

ABCB1 Genotypes Predict Cyclosporine-Related Adverse Events and Kidney Allograft Outcome

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ABSTRACT

Cyclosporine A (CsA) is a substrate of P-glycoprotein, an efflux transporter encoded by the *ABCB1* gene. Compared with carriers of the wild-type gene, carriers of T allelic variants in exons 21 or 26 have reduced P-glycoprotein activity and, secondarily, increased intracellular concentration of CsA; therefore, carriers of T variants might be at increased risk for CsA-related adverse events. We evaluated the associations between *ABCB1* genotypes (in exons 12, 21, and 26) and CsA-related outcomes in 147 renal transplant recipients who were receiving CsA-based immunosuppression and were included in the Mycophenolate Steroids Sparing study. During a median of 65.5 mo follow-up, carriers of T allelic variants in exons 21 or 26 had a three-fold risk for delayed graft function (DGF), a trend to slower recovery of renal function and lower GFR at study end, and significantly higher incidences of new-onset diabetes and cytomegalovirus reactivation compared with carriers of the wild-type genotype. T variants in both exons 21 and 26 were independently associated with 3.8- and 3.5-fold higher risk for DGF, respectively ($P = 0.022$ and $P = 0.034$). The incidence of acute rejection and the mean CsA dose and blood levels were comparable in genotype groups. In conclusion, renal transplant recipients with T allelic variants in *ABCB1* exons 21 or 26 are at increased risk for CsA-related adverse events. Genetic evaluation may help to identify patients at risk and to modulate CsA therapy to optimize graft and patient outcomes.

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The introduction of cyclosporine A (CsA) therapy in the early 1980s opened a new era in organ transplantation. Compared with steroid- and azathioprine-based regimens, immunosuppressive protocols including this inhibitor of calcineurin—a key enzyme involved in T cell activation¹—decreased the incidence of acute rejections from 40% to 50% to 20% to 30% and increased one-year survival rates of the grafts from 60% to between 80% and 90%.² Thirty years later, CsA remains a cornerstone of immunosuppressive therapy for recipients of

both renal and nonrenal transplants worldwide. However, standard recommended doses are associ-

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Table 1. Patient/donor characteristics according to SNPs in the exon 12, 21 and 26 of the *ABCB1* gene

Characteristic	Exon 12		Exon 21 ^a		Exon 26	
	CC (n = 54)	CT/TT (n = 93)	GG (n = 54)	GT/TT (n = 92)	CC (n = 52)	CT/TT (n = 95)
Recipients						
men	33 (61.1)	61 (65.6)	33 (61.1)	61 (66.3)	31 (59.6)	63 (66.3)
age (years)	41.6 (12.3)	45.1 (11.8)	40.6 (12.4)	45.6 (11.5) ^b	39.0 (11.9)	46.5 (11.3) ^c
weight (kg)	65.6 (11.7)	67.2 (11.5)	65.6 (11.9)	67.3 (11.4)	64.7 (12.8)	67.7 (10.7)
height (cm)	168 (9.0)	168.4 (8.9)	167.9 (8.9)	168.4 (9.0)	167.4 (9.2)	168.7 (8.9)
Serology						
CMV positive	37 (68.5)	75 (81.5)	40 (74.4)	71 (78.0)	37 (72.5)	75 (78.9)
hepatitis B positive	4 (7.5)	7 (7.6)	4 (7.5)	7 (7.7)	3 (5.9)	8 (8.5)
hepatitis C positive	5 (9.4)	6 (6.5)	5 (9.4)	6 (6.6)	4 (7.8)	7 (7.4)
Primary disease						
diabetes mellitus	1 (1.8)	0 (0)	1 (1.8)	0 (0)	1 (1.9)	0 (0)
hypertension	2 (3.7)	5 (5.4)	2 (3.7)	5 (5.4)	1 (1.9)	6 (6.3)
glomerulonephritis	23 (45.6)	43 (46.2)	24 (44.4)	42 (45.7)	24 (46.1)	42 (44.2)
polycystic kidney disease	3 (5.6)	10 (10.7)	3 (5.6)	9 (9.8)	3 (5.8)	10 (10.5)
pyelonephritis	9 (16.7)	10 (10.7)	8 (14.8)	11 (12.0)	9 (17.3)	10 (10.5)
other	5 (9.3)	5 (5.4)	5 (9.3)	5 (5.4)	5 (9.6)	5 (5.3)
uncertain	11 (20.4)	20 (21.5)	11 (20.4)	20 (21.7)	9 (17.3)	22 (23.2)
Donors						
men	31 (57.4)	49 (52.7)	35 (64.8)	45 (48.9)	33 (63.5)	47 (49.5)
age (years)	40.0 (16.7)	43.6 (15.0)	37.8 (16.6)	44.8 (14.7) ^c	36.7 (15.7)	45.4 (14.9) ^c
weight (kg)	68.3 (9.8)	69.9 (11.6)	68.3 (9.4)	69.7 (11.8)	67.8 (8.9)	70.2 (11.9)
height (cm)	169.8 (8.4)	168.7 (8.2)	170.3 (8.3)	168.4 (8.3)	169.4 (8.7)	168.9 (8.0)
Serology						
CMV positive	38 (70.4)	62 (66.7)	35 (64.8)	64 (69.6)	35 (67.3)	65 (68.4)
hepatitis B positive	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
hepatitis C positive	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
B-cell crossmatch						
negative	54 (100)	91 (98.9)	54 (100)	90 (98.9)	52 (100)	93 (98.9)
positive	0 (0)	1 (1.1)	0 (0)	1 (1.1)	0 (0)	1 (1.1)
not done	0 (0)	1 (1.1)	0 (0)	0 (0)	0 (0)	0 (0)
HLA mismatches						
0	1 (1.8)	4 (4.3)	1 (1.8)	4 (4.3)	0 (0)	5 (5.3)
1	13 (24.1)	23 (24.7)	11 (20.4)	24 (26.1)	11 (21.1)	25 (26.3)
2	27 (50.0)	45 (48.4)	27 (50.0)	45 (48.9)	28 (53.9)	44 (46.3)
3	13 (24.1)	21 (22.6)	15 (27.8)	19 (20.7)	13 (25.0)	21 (22.1)
Treatment						
mycophenolate mofetil	30 (55.6)	42 (45.2)	31 (57.4)	41 (44.6)	28 (53.9)	44 (46.3)
azathioprine	24 (44.4)	51 (54.8)	23 (42.6)	51 (55.4)	24 (46.1)	51 (53.7)
Cold ischemia (h)	18.4 (6.9)	17.5 (7.3)	18.1 (6.9)	17.7 (7.3)	18.1 (7.0)	17.7 (7.2)
Warm ischemia (min)	40.6 (16.9)	40.7 (16.7)	40.4 (16.7)	40.8 (16.9)	41.9 (17.0)	39.8 (16.6)

