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Original Paper

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The effect of *CYP3A5* and *ABCB1* single nucleotide polymorphisms on tacrolimus dose requirements in Caucasian liver transplant patients

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Summary

Background: Tacrolimus is a substrate of cytochrome P-450 (CYP) 3A enzyme and of the drug transporter *ABCB1*. We have investigated the effects of possible relevant *CYP3A5* and *ABCB1* single nucleotide polymorphisms (SNPs) present in both donors and recipients on tacrolimus blood levels achieved in a population of 32 Caucasian liver transplant patients.

Material/Methods: At 1, 3 and 6 months after transplantation, tacrolimus doses (mg/kg/day) and trough blood levels (C_0) were determined. Polymerase chain reaction followed by restriction fragment length polymorphism analysis was used for genotyping *CYP3A5*3* [6986A>G] as well as *ABCB1* at exons 21 [2677G>T] and 26 [3435C>T].

Results: 87.5% of the population showed a *CYP3A5**3/*3 genotype. For the *ABCB1* SNPs, in the case of 3435C>T the total frequency observed for the allelic variant was 50%. For the 2677G>T, the total frequency of the allelic variant was 12.5%, lower than in other Caucasian populations and without any significant linkage with 3435C>T. At 3 and 6 months after transplantation, tacrolimus dose requirements were significantly higher in patients receiving a liver with one copy of the *1 allele compared to those homozygous for the *3 allele (0.111±0.057 vs. 0.057±0.030 [P<0.05] at 3 month and 0.086±0.051 vs. 0.044±0.025 [P<0.05] at 6 month). For the recipients' genotypes, the presence of at least one *1 copy tended, though not statistically significantly, to increase tacrolimus doses. With regard to the *ABCB1* SNPs, they did not show any influence on tacrolimus dosing requirements.

Conclusions: Pharmacogenetic analysis of CYP3A5 in the donor could contribute to determine the appropriate initial dosage of tacrolimus in liver transplant patients.

Key words tacrolimus • single nucleotide polymorphisms • *CYP3A5* • *ABCB1* • liver transplant

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Background

Tacrolimus is an immunosuppressant widely used in liver transplant patients. Due to its narrow therapeutic index and high inter-and intra-individual pharmacokinetic variability, the administration regimens of this drug need to be closely monitored [1]. In fact, achieving the desired target blood concentrations immediately after transplantation is of critical importance to avoid rejection or excessive immunosuppression and to limit important dose-related adverse reactions such as nephrotoxicity, neurotoxicity and hyperglycemia [1].

To date the most widely used parameter for the therapeutic monitoring of tacrolimus is its trough whole blood concentration (C_0), that is measured 12 hours after the dose administration and correlates well with the area under the concentration-time curve [2,3].

Many factors of pharmacokinetic and pharmacodynamic nature may influence the activity of the drug [1,2,4,5]. Also the genetic background may influence the inter-individual variability in the pharmacokinetics of tacrolimus [6].

After oral administration, tacrolimus has limited bioavailability (~25%, but it can vary from 4% to 93%) and is extensively metabolized by the oxidative enzymes cytochromes P4503A (CYP3A4 and CYP3A5) in the intestinal wall and liver [1,7]. CYP3A constitute the most abundant group of the cytochrome P450 family and are responsible for the metabolism of more than half of clinically used drugs [8,9]. Previous studies have shown that a single nucleotide polymorphism (SNP), that is 6986A>G at intron 3, correlates with CYP3A5 protein production and enzyme activity [10]. Individuals with at least one wild-type allele (*1) express hepatic and intestinal CYP3A5, whereas the subjects homozygous for the mutant variant (*3/*3) do not present a functional protein [10,11]. Accordingly, several studies have indicated that this *CYP3A5* SNP can be associated with changes in tacrolimus dose requirements [12–14].

