-	1.3 (2/152)
<i>ABCB1</i> 2677G>T/A (Exon 21)	G/G	15 (29.4)	18 (35.3)	15 (30)	31.6 (48/152)
	G/T	27 (52.9)	23 (45.1)	18 (36)	44.7 (68/152)
	G/A	1 (2)	-	3 (6)	2.6 (4/152)
	T/T	6 (11.7)	8 (15.7)	13 (26)	17.8 (27/152)
	T/A	2 (4)	2 (4)	1 (2)	3.3 (5/152)
<i>ABCB1</i> 3435C>T (Exon 26)	C/C	12 (23.5)	9 (17.6)	11 (22)	21 (32/152)
	C/T	26 (51)	26 (51)	26 (52)	51.3 (78/152)
	T/T	13 (25.5)	16 (31.4)	13 (26)	27.7 (42/152)

subjected to 3% agarose gel electrophoresis and detected by staining with ethidium bromide.

Genotype identification by PCR-RFLP was confirmed by random evaluation of at least 10 samples per gene SNP through sequencing. The PCR products were purified using the Wizard PCR Preps DNA Purification System kit (Promega, Madison, WI).

Direct sequencing of the amplified and purified amplicons was performed using a Cycle Sequencing Termination kit (ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kits, version 1.1) in an ABI PRISM 310 apparatus (Applied Biosystems, Foster City, CA).

**Statistical analysis.** All values were expressed as means  $\pm$  SD and the two-tailed Mann-Whitney U test was employed to determine the difference in continuous values among groups. The  $\chi^2$  test was used to analyze differences in the frequencies between Sicilian and non-Sicilian subjects. The allele and genotype frequencies of the *CYP3A5* and *ABCB1* polymorphisms were assessed for deviation from the Hardy-Weinberg equilibrium using the  $\chi^2$  test. P-values <0.05 were considered to indicate statistically significant differences.

## Results

**Frequency of *CYP3A5* and *ABCB1* variants in liver transplant recipients and donors and in kidney recipients.** As shown in Table I, among the 51 liver transplant recipients involved in the study, the *CYP3A5*\*3/\*3 genotype was observed in 44 (86.3%) recipient cases, *CYP3A5*\*1/\*3 in 6 (11.7%) cases and *CYP3A5*\*1/\*1 in 1 (2%) case. For the corresponding donors, *CYP3A5*\*3/\*3 was present in 40 (78.4%) cases, *CYP3A5*\*1/\*3 in 10 (19.6%) cases and *CYP3A5*\*1/\*1 in 1 (2%) case (Table I).

Among the 50 kidney transplant recipients, the *CYP3A5*\*3/\*3 genotype was observed in 45 cases and *CYP3A5*\*1/\*3 in 5 cases. Overall, the frequency of *CYP3A5*\*3/\*3 was 84.9% (129/152 subjects), that of \*1/\*3 13.8% (21/152 subjects) and that of \*1/\*1 1.3% (2/152 subjects). The total allelic frequency was 91.7% for *CYP3A5*\*3 and 8.3% for *CYP3A5*\*1.

As for the *ABCB1* SNP at exon 21 (2677G>T/A), the G/G, G/T, T/T and T/A genotypes were found in 18 (35.3%), 23 (45.1%), 8 (15.7%) and 2 (4%) of the liver transplant recipients, respectively. The G/G, G/T, G/A, T/T and T/A genotypes were found in 15 (29.4%), 27 (52.9%), 1 (2%), 6 (11.7%) and 2 (4%) of the donors, respectively. For the kidney recipients, the G/G, G/T, G/A, T/T and T/A genotypes were observed in 15 (30%), 18 (36%), 3 (6%), 13 (26%) and 1 (2%) of the patients (Table I). The overall percentages of expression of G/G, G/T, G/A, T/T and T/A were 31.6 (48/152 subjects), 44.7 (68/152), 2.6 (4/152), 17.8 (27/152) and 3.3% (5/152), respectively (Table I). The total allelic frequencies of the G, T and A variants were 55.3, 41.7 and 3%, respectively.

For the SNP of *ABCB1* at exon 26 (3435C>T), among the liver transplant recipients, the C/C, C/T and T/T genotypes were observed in 9 (17.7%), 26 (51%) and 16 (31.3%) cases, respectively. Among the donors, the C/C, C/T and T/T genotypes were observed in 12 (23.5%), 26 (51%) and 13 (25.5%) cases, respectively. For the kidney recipients, the C/C, C/T and T/T genotypes were identified in 11 (22%), 26 (52%) and 13 (26%) cases, respectively (Table I).