Data are shown as number (percentage), except for ischemia time, which is mean (SE).

^aOne patient was not characterized for exon 21.

^b $P < 0.05$, ^c $P < 0.01$ vs GG (exon 21) or CC (exon 26) genotype.

ated with nephrotoxicity, resulting in delayed graft function (DGF) and progressive renal function deterioration in the long term.^{3,4} Moreover, CsA worsens glucose tolerance and lipid profile; increases systemic BP; and, similarly to other immunosuppressants, enhances the risk of opportunistic infections, lymphoproliferative disorders, and cancer.^{1,5}

To minimize side effects without increasing the risk of rejection, treatment is titrated to target CsA blood levels according to well established guidelines.⁶ However, the therapeutic index remains narrow, whereas the frequency and severity of

CsA-related adverse effects are considerably variable among patients, even at comparable CsA levels.^{1,6} This suggests the possibility that a heterogeneous individual susceptibility may result in increased risk in some patients despite exposure to CsA levels that in the majority of cases are devoid of significant toxicity.⁶ Thus, identifying markers or predictors of individual response to CsA therapy might help to tailor CsA therapy and optimize the risk/benefit profile of CsA-based immunosuppression.

Drug efficacy and tolerability are influenced by several fac-

Table 2. Patients with events according to immunosuppressive regimen concomitant with cyclosporine A

	Overall			Patients who completed steroid withdrawal			Patients who did not complete steroid withdrawal		
	Azathioprine (n = 75)	MMF (n = 72)	P	Azathioprine (n = 50)	MMF (n = 47)	P	Azathioprine (n = 25)	MMF (n = 25)	P
Delayed graft function	17 (22.7)	16 (22.2)	0.95	12 (24.0)	9 (19.2)	0.56	5 (20.0)	7 (28.0)	0.51
Proteinuria	19 (25.3)	19 (26.4)	0.88	14 (28.0)	11 (23.4)	0.60	5 (20.0)	8 (32.0)	0.33
Diabetes	14 (18.7)	10 (13.9)	0.43	11 (22.0)	4 (8.5)	0.07	3 (12.0)	6 (24.0)	0.46
Rejection	43 (57.3)	30 (41.7)	0.06	27 (54.0)	17 (36.2)	0.08	16 (64.0)	13 (52.0)	0.39
Graft loss	3 (4.0)	3 (4.2)	0.99	1 (2.0)	0 (0)	0.99	2 (8.0)	3 (12.0)	0.99
Cardio/cerebro-vascular ^a	11 (14.5)	14 (19.4)	0.44	8 (16.0)	8 (17.0)	0.89	3 (12.0)	6 (24.0)	0.46
Infections ^a	54 (72.0)	43 (59.7)	0.12	37 (74.0)	27 (57.4)	0.09	17 (68.0)	16 (64.0)	0.77
cytomegalovirus	27 (36.0)	23 (31.9)	0.60	20 (40.0)	15 (31.9)	0.41	7 (28.0)	8 (32.0)	0.76
urinary tract infection	23 (30.7)	26 (36.1)	0.49	18 (36.0)	16 (34.0)	0.84	5 (20.0)	10 (40.0)	0.12
other causes	38 (50.7)	29 (40.2)	0.21	24 (48.0)	16 (34.0)	0.16	14 (56.0)	13 (52.0)	0.78
Cancers ^a	9 (12.0)	5 (6.9)	0.30	7 (14.0)	2 (4.3)	0.16	2 (8.0)	3 (12.0)	0.99

Data are n (%). MMF, mycophenolate mofetil.

^aSome patients have recorded >1 event.

Table 3. Patients with events according to different genotypes in the exons 12, 21, and 26 of *ABCB1*

Event	Exon 12		Exon 21 ^a		Exon 26	
	CC (n = 54)	CT/TT (n = 93)	GG (n = 54)	GT/TT (n = 92)	CC (n = 52)	CT/TT (n = 95)
Delayed graft function	9 (17%)	24 (26%)	6 (11%)	27 (29%) ^e	6 (11%)	27 (28%) ^e
Proteinuria	14 (26%)	24 (26%)	14 (26%)	24 (26%)	10 (21%)	28 (29%)
Diabetes	7 (13%)	17 (18%)	7 (13%)	17 (18%)	4 (8%)	20 (21%) ^e
Rejection	30 (56%)	43 (46%)	29 (54%)	43 (47%)	27 (52%)	46 (48%)
Time of the onset of AR (days)						
median (IQ)	24 (10–330)	30 (16–282)	23 (10–330)	30 (17–282)	22 (10–288)	33 (15–308)
Graft loss	2 (4%)	4 (4%)	1 (2%)	5 (5%)	1 (2%)	5 (5%)
Cardio/cerebrovascular ^b	9 (17%)	16 (17%)	7 (13%)	18 (20%)	8 (15%)	17 (18%)
CMV disease	13 (24%)	37 (40%)	9 (17%)	40 (43%) ^f	11 (21%)	39 (41%) ^e
ganciclovir-treated	13 (24%)	33 (35%)	9 (17%)	36 (39%) ^f	11 (21%)	35 (37%)
primary CMV infection ^c	6 (11%)	5 (5%)	3 (6%)	8 (9%)	4 (8%)	7 (7%)
reactivation ^d	6 (11%)	32 (34%) ^f	5 (9%)	32 (35%) ^f	6 (11%)	32 (34%) ^f
Other infections ^b						
urinary tract	16 (30%)	35 (38%)	16 (30%)	35 (38%)	18 (35%)	33 (35%)
viral	5 (9%)	10 (11%)	7 (13%)	8 (9%)	6 (11%)	9 (9%)
pneumonias	7 (13%)	9 (10%)	5 (9%)	11 (12%)	6 (11%)	10 (10%)
other	7 (13%)	27 (29%) ^e	6 (11%)	28 (30%) ^f	6 (11%)	28 (29%) ^e
Cancer ^b	2 (4%)	12 (13%)	2 (4%)	12 (13%)	2 (4%)	12 (13%)