In addition, similarly to other drugs commonly used in transplantation, tacrolimus is also a substrate of P-glycoprotein (P-gp), a membrane drug efflux transporter encoded by the multi- drug resistance *ABCB1* (*MDR1*) gene [15]. P-gp is present in several human tissues and organs like the intestine, adrenal gland, kidney, liver, blood-brain barrier, placenta, lymphocytes, ovary and testis [16]. It influences the disposition of several xenobiotics by limiting their absorption from the gut lumen and increasing their biliary and urinary excretion [17,18].

It has been suggested that some SNPs of the *ABCB1* gene, like 2677G>T at exon 21 and 3435C>T at exon 26, may cause altered P-gp expression and function [19,20]. Although 3435C>T is a silent polymorphism, that does not result in an amino acid change in the encoded protein, it may be in linkage disequilibrium with other functional polymorphisms of the *ABCB1* gene, including 2677G>T, which causes a serine-alanine substitution and results in a low expression of intestinal P-gp [21]. In addition, recent data have suggested that the 3435C>T substitution may reduce *ABCB1* mRNA stability in the liver [22]. People with the 2677GG or 3435CC genotypes would have greater function of the drug transporter associated with lower tacrolimus bioavailability and level/dose ratios [21,23,24]. However, the conclusions drawn so far on the real importance of *ABCB1* SNPs on tacrolimus or cyclosporine pharmacokinetics are largely controversial [14,21,23–26].

Importantly, the allele frequencies of the aforementioned *CYP3A5* and *ABCB1* SNPs can widely differ among ethnic groups [27–29]. The objec-

tive of this exploratory study was to analyze the possible influence of these SNPs on tacrolimus dose requirements in a population of Caucasian, mainly Sicilian, liver transplant patients.

Material and Methods

Subjects

A total of 32 subjects (24 males, 8 females) who underwent liver transplantation at the Mediterranean Institute for Transplantation and Advanced Specialized Therapies (ISMETT) and their respective donors were consecutively included in this study. The average age of the liver transplant recipients was 53.53±12.38 (15–66) years; all them were Caucasians and in particular 29 (90.6%) were Sicilians. The average age of the transplant donors was 39.25±18.99 (15–82); 13 were males and 19 females and all Caucasians (65.6% Sicilians). After transplantation, the recipients were treated with tacrolimus, alone or in combination with steroids (mostly during the first month) and/or mycophenolate mofetil.

They were checked to make sure that they were not taking any drug known to interact with tacrolimus. The average initial tacrolimus dose was 0.015 mg/kg every 12 hours subsequently adjusted according to the whole blood trough (C_0) levels. The target C_0 level was set between 5 and 12 ng/mL.

Body weight, laboratory data (including 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 of ISMETT and informed consent was obtained for all patients.

Genotype identification

Recipient and donor genotypes were determined by analysing 200 µL 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 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 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 µL with 40 picomoles of each primer, 0.2 mM dNTP, 1 X High Fidelity PCR Buffer (600 mM TRIS-SO_4 pH 8.9 and 180 mM NH SO_4), 2 mM Mg SO_4 and 1.5 U of Taq DNA 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 µL for each samples) were analyzed by electrophoresis on 1% agarose gel. The enzymatic digestion was performed for 2 hours at 37°C using specific restriction enzymes. For *CYP3A5**3 we used 15 µL of PCR product and 10 U of SspI plus 1 X Buffer (50 mM NaCl, 100 mM TRIS-HCl, 10 mM $MgCl_2$ and 0.025% Triton X-100) in a total volume of 20 µL; for 2677G>T, we used 15 µL of PCR product and 20 U of XbaI plus 1 X Buffer (50 mM NaCl, 10 mM TRIS-HCl, 10 mM $MgCl_2$ and 1 mM DTT) in a total volume of 20 µL; and for 3435C>T we used 10 µL of PCR product and 10 U of DpnII plus 1 X Buffer (100 mM NaCl, 50 mM BIS TRIS-HCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol) in a total volume of 15 µL. The digestion products were then subjected to 3% agarose gel electrophoresis and detected by staining with ethidium bromide.