The overall percentages of expression were 21 (32/152), 51.3 (78/152) and 27.7% (42/152) for the C/C, C/T and T/T genotypes, respectively (Table I). The total allelic frequency observed for the variant T was 46.7%.

The data showed strong linkage (49.3%) between the two *ABCB1* polymorphisms. The *CYP3A5* and *ABCB1* (exons 21

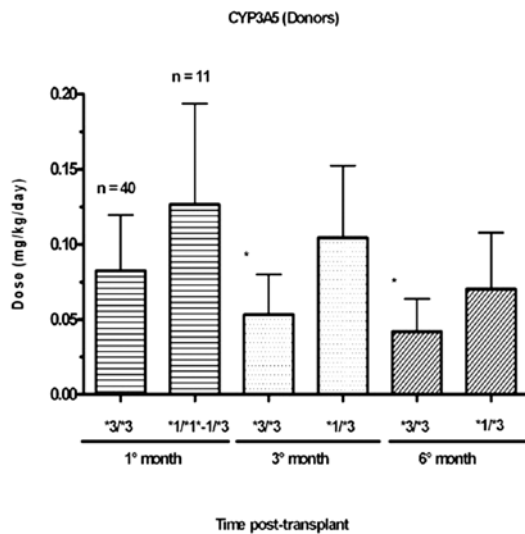


Figure 1. The tacrolimus doses (mg/kg/day) according to donors *CYP3A5* genotypes at 1, 3 and 6 months after liver transplantation. \*Designates that the differences between the groups were statistically significant ( $P<0.05$ ).

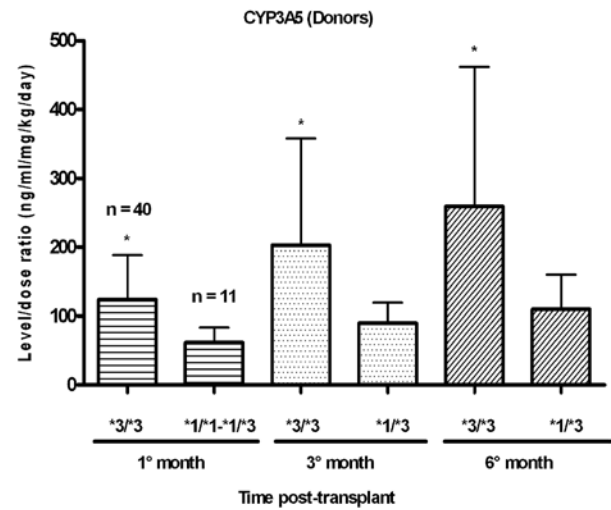


Figure 2. The tacrolimus blood level/dose ratios (ng/ml/mg/kg/day) according to donors *CYP3A5* genotypes at 1, 3 and 6 months after liver transplantation. \*Designates that the differences between the groups were statistically significant ( $P<0.05$ ).

Table II. Tacrolimus blood L/D ratio (ng/ml per mg/kg/day) in different groups of combined *CYP3A5* genotypes in both donors and recipients at 1, 3 and 6 months after liver transplantation.

Group comparison	n	L/D ratio (ng/ml/mg/kg/day)		
		1 month	3 months	6 months
Non-expressor donors - Expressor vs. non-expressor recipients	3	117.23±27.05	144.49±81.84	229.50±120.31
	37	124.73±66.63	207.53±159.25	261.41±208.97
Non-expressor recipients - Expressor vs. non-expressor donors	11	61.42±21.93 <sup>a</sup>	89.91±29.75 <sup>a</sup>	110.03±50.07 <sup>a</sup>
	37	124.73±66.63	207.53±159.25	261.41±208.97

<sup>a</sup>The differences between the groups were statistically significant ( $P<0.05$ ).

and 26) genotype frequencies were not significantly different from those predicted by the Hardy-Weinberg equilibrium.

*Effect of CYP3A5 and ABCB1 SNPs on tacrolimus dosage and level-dose ratio (L/D) in the liver transplant recipients.* At 1, 3 and 6 months after liver transplantation, the average tacrolimus doses (mg/kg/day) were 0.092 (0.025-0.257), 0.064 (0.014-0.21) and 0.048 (0.006-0.177), respectively.