AR, acute rejection; IQ, interquartile range; CMV, cytomegalovirus.

^aOne patient was not characterized for exon 21.

^bSome patients with more than one event.

^cPrimary CMV infection was defined by the presence of viral antigenemia in recipients without evidence of previous viral exposure (negative serology) at transplantation who received a graft from a seropositive donor.

^dCMV reactivation was defined by the presence of viral antigenemia in recipients with evidence of previous viral exposure (positive serology) at transplantation.

^eP < 0.05, ^fP < 0.01 versus GG (exon 21) or CC (exon 26) genotype.

tors, including the activity of proteins and enzymes involved in drug transport and metabolism.⁷ CsA is a substrate of an efflux transporter—the P-glycoprotein (P-gp) encoded by the multidrug resistance-1 gene (now referred as *ABCB1*)—which actively transports lipophilic drugs and other xenobiotics from the intracellular to the extracellular domain.⁸ This transporter is expressed in lymphocytes,⁹ and in other leukocytes,¹⁰ as well as in hepatocytes and on the brush border of enterocytes and proximal tubular cells.⁸ Reduced expression or functional inhibition of this efflux-pump invariably results in increased in-

tracellular and tissue drug concentrations,^{8,9} but may have unpredictable effects on CsA blood levels. Indeed, CsA blood levels increased, decreased, or did not change in different settings, likely because of different balances between enhanced distribution into the tissue compartment and decreased excretion into the gastrointestinal lumen or urinary tract.⁷ Increased CsA concentrations in lymphocytes,¹¹ polymorphonuclear cells,¹² and other circulating leukocytes¹³ have been associated with increased production and release of reactive oxygen species (ROS). ROS production by leukocytes is the primary de-

Table 4. Univariate logistic regressions using DGF as the dependent variable

Univariate Analyses	Estimate	Standard Error	Odds Ratio	CI	P
Cold ischemia time	0.0015	0.0001	1.002	1.000–1.003	0.0037
Age recipient	0.0276	0.0172	1.028	0.994–1.063	0.1085
Weight recipient	0.0222	0.0172	1.022	0.989–1.057	0.1948
Sex recipient	−0.0771	0.2086	0.857	0.378–1.942	0.7118
Age donor	0.0371	0.0140	1.038	1.010–1.067	0.0079
Weight donor	−0.0221	0.0187	0.978	0.943–1.015	0.2369
Sex donor	0.1537	0.1981	1.360	0.626–2.956	0.4377
MMF or azathioprine	0.0128	0.1977	1.026	0.473–2.227	0.9485
B-cell crossmatch	7.7594	623.6	Not estimable	-	0.9901
Primary disease					0.8117
diabete mellitus vs uncertain	−8.2308	176.2	Not estimable	-	0.9627
hypertension vs uncertain	1.5041	29.3718	1.371	0.217–8.664	0.9592
glomerulonephritis vs uncertain	0.9163	29.3645	0.762	0.267–2.175	0.9751
polycystic kidney disease vs uncertain	1.9504	29.3672	2.143	0.529–8.681	0.9470
pyelonephritis vs uncertain	1.0986	29.3671	0.914	0.228–3.662	0.9702
other vs uncertain	1.5731	29.3691	1.469	0.299–7.228	0.9573
HLA mismatches					0.9103
1 vs 0	−0.0351	1.1947	0.966	0.093–10.039	0.9766
2 vs 0	0.2877	1.1507	1.333	0.140–12.717	0.8026
3 vs 0	0.0364	1.1958	1.037	0.100–10.805	0.9757

CI, 95% confidence interval.

Table 5. Multivariate logistic regressions using DGF as the dependent variable

Multivariate Analyses	Estimate	Standard Error	Odds Ratio	CI	P
Exon 12					
CC vs CT/TT	0.7633	0.5255	2.145	0.766–6.010	0.1464
cold ischemia time	0.0002	0.0001	1.002	1.001–1.003	0.0028
age donor	0.0416	0.0172	1.042	1.008–1.078	0.0157
Exon 21					
GG vs GT/TT	1.3307	0.5836	3.784	1.205–11.876	0.0226
cold ischemia time	0.0017	0.0006	1.002	1.001–1.003	0.0026
age donor	0.0375	0.0174	1.038	1.003–1.074	0.0314
Exon 26					
CC vs CT/TT	1.2320	0.5841	3.428	1.091–10.771	0.0349
cold ischemia time	0.0017	0.0006	1.002	1.001–1.003	0.0026
age donor	0.0378	0.0174	1.039	1.004–1.074	0.0293

CI, 95% confidence interval.

fense against invading micro-organisms, but has also been involved in the pathogenesis of ischemia-reperfusion damage of engrafted tissues or organs.^{3,14} Thus, increased intracellular CsA disposition with enhanced ROS production might amplify oxidative stress and tissue damage to the graft after reperfusion.¹⁵ Increased intralymphocyte drug concentration is also associated with more effective inhibition of lymphocyte proliferation by CsA *in vitro*,¹⁶ an effect that, *in vivo*, might translate into more effective protection against graft rejection,¹⁷ but also into excess risk of opportunistic infections, lymphoproliferative disorders, or cancer.