Statistical analysis

All values were expressed as means ± SD and the two-tailed Mann-Whitney U test was employed to determine the difference in continuous values among groups. The χ^2-square test was used to analyse differences between Sicilian and non-Sicilian subjects.

Table 1. Distribution of *CYP3A5* and *ABCB1* genotypes in liver transplant donors and recipients.

Recipients' genotype	Allelic status	n. of pts	Donors' genotype	Allelic status	n. of pts	Total percentage of expression
CYP 3A5*3	*3/*3	29 (90.7%)	CYP3A5*3	*3/*3	27 (84.4%)	87.5 (56/64)
	*1/*3	2 (6.2%)		*1/*3	5 (15.6%)	10.9 (7/64)
	*1/*1	1 (3.1%)		*1/*1	–	1.6 (1/64)
ABCB1 2677G>T (Exon 21)	G/G	27 (84.4%)	ABCB1 2677G>T (Exon 21)	G/G	23 (71.9%)	78.0 (50/64)
	G/T	5 (15.6%)		G/T	7 (21.9%)	19.0 (12/64)
	T/T	–		T/T	2 (6.2%)	3.0 (2/64)
ABCB1 3435C>T (Exon 26)	C/C	5 (15.6%)	ABCB1 3435C>T (Exon 26)	C/C	10 (31.2%)	23.4 (15/64)
	C/T	19 (59.4%)		C/T	15 (46.8%)	53.2 (34/64)
	T/T	8 (25.0%)		T/T	7 (22.0%)	23.4 (15/64)

The allele and genotype frequencies of the *CYP3A5* and *ABCB1* polymorphisms were assessed for deviation from the Hardy-Weinberg equilibrium using the χ^2-square test. P values <0.05 were considered statistically significant.

Results

Frequency of *CYP3A5* and *ABCB1* variants in liver transplant recipients and donors

As shown in Table 1, among the 32 liver transplant recipients involved in the study, *CYP3A5**3/*3 genotype was observed in 29 (90.7%) cases, *CYP3A5**1/*3 in 2 (6.2%) cases and *CYP3A5**1/*1 in 1 (3.1%) case. For the corresponding donors, *CYP3A5**/3*3 genotype was present in 27 (84.4%) cases and *CYP3A5**1/*3 in 5 (15.6%) cases. Overall, the frequency of *CYP3A5**/3*3 was 87.5% (56/64 subjects), that of *1/*3 10.9% (7/64 subjects) and that of *1/*1 1.6% (1/64 subjects). The total allelic frequency was 93% for *CYP3A5**3 and 7% for *CYP3A5**1.

As for the *ABCB1* SNP at exon 21 (2677G>T), the GG and GT genotypes were found in 27 (84.4%) and 5 (15.6%) of the transplant recipients, respectively. The GG, GT and TT genotypes were found in 23 (71.9%), 7 (21.9%) and 2 (6.2%) of the donors, respectively. The overall percentages of expression of GG, GT and TT were 78.1, 18.8 and 3.1, respectively. The total frequency of the allelic variant was 12.5%.

For the SNP of *ABCB1* at exon 26 (3435C>T), among the recipients, the CC, CT and TT genotypes were observed in 5 (15.6%), 19 (59.4%) and 8 (25%) cases, respectively. Among the donors, the CC, CT and TT genotypes were observed in 10 (31.2%), 15 (46.8%) and 7 (22%) cases, respectively. The overall percentages of expression were 23.4, 53.2 and 23.4 for the wild-type, heterozygous and homozygous mutated genotypes, respectively. The total frequency observed for the allelic variant was 50%.

The data showed no significant association between the two *ABCB1* polymorphisms. The *CYP3A5* and *ABCB1* genotype frequencies were not significantly different from those predicted by the Hardy-Weinberg equilibrium, thus indicating the reliability of the genotype determinations.