The tacrolimus doses and blood L/D ratios of liver recipients in relation to donors genotype are shown in Figs. 1 and 2. After 3 and 6 months of transplantation, at equivalent tacrolimus blood levels, the doses of the immunosuppressive agent required to reach the desired trough concentrations were significantly higher ( $P<0.05$ ) in the group of subjects receiving a liver with at least one copy of allele \*1 compared to the subjects receiving a liver homozygous for allele \*3 (Fig. 1).

A similar, though of borderline statistical significance ( $P=0.0503$ ), trend was observed one month after transplantation (Fig. 1); in addition, at that time the tacrolimus  $C_0$  levels were substantially lower in the patients receiving livers with \*1/\*3 or \*1/\*1 alleles rather than with the \*3/\*3 allele.

A statistically significant difference was also evidenced by relating L/D ratios with the *CYP3A5* genotypes of the liver donors at 1, 3 and 6 months after transplantation (Fig. 2).

Also for the recipient genotypes, the presence of at least one \*1 copy tended to increase tacrolimus doses and to lower dose-adjusted  $C_0$  levels, though the differences with respect to the \*3/\*3 genotypes were statistically significant only at the third month (data not shown).

For further investigation we analyzed the effects of combining the *CYP3A5* genotypes of the donor and of the recipient (Table II). At 1, 3 and 6 months after liver transplantation, only the presence of the *CYP3A5*\*1 allele in donors, but not in recipients, had a statistically significant effect ( $P<0.05$ ) on the tacrolimus L/D ratio.

Table III shows that, at equivalent tacrolimus trough blood levels, the presence, either in the liver donors or in the liver recipients, of the two examined *ABCB1* polymorphisms, did not have any significant effect on the tacrolimus doses or L/D ratios during the 6 months of observation.

Furthermore, we evaluated the influence of the *ABCB1* genotypes (according to both the donors and recipients

Table III. Relationship of *ABCB1* SNPs to tacrolimus  $C_0$  blood levels, dose requirements and L/D ratio after liver transplantation.

	1 month			3 months			6 months		
	Level (ng/ml)	Dose (mg/kg/day)	L/D (ng/ml/mg/kg/day)	Level (ng/ml)	Dose (mg/kg/day)	L/D (ng/ml/mg/kg/day)	Level (ng/ml)	Dose (mg/kg/day)	L/D (ng/ml/mg/kg/day)
<b>Donor genotype</b>									
<i>ABCB1</i> Ex-21									
G/G (n=41)	8.83±3.53	0.096±0.05	112.45±68.24	8.20±2.89	0.063±0.04	190.42±159.43	7.89±2.60	0.046±0.03	244.95±207.77
G/T (n=7)	7.23±3.33	0.069±0.02	109.28±44.2	8.31±2.41	0.068±0.02	127.12±29.21	7.31±1.40	0.050±0.01	156.73±57.93
T/T (n=3)	6.81±1.90	0.076±0.01	88.93±18.24	8.83±1.80	0.069±0.01	134.61±46.00	8.20±0.75	0.062±0.02	143.58±48.96
G/T+T/T (n=10)	7.10±2.87	0.071±0.02	103.17±38.38	8.46±2.16	0.068±0.02	123.37±32.43	7.57±1.27	0.053±0.01	152.78±53.01
<b>Recipient genotype</b>									
<i>ABCB1</i> Ex-21									
G/G (n=43)	8.67±3.59	0.092±0.04	109.32±57.22	8.17±2.80	0.062±0.03	184.53±154.55	7.86±2.48	0.046±0.02	236.62±202.38
G/T (n=6)	7.33±2.96	0.103±0.06	85.60±54.80	8.84±3.00	0.087±0.03	116.60±58.47	7.79±2.36	0.062±0.03	137.72±73.78
T/T (n=2)	8.10±0.14	0.050±0.03	213.94±120.20	8.30±0.84	0.038±0.01	233.40±73.22	7.40±0.00	0.028±0.01	285±120.20
G/T+T/T (n=8)	7.52±2.53	0.090±0.06	117.69±94.27	8.70±2.57	0.075±0.04	145.80±78.30	7.69±2.00	0.054±0.03	174.54±102.95
<b>Donor genotype</b>									
<i>ABCB1</i> Ex-26									
C/C (n=12)	8.12±3.25	0.087±0.05	106.71±43.78	7.76±1.86	0.068±0.03	167.97±174.78	8.29±2.01	0.054±0.02	226.29±267.65
C/T (n=26)	8.19±3.31	0.089±0.048	115.97±76.11	8.37±2.22	0.066±0.043	167.45±100.13	8.17±2.53	0.048±0.033	222.50±116.48
T/T (n=13)	9.10±3.50	0.102±0.047	96.31±37.00	8.50±4.15	0.061±0.026	176.03±181.08	6.73±2.26	0.041±0.017	224.60±230.63
C/T+T/T (n=39)	8.49±3.38	0.093±0.047	109.42±65.82	8.41±2.99	0.064±0.038	170.31±130.26	7.69±2.51	0.046±0.028	223.20±160.39
<b>Recipient genotype</b>									
<i>ABCB1</i> Ex-26									
C/C (n=9)	7.50±2.37	0.089±0.05	114.50±88.70	8.29±2.70	0.073±0.03	139.35±75.76	7.69±1.87	0.054±0.02	170.99±96.89
C/T (n=26)	9.00±3.64	0.086±0.035	121.28±64.05	8.44±3.03	0.061±0.040	208.54±188.34	8.31±2.53	0.048±0.033	270.92±247.58
T/T (n=16)	8.24±3.67	0.102±0.060	91.15±41.26	7.93±2.42	0.063±0.036	151.56±70.54	7.13±2.38	0.044±0.017	186.76±85.06
C/T+T/T (n=42)	8.71±3.63	0.092±0.046	109.80±57.82	8.24±2.79	0.062±0.038	186.83±155.67	7.86±2.51	0.046±0.028	238.86±204.29