P-glycoprotein expression and activity are reduced in carriers of one or two T allelic variants in exons 12, 21, and 26 as compared with carriers of the wild-type *ABCB1* gene.^{8,18,19} Thus, at comparable CsA exposure, carriers of the allelic variants are expected to have higher intracellular and tissue CsA

levels than wild-type carriers and, conceivably, should be exposed to more, and more severe, CsA-related events. We formally tested this hypothesis in a large cohort of renal transplant recipients prospectively monitored in the setting of a randomized, controlled, clinical trial, the Mycophenolate Steroids Sparing (MYSS) study, which aimed to compare the risk/benefit profile of mycophenolate mofetil and azathioprine therapy in immunosuppressive regimens including the CsA micro-emulsion Neoral.²⁰

RESULTS

Donor and Recipient Characteristics at Inclusion

One hundred forty-seven of the 336 MYSS patients consented to enter the present study. Gender distribution and weight of

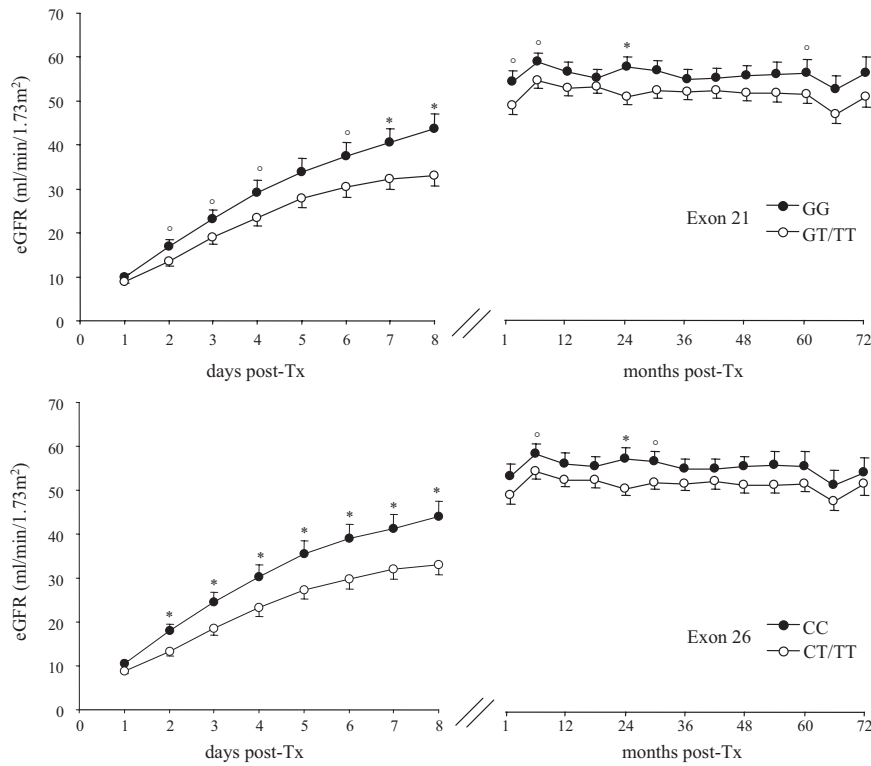


Figure 1. Time-course of estimated GFR in 147 kidney transplant recipients during the first 8 d post-transplant (left panels) and up to the last available follow-up (right panels), divided according to *ABCB1* genotypes in exon 21 (upper panels) and exon 26 (lower panels). * $P < 0.01$ and ° $P < 0.05$ versus GT/TT (exon 21) or CT/TT (exon 26).

recipients and corresponding donors at the time of transplantation in different considered *ABCB1* genotype groups were similar, as was the distribution of primary renal diseases in recipients, the percentage of B cell negative cross-matches, the number of HLA mismatches, allocation to mycophenolate mofetil or azathioprine treatment, and cold/warm ischemia times (Table 1). However, recipients carrying the allelic “T” variants in exons 21 and 26 happened to be significantly older and to receive the graft from older donors compared with those with the wild-type genotypes, whereas no differences were observed between the exon 12 genotypes (Table 1). Baseline characteristics of patients considered in the present study were similar to those observed in the general population of patients included in the MYSS trial. Moreover, all of the considered safety and efficacy outcome variables were independent of patient randomization to MMF or azathioprine therapy, as well as of steroid discontinuation (Table 2).

Thirty-seven percent of the patients had the wild-type *ABCB1* genotype in exon 12 or 21 and 36% in exon 26. The other patients carried at least one allelic variant. These frequencies were in agreement with those reported in the literature.^{8,21,22} All of the single nucleotide polymorphisms (SNPs) in the *ABCB1* gene were in linkage disequilibrium ($r^2 \geq 0.44$). No deviation from the expected proportions of *ABCB1* genotypes predicted by the Hardy-Weinberg equilibrium was detected.

ABCB1 Genotypes and Frequency of DGF

The incidence of DGF was almost three-fold higher in carriers of the T variant in exon 21 and 26 of the *ABCB1* gene compared with those with the wild-type genotype, and the difference between genotype groups was statistically significant (Table 3). Conversely, there was no significant association between exon 12 genotype and incidence of DGF. Mean CsA C_0 values during the first week after transplantation in patients with or without delayed graft function (262 ± 61 versus 273 ± 119 ng/ml, respectively; $P = 0.92$), as well as in those with the wild-type or mutated *ABCB1* in exon 21 (274 ± 103 versus 276 ± 86 ng/ml, respectively; $P = 0.94$) or in exon 26 (263 ± 85 versus 280 ± 96 ng/ml, respectively; $P = 0.60$) were similar. C_2 levels over the same observation period were also similar between those with or without DGF (1120 ± 319 versus 1345 ± 433 ng/ml respectively, $P = 0.22$). Multiple logistic regressions considering donor and recipient variables that were significantly associated with DGF at univariate analyses showed that the presence of one or two T alleles in exon 21 (odds ratio [OR] and 95% confidence interval [CI]: 3.8 [1.2 to 11.9], $P = 0.022$) or 26 (3.4 [1.1 to 10.8], $P = 0.034$) significantly predicted the risk of DGF, in addition to cold ischemia time and donor age (Tables 4 and 5).