Relationship of *CYP3A5* and *ABCB1* SNPs to tacrolimus C_0 blood levels and dose requirements

At 1, 3 and 6 months after liver transplant the average tacrolimus doses (mg/kg/day) were 0.083 (0.025–0.157), 0.066 (0.014–0.21) and 0.051 (0.006–0.177), respectively. Table 2 shows the tacrolimus doses needed to obtain a given blood C_0 level according to the donors' and recipients' genotypes. The data (Table 2) show an influence of the donors' genotypes on tacrolimus dose requirements only for the *CYP3A5* gene. In fact, 3 and 6 months after transplantation, the daily doses of tacrolimus needed to reach target blood levels were significantly (P<0.05) higher in the patients receiving a liver with one copy of the *1 allele compared to those homozygous for

Table 2. Relationship between genotypes, blood levels and tacrolimus doses.

	Level 1st month (ng/ml)	Dose 1st month (mg/kg/ day)	L/D 1st month (ng/ml/ mg/kg/ day)	Level 3rd month (ng/ml)	Dose 3rd month (mg/kg/ day)	L/D 3rd month (ng/ml/ mg/kg/ day)	Level 6th month (ng/ml)	Dose 6th month (mg/kg/ day)	L/D 6th month (ng/ml/ mg/kg/ day)
Donors									
CYP3A5*3/*3(n=27)	8.07 ±3.52	0.081 ±0.041	114.92 ±61.77	8.15 ±3.23	0.057 ±0.030	196.15 ±176.39	7.63 ±2.36	0.0449 ±0.025	251.1 ±233.9
*1/*3(n=5)	6.51 ±2.17	0.097 ±0.044	71.86 ±18.83	8.45 ±2.23	0.111* ±0.057	88.46 ±38.25	6.79 ±1.51	0.086* ±0.051	95.44* ±37.47
Recipients									
CYP3A5*3/*3(N=29)	7.67 ±3.31	0.081 ±0.041	109.54 ±60.59	8.33 ±3.19	0.061 ±0.029	186.61 ±171.82	7.45 ±2.3	0.0473 ±0.024	232.31 ±228.74
*1/*3(N=2)	10.71 ±4.96	0.127 ±0.042	96.22 ±71.24	6.78 ±1.01	0.118 ±0.129	130.25 ±133.7	7.68 ±2.80	0.102 ±0.106	194.95 ±230.16
*1/*1(n=1)	6.60	0.071	92.95	7.14	0.108	66.1	8.57	0.066	129.8
*1/*3+*1/*1(n=3)	9.34 ±4.23	0.108 ±0.044	95.13 ±50.41	6.90 ±0.74	0.115 ±0.091	108.87 ±101.54	7.97 ±2.04	0.09 ±0.078	173.23 ±167.04