Table IV. Relationship of *CYP3A5* and *ABCB1* SNPs to tacrolimus C<sub>0</sub> blood levels, dose requirements and L/D ratio after kidney transplantation.

Kidney transplant recipients	1 month			3 months			6 months		
	Level (ng/ml)	Dose (mg/kg/day)	L/D (ng/ml/mg/kg/day)	Level (ng/ml)	Dose (mg/kg/day)	L/D (ng/ml/mg/kg/day)	Level (ng/ml)	Dose (mg/kg/day)	L/D (ng/ml/mg/kg/day)
<i>CYP3A5</i> <sup>*</sup> 3/3 (n=45)	12.81±3.55	0.119±0.098	160.41±114.19 <sup>a</sup>	11.51±3.16	0.087±0.066 <sup>a</sup>	187.13±98.17 <sup>a</sup>	9.34±2.57	0.070±0.051	188.70±114.87 <sup>a</sup>
<i>CYP3A5</i> <sup>*</sup> 1/3 (n=5)	8.04±3.15	0.131±0.088	76.94±35.06	9.46±2.04	0.119±0.027	79.92±9.03	6.25±2.01	0.091±0.040	72.75±20.69
<i>ABCB1</i> Ex-21									
G/G (n=15)	12.24±3.26	0.082±0.03	171.26±71.06	11.01±3.03	0.058±0.03	239.58±109.99	8.69±2.21	0.047±0.02	235.38±126.53
G/T (n=18)	12.21±4.27	0.106±0.07	148.54±85.56	11.09±2.66	0.098±0.06 <sup>a</sup>	161.23±98.80 <sup>a</sup>	9.20±3.41	0.087±0.06 <sup>a</sup>	159.06±120.80 <sup>a</sup>
G/A (n=3)	16.30±6.78	0.327±0.27	74.42±50.29 <sup>a</sup>	16.45±6.27	0.212±0.11 <sup>a</sup>	90.74±38.49 <sup>a</sup>	10.36±2.05	0.086±0.04	139.54±72.84
T/A (n=1)	10.64	0.014	714.09	8.35	0.052	159.96	8.4	0.058	142.86
T/T (n=13)	11.82±2.75	0.142±0.05 <sup>a</sup>	92.83±34.13 <sup>a</sup>	10.98±2.13	0.089±0.04	146.34±59.82 <sup>a</sup>	8.94±2.35	0.078±0.03 <sup>a</sup>	146.16±84.41
G/T+G/A+T/T+T/A (n=35)	12.37±4.01	0.136±0.10 <sup>a</sup>	137.65±123.19 <sup>a</sup>	11.43±3.18	0.103±0.06 <sup>a</sup>	149.62±81.34 <sup>a</sup>	9.18±2.86	0.083±0.05 <sup>a</sup>	152.13±100.89 <sup>a</sup>
<i>ABCB1</i> Ex-26									
C/C (n=11)	11.32±2.04	0.107±0.052	132.45±67.78	10.78±2.41	0.081±0.044	167.47±87.69	9.24±1.92	0.069±0.036	162.22±69.95
C/T (n=26)	12.53±4.15	0.115±0.07	157.11±137.27	10.74±2.42	0.090±0.05	162.82±89.97	9.21±3.16	0.078±0.05	168.37±111.30
T/T (n=13)	12.80±4.16	0.140±0.15	151.77±91.63	12.87±4.35	0.097±0.08	211.85±121.68	8.49±2.19	0.062±0.04	207.25±146.42
C/T+T/T (n=39)	12.62±4.10	0.123±0.106	157.59±121.25	11.45±3.29	0.092±0.068	178.93±102.35	8.97±2.87	0.073±0.054	181.30±124.68