ABCB1 Genotypes and Post-Transplant Estimated GFR

At each considered time point after transplantation up to study end, estimated GFR (eGFR) was lower in carriers of the allelic

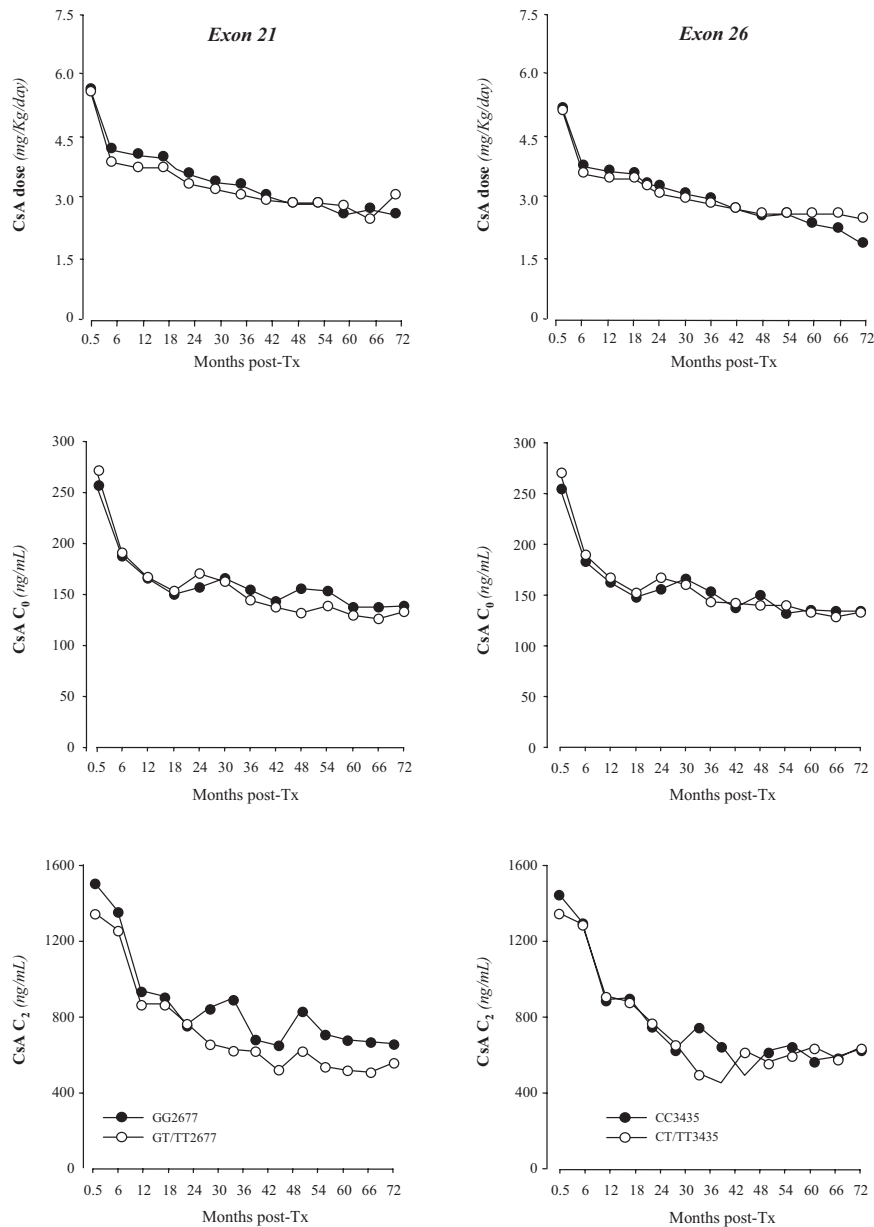


Figure 2. Median daily cyclosporine A (CsA) C₀ doses and C₂ blood CsA concentrations at different time points throughout the study period according to different genotypes in exon 21 and 26 of the *ABCB1* gene.

T variants in exon 21 or 26 than in carriers of the *ABCB1* wild genotype (Figure 1), whereas no differences were observed in eGFRs among different exon 12 genotypes (data not shown). During the first week after transplantation, there was a trend to slower eGFR increase in carriers of the T allelic variants compared with carriers of the wild-type genotype in exon 21 (Δ eGFR: 3.7 ± 0.3 ml/min per day versus 5.1 ± 0.4 ml/min per day, respectively; $P = 0.07$) as well as in exon 26 (Δ eGFR: 3.7 ± 0.3 ml/min per day versus 5.1 ± 0.4 ml/min per day, respectively, $P = 0.08$). At final visits carriers of the T variants tended also to have lower eGFRs than carriers of the wild-type genotype in exon 21 (eGFR: 54.8 ± 15.5 ml/min/1.73 m² versus

49.4 ± 16.3 ml/min/1.73 m², respectively; $P = 0.05$) and in exon 26 (54.3 ± 16.5 ml/min/1.73 m² versus 49.8 ± 15.7 ml/min/1.73 m², respectively; $P = 0.11$).

Mean CsA C₀ and C₂ values and daily drug doses during the whole follow-up period in patients with mutated or wild-type *ABCB1* in exon 21 or exon 26 were similar (Figure 2).