Donors									
ABCB1 Ex-21GG (n=23)	8.16 ±3.50	0.088 ±0.046	110.35 ±65.66	8.11 ±3.36	0.064 ±0.045	201.14 ±192.87	7.46 ±2.55	0.049 ±0.038	257.78 ±254.85
GT (n=7)	7.23 ±3.33	0.069 ±0.024	109.28 ±44.2	8.31 ±2.41	0.068 ±0.024	127.12 ±29.21	7.31 ±1.40	0.050 ±0.014	156.73 ±57.93
TT (n=2)	6.12 ±2.08	0.076 ±0.015	79.45 ±11.24	8.80 ±2.54	0.079 ±0.001	111.1 ±30.26	8.6 ±0.42	0.0745 ±0.0007	115.38 ±4.55
GT+TT(n=9)	6.98 ±3.01	0.070 ±0.021	102.65 ±40.67	8.42 ±2.28	0.070 ±0.021	123.56 ±28.36	7.59 ±1.35	0.055 ±0.016	147.54 ±53.4
Recipients									
ABCB1 Ex-21GG (n=27)	8.03 ±3.46	0.084 ±0.043	110.95 ±60.09	8.13 ±3.09	0.064 ±0.041	189.38 ±178.81	7.55 ±2.32	0.05 ±0.03	240.78 ±238.14
GT (n=5)	6.72 ±2.86	0.079 ±0.030	93.29 ±57.54	8.57 ±3.27	0.077 ±0.033	125.04 ±61.16	7.19 ±2.06	0.058 ±0.032	151.12 ±73.87
Donors									
ABCB1 Ex-26CC (n=10)	7.88 ±3.50	0.075 ±0.032	112.55 ±43.71	7.41 ±1.82	0.063 ±0.031	176.68 ±190.47	8.13 ±2.03	0.053 ±0.026	245 ±290.46
CT (n=15)	6.88 ±2.61	0.081 ±0.035	103.86 ±75.19	8.26 ±2.55	0.071 ±0.048	162 ±110.2	7.35 ±2.15	0.055 ±0.040	186.63 ±103.45
TT (n=7)	9.77 ±4.18	0.101 ±0.060	111.24 ±43.88	9.21 ±5.12	0.059 ±0.034	220.2 ±241.38	6.90 ±2.84	0.039 ±0.0235	288.75 ±307.81
CT+TT(n=22)	7.80 ±3.38	0.087 ±0.045	106.21 ±65.82	8.56 ±3.47	0.067 ±0.043	180.53 ±159.93	7.21 ±2.33	0.05 ±0.036	218.49 ±191
Recipients									
ABCB1 Ex-26CC (n=5)	6.72 ±2.86	0.079 ±0.030	93.29 ±57.54	8.57 ±3.27	0.077 ±0.033	125.04 ±61.16	7.19 ±2.06	0.058 ±0.032	151.12 ±73.87
CT (n=19)	8.07 ±3.21	0.078 ±0.035	121.54 ±64.38	8.47 ±3.28	0.063 ±0.045	216.46 ±205.76	8.17 ±2.34	0.051 ±0.038	274.95 ±274.51
TT (n=8)	7.93 ±4.22	0.099 ±0.059	85.78 ±41.53	7.33 ±2.60	0.065 ±0.029	125.06 ±56.28	6.09 ±1.53	0.046 ±0.022	159.63 ±78.64
CT+TT(n=27)	8.03 ±3.46	0.084 ±0.043	110.95 ±60.09	8.13 ±3.09	0.064 ±0.041	189.38 ±178.81	7.55 ±2.32	0.05 ±0.03	240.78 ±238.14