<sup>a</sup>The differences between the groups were statistically significant (P<0.05).

Table V. Tacrolimus levels, doses and level/dose ratios in liver and kidney transplant patients carrying the *CYP3A5*\*3/\*3 genotype at 1 and 3 months after transplantation treated or not treated with steroids.

	Treated with steroids				Not treated with steroids			
	n	Level	Dose	L/D	n	Level	Dose	L/D
Liver transplant	23				14			
1 month		8.59±4.23	0.082±0.042 <sup>a</sup>	115.53±51.37 <sup>a</sup>		9.13±2.34	0.079±0.029	139.83±86.21
3 months		8.57±3.29	0.050±0.025	222.50±184.29		8.26±2.22	0.059±0.030	182.94±108.21
Kidney transplant	37				8			
1 month		12.89±3.70	0.125±0.104 <sup>a</sup>	145.48±114.54 <sup>a</sup>		12.41±2.92 <sup>a</sup>	0.087±0.062	202.24±101.71
3 months		11.97±3.26	0.092±0.066	174.79±88.93		9.36±1.20	0.063±0.060	245.47±125.00

Tacrolimus levels (L) (ng/ml), doses (D) (mg/kg/day) and level/dose (L/D) ratios (ng/ml/mg/kg/day) in liver and kidney transplant patients carrying the *CYP3A5*\*3/\*3 genotype at 1 and 3 months after transplantation treated or not treated with steroids. <sup>a</sup>Within the patient groups the differences in tacrolimus levels, doses or L/D ratios between the first and third month after transplantation were statistically significant (P<0.05).

genetic profiles) by analyzing their role in association with the *CYP3A5* status. Therefore, all donors and recipients with at least one copy of the allele \*1 were excluded from the analysis. Also in this case, as regards the 37 subjects examined, the two *ABCB1* polymorphisms had no statistically significant effect on tacrolimus pharmacokinetics (data not shown).

In kidney recipients, at 1, 3 and 6 months after transplantation, the mean dose of tacrolimus was equal to 0.120 (0.014-0.316), 0.090 (0.020-0.288) and 0.074 (0.013-0.157) mg/kg/day, respectively.

Again, the *CYP3A5* genotype appeared to have a determining effect: at 1, 3 and 6 months after transplant, the L/D ratio was statistically lower (P<0.05) in the 5 patients with one copy of allele \*1 compared to patients homozygous for allele \*3 (Table IV). Also, three months after the transplant, the dose required to maintain the drug trough blood levels was statistically lower (P<0.05) in the patients homozygous for allele \*3 compared to the heterozygous patients (Table IV).

Interestingly, despite the higher doses administered to heterozygous patients, at 1 and 6 months after transplantation an equivalency of the trough blood levels was not achieved in comparison with the patients homozygous for allele \*3.

In reference to the *ABCB1* behavior in kidney recipients, during the six months of observation the C3435>T polymorphism did not affect the dosage or level/dose ratio, when the polymorphisms were evaluated among the subjects (n=45) homozygous for allele \*3 (data not shown). However, as shown in Table III, subjects carrying the 2677T/A allele required a significantly higher daily tacrolimus dose than subjects homozygous for the wild-type allele.