Other Safety Outcomes

Over a median follow-up of 65.5 months (interquartile range 52.7 to 73.6 months), incidence of new onset diabetes was significantly higher in carriers of T variants in exon 26 than in wild-type recipients (Table 3). Carriers of T variants in both

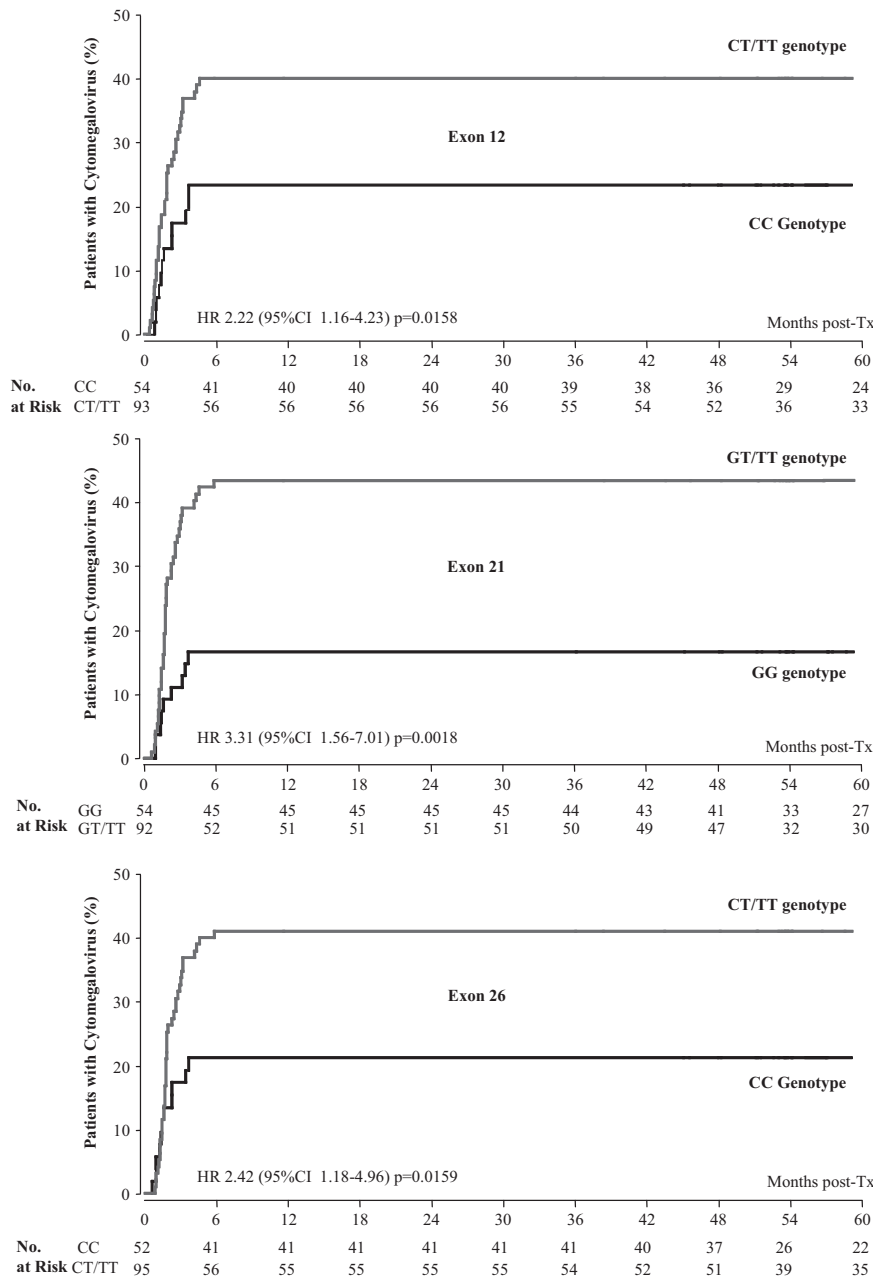


Figure 3. Kaplan-Meier survival curve showing the probability to experience cytomegalovirus antigenemia after transplantation according to different genotypes in exon 21 (upper panel) and exon 26 (lower panels) of the *ABCB1* gene in 147 kidney transplant recipients treated with cyclosporine. HR, hazard ratio; CI, confidence interval.

exons also had a significantly higher incidence of infections compared with wild-type recipients (Table 3). The difference was largely explained by a two- to three-fold excess in CMV reactivations in mutated compared with wild-type recipients (Table 3 and Figure 3). The excess risk was significant even after adjustment for predefined baseline covariates, including CMV serostatus at the time of transplant and CsA C₀ and C₂ levels. Incidence of neoplasms followed a similar trend, but numbers were small and differences between genotypes were nonsignificant.

Efficacy Outcomes

Carriers of the allelic T variants in exons 12, 21, or 26 showed a similar incidence of rejection episodes and time to the onset of the event compared with the carriers of the wild-type *ABCB1* gene (Table 3). Similarly, no significant differences in the frequency of persistent proteinuria and cardiovascular events were observed between *ABCB1* genotypes (Table 3). The number of graft losses (*n* = 6) was too limited for meaningful comparisons. The frequency of patients completing steroid withdrawal was comparable between T variant or wild-type carriers

Table 6. Patients on concomitant medications at the time of transplantation and at last visit