* statistically significant values ($P<0.05$, Mann-Whitney U-test).

the *3 allele and this in absence of significant variations of tacrolimus C_0 levels between the two groups. A similar, but statistically non-significant, trend was observed also one month after transplantation (Table 2); it can be seen that at that time the tacrolimus C_0 levels were substantially lower in the patients receiving livers with *1/*3 (6.51±2.17 ng/mL) rather than with *3/*3 (8.07±3.52 ng/mL), thus possibly hindering a stronger effect of the donors' genotype on tacrolimus dose requirements.

Also for the recipients' genotypes, the presence of at least one *1 copy tended to increase tacrolimus doses and to lower dose-adjusted C_0, though the differences with respect to the *3/*3 genotypes were not statistically significant in any of the time intervals considered (Table 2). The data of Table 2 also show that there were no significant influences of the *ABCB1* 2677G>T or 3435C>T SNPs, present either in the donors' or in the recipients' genotypes, on tacrolimus dosing requirements during the first six months after transplantation.

This was seen also when the possible effects of the same *ABCB1* SNPs were analysed taking into account the presence or not of the *1 CYP3A5 allele in the donors' or in the recipients' genotypes (not shown).

Finally, also according to previous reports [30–34], tacrolimus dosing requirements were not different according to the sex of either the donors or of the recipients (not shown). It is debated whether co-exposure to steroids may influence tacrolimus clearance at CYP3A level [35–37]; however, in this series we did not find any significant differences in the tacrolimus dosages needed by the patients receiving steroids with respect to those steroid-free (analysis not shown), also when the different genotypes of the recipients and of the donors were considered. This may depend on the fact that steroids, according to the patient's clinical condition and in the absence of rejection, were rapidly tapered and then discontinued during the first month after transplantation [5,33].

Discussion

Analysis of *CYP3A5* and *ABCB1* SNPs revealed a high overall percentage of homozygosity for the CYP3A5*3 variant (6986A>G at intron 3) in the present population composed entirely of Caucasian subjects. This result is in good agreement with other reports which have indicated that up to 90% of Caucasians have such genotype [10,38–40]; in other races, in particular in Africans or African-Americans, the percentage of subjects with the *3/*3 genotype is much lower (about 30%) [10,38,40,41]. As for the *ABCB1* SNP 2677G>T at exon 21, we observed a total frequency of the allelic variant T (12.5%) lower than that (40–50%) observed in other Caucasian populations [28,42–44] and more similar to that (13%) of African-American individuals [27,29,44]. This finding might perhaps represent a peculiarity of our population mainly made up of Sicilians; however the difference in the frequencies of T between Sicilians (9%, n=50) and non-Sicilians (25%, n=14) was not statistically significant.

As for the *ABCB1* 3435C>T SNP at exon 26, the overall frequency of the allelic variant T was 50%, in accordance with other studies which have reported percentages in Caucasians between 33% and 65% [42,44,45]. In our population there was not the linkage between the 2677G>T and 3435C>T SNPs suggested by other authors [21,44,46].

Considering the relationship between genotypes and tacrolimus dose requirements, it has been shown that subjects homozygous for the *3 variant do not express functional CYP3A5 and exhibit lower clearance of the drug [11,29,47,48]. For this reason, they require lower doses of tacrolimus to reach target blood levels compared with the subjects with at least one *1 allele (expressors). Results of this kind have been found checking the genotypes of kidney, heart and lung transplanted patients [13,49–51]. As for liver transplants, our data confirm those of other authors who have indicated the main importance of the liver donors' genotype with regard to the expression of CYP3A5 [52]. They trend to suggest also some influence of the recipients' genotype, implying the possible role that CYP3A5 may play also in extrahepatic tissues like the intestinal wall, as others have reported [53].

With regard to *ABCB1* SNPs, previous studies have not produced consistent results: some authors have indicated that the subjects homozygous for the variant allele 3435T at exon 26 present a reduction in intestinal P-gp, with an effect on the bioavailability of P-gp substrates like cyclosporine and tacrolimus [19,21,24,50,54]. Nevertheless, several other studies performed in kidney, lung and heart transplant patients have excluded that *ABCB1* genetic polymorphisms play any relevant role in determining cyclosporine or tacrolimus

pharmacokinetics [14,25,55–59]. Though our present data need to be corroborated in a larger population of patients, they are in agreement with the latter conclusion, since there were no significant influences of the *ABCB1* 2677G>T or 3435C>T SNPs, present in the recipients' or also in the donors' genotypes, on tacrolimus dosing requirements during the first six months after transplantation.

Finally, we observed a very wide variability of the tacrolimus doses required to reach target blood levels even among the patients carrying the same genotype (in particular of *CYP3A5*, whose SNP appeared to be functionally active) and with a high overlap of the values between the wild-type and mutant genotypes. This further underlines that genetic polymorphism is only one of the possible factors which may influence tacrolimus pharmacokinetics [34,60–62].

Conclusions

The results of the present study suggest that pharmacogenetic analysis cannot substitute therapeutic drug monitoring, even though genotyping the CYP3A5*3 polymorphism of the donor can represent a useful tool to evaluate the appropriate initial dosage of tacrolimus in liver transplant patients.

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