We also found that after liver or kidney transplant there were increases in plasma albumin concentrations and in hematocrit values and statistical analysis showed that they were significantly associated with a reduction in the tacrolimus doses needed to reach the target dose levels (data not shown).

For steroids, in the present study these agents were used only in a part of the patients; they were administered in moderate doses and in general suspended one month after transplant. The results did not show any difference in the

tacrolimus doses at 1 month and further between patients treated with steroids and not, also considering the different *CYP3A5* and *ABCB1* genotypes (data not shown). However, in the group of patients treated with steroids, the reduction of the doses between the first and third month (usually required to maintain the target blood levels) was significantly lower than in the patients without steroids. This was observed only in patients without *CYP3A5* expression (Table V).

## Discussion

The findings on the *CYP3A5* gene in our population are in agreement with literature data, according to which up to 90% of Caucasian subjects are homozygous for the non-functional variant *CYP3A5*\*3 (10,11,24,25): in our series the percentage of *CYP3A5*\*3/\*3 subjects was 84.9 and the total frequency of the variant \*3 was 91.7%. The percentage of *CYP3A5*\*3/\*3 subjects is lower (~20-30%) in other ethnic groups, in particular among Africans and African Americans (10,11,25-27). Regarding the *ABCB1* gene polymorphisms, in the case of G2677T/A at exon 21, the total frequency of the variant T in our patients (41%) was similar to that of other Caucasian populations (40-50%) (19,20,28-30) and different from that recorded in Africans and African Americans (0.9-13%) (30-33). The allelic frequency of 2677A was 3%. In Caucasians the frequency of this allele is low (1-4%) and different from that reported for Asian populations like the Chinese (~6%) and Japanese (~20%) ones (20). As regards C3435T at exon 26, the total frequency of the variant T was 46.7%, also in agreement with data of other authors who have indicated that it is 33-65% among Caucasian populations (28-30,34-36).

Our data confirmed strong linkage between the two *ABCB1* polymorphisms, (12,14,30,37). The *ABCB1* 2677T-3435T (T-T) haplotype is present in ~32% of Caucasians, 62% of European Americans, 27% of Asian Americans and 35% of Mexican Americans (14,38). In other ethnic groups, like the African and African American populations, a lower T/T frequency and even the absence of a linkage disequilibrium between the two *ABCB1* polymorphisms have been reported (14,31,38,39).



Considering the relationship between genotype and tacrolimus dosage and L/D ratios, our data in both the liver and kidney transplant situations have confirmed the significant influence of *CYP3A5* on tacrolimus pharmacokinetics. Subjects homozygous for the variant \*3, likely owing to the lower metabolic drug clearance, require a lower tacrolimus dose to reach the desired therapeutic levels compared to the subjects with at least one \*1 copy who are considered to express the functional enzyme (21,40,41). Similar results have been previously reported for liver (8,41-43), kidney (12,44-47), heart (48), and lung recipients (49). In particular, as regards to liver transplantation, our data support the primary importance of the donor with respect to the *CYP3A5* genotype (8,43,50-53); however, they suggest a possible influence of the recipient genotype also (52), which might be corroborated by the known metabolic role of *CYP3A5* in extra-hepatic tissues such as the intestine (51,53).

Data from literature concerning *ABCB1* suggest that subjects homozygous for the exon 26 3435T variant present a 2-fold reduction of intestinal P-gp, (54-56) which might influence the bioavailability of P-gp substrates such as tacrolimus and cyclosporine (14,50,56,57). Nonetheless, other studies focusing on this polymorphism in the context of kidney, liver and lung transplants, have generally excluded this role of T/T or at most indicated a minor influence of such recipient genotype in the disposition of tacrolimus and cyclosporine (14,17,18,43,49,52). Our data support these findings, confirming that the presence of the T allele in liver and kidney recipients does not affect the dose required to achieve the target levels of tacrolimus. On the other hand, the exon 21 G2677T/A polymorphisms, did not affect tacrolimus dose in our liver transplant patients, but increased it in kidney transplant patients, in contrast with other studies reporting that carriers of the G/G wild-type genotype require higher daily tacrolimus doses (13). We have no explanation for the last result; overall, however, as other authors have shown (58), the effect of *ABCB1* polymorphisms on tacrolimus dosage was not as evident as it was for the *CYP3A5*\*3 polymorphism.