Concomitant medication	Baseline						Final visit					
	Exon 12		Exon 21 ^a		Exon 26		Exon 12		Exon 21 ^a		Exon 26	
	CC (n = 54)	CT/TT (n = 93)	GG (n = 54)	GT/TT (n = 92)	CC (n = 52)	CT/TT (n = 95)	CC (n = 54)	CT/TT (n = 93)	GG (n = 54)	GT/T (n = 92)	CC (n = 52)	CT/TT (n = 95)
Antihypertensive agents												
any	38 (70.4)	60 (64.5)	35 (64.8)	62 (67.4)	34 (65.4)	64 (67.4)	53 (98.1)	93 (100)	53 (98.2)	92 (100)	51 (98.1)	95 (100)
diuretic	25 (46.3)	39 (41.9)	26 (48.2)	38 (41.3)	23 (44.2)	41 (43.2)	38 (70.4)	68 (73.1)	36 (66.7)	70 (76.1)	33 (63.5)	73 (76.8)
beta blocker	1 (1.8)	2 (2.1)	0	3 (3.3)	0	3 (3.2)	24 (44.4)	50 (53.8)	25 (46.3)	48 (52.2)	25 (48.1)	49 (51.6)
calcium channel blocker	22 (40.7)	30 (32.3)	18 (33.3)	33 (35.9)	20 (38.5)	32 (33.7)	53 (98.1)	85 (91.4)	53 (98.2)	84 (91.3)	51 (98.1)	87 (91.6)
Nifedipine	17 (31.5)	18 (19.4)	14 (25.9)	20 (21.7)	15 (28.8)	20 (21.0)	46 (85.2)	65 (69.9) ^b	45 (83.3)	65 (70.6)	43 (82.7)	68 (71.6)
Diltiazem	5 (9.3)	9 (9.7)	4 (7.4)	10 (10.9)	4 (7.7)	10 (10.5)	5 (9.3)	11 (11.8)	4 (7.4)	12 (13.0)	5 (9.6)	11 (11.6)
Verapamil	0	0	0	0	0	0	1 (1.8)	0	0	1 (1.1)	0	1 (1.0)
Amlodipine	0	3 (3.2)	0	3 (3.3)	1 (1.9)	2 (2.1)	13 (24.1)	27 (29.0)	15 (27.8)	25 (27.2)	14 (26.9)	26 (27.4)
Felodipine	0	0	0	0	0	0	3 (5.6)	3 (3.2)	4 (7.4)	2 (2.2)	4 (7.7)	2 (2.1)
Lacidipine	0	0	0	0	0	0	0	1 (1.1)	0	1 (1.1)	0	1 (1.0)
Sympatholytic agent	5 (9.3)	10 (10.7)	5 (9.3)	10 (10.9)	3 (5.8)	12 (12.6)	19 (35.2)	43 (46.2)	17 (31.5)	44 (47.8)	16 (30.8)	46 (48.4) ^b
ACE inhibitor/ARBs	0	0	0	0	0	0	10 (18.5)	16 (17.2)	9 (16.7)	16 (17.4)	8 (15.4)	18 (19.0)
Lipid-lowering agents	0	0	0	0	0	0	10 (18.5)	19 (20.4)	11 (20.4)	18 (19.6)	11 (21.2)	18 (19.0)
Antiplatelet agent	15 (27.8)	28 (30.1)	15 (27.8)	27 (29.4)	15 (28.9)	28 (29.5)	38 (70.4)	64 (68.8)	37 (68.5)	64 (69.6)	35 (67.3)	67 (70.5)

Data are shown as number (percentage). ACE, angiotensin converting enzyme; ARB, angiotensin II receptor blockers.

^aOne patient was not characterized for exon 21.

^bP < 0.05 vs the CC/GG genotype.

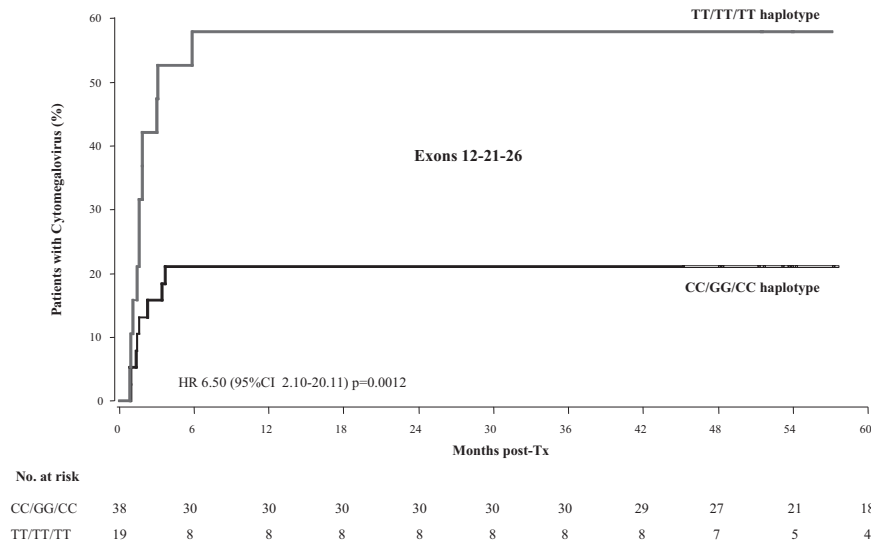


Figure 4. Kaplan-Meier survival curve showing the probability to experience cytomegalovirus antigenemia after transplantation according to TT-TT-TT or CC-GG-CC haplotypes of the ABCB1 gene. HR, hazard ratio; CI, confidence interval.

in exon 21 (67% versus 65%, respectively; P = 0.86) or in exon 26 (62% versus 68%, respectively; P = 0.40).

Concomitant Antihypertensive and Lipid-Lowering Therapy

At baseline evaluation, the proportion of patients on different concomitant medications was similar across genotype groups (Table 6). Conversely, at final visit, a trend to increased need for antihypertensive treatment with sympatholytic drugs was observed in patients with the T variants.

Haplotype Analyses

Haplotype analyses comparing CC-GG-CC (n = 38) and TT-TT-TT (n = 19) groups showed that the “T” haplotype was associated with a 6.5-fold excess CMV infection compared with fully wild-type genotype (Figure 4). The excess risk was significant even after adjustment for predefined baseline covariates. Incidence of DGF followed a similar trend (26.3% versus 10.5%), but the number of events was small and the differences between the two haplotypes failed to achieve the statistical significance (P = 0.201).