Since the *CYP3A* activity may influence the contribution of P-gp on tacrolimus disposition, we analyzed the effect of the *ABCB1* polymorphisms only in the subjects with the *CYP3A5*\*3/\*3 genotype. Also in this instance, both in liver and kidney recipients, the two *ABCB1* polymorphisms, with the exception of the G2677T/A polymorphism in kidney transplanted patients, did not show any statistically significant effect on the tacrolimus dose.

It has been proposed that in liver transplant patients, regardless of the status of the donors with respect to *CYP3A5*, the doses needed to reach the target trough blood levels may increase along time. This may be due to the fact that, early after transplantation, the liver cannot fully perform its metabolic function owing to the damage associated with ischemia and hepatic reperfusion (51).

However, our results did not show such time-related dose increases, but rather progressive reductions. This behavior was especially evident in the liver transplanted patients. Indeed, another multi-center study on liver transplant recipients suggested that, as time elapses, lower doses of tacrolimus are necessary to reach the same target concentrations in whole blood (59). The data of this study revealed that the low

concentrations of plasma albumin and total proteins recorded soon after transplantation underwent subsequent constant increases and resumed normal levels within 4-8 weeks. The restore of plasma albumin and total protein concentrations could lead to a higher rate of bound tacrolimus in plasma and therefore to a reduction in tacrolimus clearance. Our data support the afore-mentioned findings; in particular, we found that after transplantation there were increases in plasma albumin concentrations and in hematocrit values. Statistical analysis showed that they were significantly associated with a reduction in the tacrolimus doses needed to reach the target dose levels (data not shown). A previous study also provided evidence of the influence of plasma albumin concentrations and hematocrit on the clearance of tacrolimus (60).

As regards to the influence of steroids, which were administered in not intensive doses, there was no difference in the tacrolimus doses between patients treated with these agents and not, also considering the different *CYP3A5* and *ABCB1* genotypes. However, the reduction in the doses between the first and third month in the *CYP3A5*\*3/\*3 subjects treated with steroids was significantly lower than that in the patients with the same genotype but not treated with steroids. This result may perhaps support that the subjects lacking *CYP3A5*\*1 can undergo some inductive effects of these drugs on tacrolimus metabolism (23).

Some studies have evidenced that the *CYP3A* enzyme activity is low immediately after birth and that then it progressively increases reaching a peak during youth and adulthood and eventually decreasing in old age (61). Other studies have demonstrated that the P-gp activity in peripheral blood lymphocytes is greater in the umbilical cord, and that it steadily decreases with age (62). On the contrary, other authors have reported that the P-gp activity does not decline in old age (63). However, our data did not show any influence of age on the tacrolimus doses used during the six-month observation period (data not shown). Furthermore, it has been proposed, that drugs that are a substrate of both *CYP3A* and P-gp, such as tacrolimus, may undergo a greater clearance in women than in men following i.v. administration. Nevertheless our data did not suggest any statistically significant difference in the dosage between male and female patients, also taking into account the genotypes of both donors and recipients (data not shown).

Neurotoxicity is an important dose-limiting effect of calcineurin inhibitors and corticosteroids. On the other hand, P-gp is highly expressed in the hematoencephalic barrier, where its function is to limit drug access into the central nervous system. In a study carried out on 17 liver recipients treated with tacrolimus, the presence of the mutant T allele in exon 21 was a predictor of tacrolimus-related neurotoxicity (64). In our population only 1 patient developed neurotoxicity ascribable to the administration of tacrolimus. Interestingly, this patient exhibited a mutant T homozygous genotype at exon 21.

In conclusion, our study has demonstrated the high variability of the tacrolimus doses required to maintain the desired drug levels, even in subjects expressing the same genotype (with particular reference to the expression of functional *CYP3A5*), and the noticeable overlap of such doses in subjects with the wild-type and mutant genotypes.

This shows that genetic polymorphism is only one of the factors influencing tacrolimus pharmacokinetics, which, in



fact, can be modified by many other variables (51,65,66). Thus, genotyping cannot substitute but only support the therapeutic monitoring of the immunosuppressor blood concentrations. Nonetheless, *CYP3A5* genotyping may represent a tool especially useful to evaluate the appropriate initial dose of tacrolimus for transplant recipients, even in populations like the Caucasians which are fairly homogenous with respect to the lack of *CYP3A5*<sup>\*1</sup>.

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