DISCUSSION

Here we found that in renal transplant recipients who received CsA-based maintenance immunosuppressive therapy, the allelic T variants of exons 21 and 26 of the *ABCB1* gene predicted increased incidence of DGF, slower post-transplant renal function recovery, lower final eGFRs, a significant increase in new cases of diabetes, CMV infections, and a trend to more neoplasms. These findings were not explained by different CsA exposure in different genotype groups, as the CsA dose and blood levels throughout the whole observation period were similar in carriers of the T variant or wild-type *ABCB1* gene. The older age of donors of the T allele carriers did not explain the observed associations between *ABCB1* genotypes and DGF or other considered outcomes, because at multiregression analyses different genotypes in exons 21 and 26 retained their predictive value even after adjustments for this potential confounding factor. This is consistent with evidence that age has an appreciable effect on the rate of renal function recovery among recipients of grafts from donors aged more than 50 to 55 yr.^{23,24} On the same line, a recent analysis from the USRDS registry involving more than 64,000 renal transplant recipients found that the risk of opportunistic infections increased only among recipients who were more than 50 yr old, whereas there was no relationship between outcome and age among recipients 18 to 50 yr old.³⁶ That the large majority of donors and recipients in the present study were younger than 50 yr at transplantation provides additional evidence that age did not affect the study findings to any appreciable extent. All considered outcomes were also independent of patient randomization to mycophenolate mofetil or azathioprine therapy (see Table 2).

We suggest that delayed and impaired kidney function recovery in carriers of T allelic variants in *ABCB1* exons 21 or 26 might be the consequence of a more severe reperfusion injury. Conceivably, oxidative stress in grafts given to carriers of the T allelic variants could have been amplified by ROS generated and released in increased amounts by infiltrating lymphocytes, polymorphonuclear cells, and other host leukocytes.^{11–13} This effect might be sustained by increased intracellular CsA concentration secondary to depressed CsA efflux *via* P-glycoprotein transport in this population.^{9,10} Although this is consistent with findings that both acute and chronic CsA nephrotoxicity

are associated with oxidative stress and can be limited by free radical scavengers acting to block ROS,^{25–26} *ad hoc* studies using mass spectrometry technology to measure intracellular CsA concentration are needed to specifically address this working hypothesis.

Increased intracellular CsA concentration is also associated with excess inhibition of lymphocyte function *in vivo*,¹⁷ which is likely to underlie the significant increase in infections and CMV reactivations as well as the six-fold increase in neoplasms we observed during the study period in T variant compared with wild-type carriers. Differences in the incidence of neoplasms, however, were only marginally significant, most probably because the small number of events reduced the statistical power of comparative analyses. Whether the excess incidence of diabetes in carriers of the T allelic variant may reflect more severe CsA toxicity to the pancreatic β cells²⁷ or, rather, increased tissue resistance to insulin²⁸ is matter of investigation.

In our population the excess risk observed in carriers of the T alleles largely exceeded the potential benefits. This finding can be taken to suggest that CsA therapy might be optimized in this population by titrating CsA dose to lower than the usually recommended target blood levels. Further investigation is needed to address this hypothesis. Ideally, treatment should be titrated to intracellular CsA levels.¹⁷ More pragmatically, CsA dose could be titrated by considering the *ABCB1* genotype. Because delayed or partial renal function recovery after kidney transplantation strongly predict decreased graft survival in the long term,^{3,29} this approach might help to improve long-term allograft survival in patients at increased risk of CsA nephrotoxicity because of their genotype. Again, prospective *ad hoc* designed trials are needed to address whether analysis of the *ABCB1* genotype may serve to guide CsA therapy to optimize renal graft outcomes.

All previous studies evaluating the relationships between *ABCB1* genotypes and graft outcomes were observational and retrospective,^{30,31} and the results were often confounded by heterogeneity in patient characteristics and follow-up duration, and by changes in monitoring and treatment strategies introduced during the observation period.³¹ Here data were prospectively retrieved over a long follow-up period in the setting of a randomized, controlled trial with standardized treatments and predefined serial evaluations of patient and graft

Table 7. Primer sequences and analytical conditions for *ABCB1* SNPs detection by dHPLC

Primer Name	Sequence (5' to 3')	Product Size (bp)	Temperature (°C)	Gradient (%B)
<i>ABCB1</i> exon 12	(F) AGTCAGTTCCTATATCCTGTGTCTGTGA	251	60.6	54.1–64.1
	(R) GCAGTCACATTGCACATCTTTCT			
<i>ABCB1</i> exon 21	(F) ATAGGTCCAGGCTTGCTGTAATT	279	54.6	56.1–66.1
	(R) AATCAGTGTTATTTTGTACTCCCTACTG			
<i>ABCB1</i> exon 26	(F) GACTGCAGCATTGCTGAGAACA	221	60.8	52.8–62.8
	(R) GGTCTCCCAGAAGTGAAGAGAAATT			

dHPLC analysis was performed using the WAVE Systems (WAVE DNA Fragment Analysis System, model MD4000plus; Transgenomics, Cedex, France), equipped with a DNA-Sep column (Transgenomics). Solvent gradient was obtained with buffer A 100 mM triethylammonium acetate pH 7.0 (TEAA, Transgenomics) and buffer B 100 mM TEAA pH 7.0 and 25% acetonitrile pumped at a flow rate of 1.5 ml/min. UV detector was set at 260 nm. SNP, single nucleotide polymorphism. F, forward; R, reverse; bp, base pair.

outcomes. A limitation is that we did not evaluate the *ABCB1* genotype in kidney donors of our present patients. On the basis of recent evidence, donor *ABCB1* genotype may also predict chronic CsA nephrotoxicity on the graft.³² Indeed, P-glycoprotein is also localized on the brush border of proximal tubular cells, and its reduced activity in grafts from donors with the T allelic variants would result in increased intracellular CsA concentration and toxicity. Thus, different interactions between donor and recipient genotypes might also affect graft susceptibility to CsA nephrotoxicity. Moreover, *ad hoc* prospective studies are needed to definitively establish the predictive role of *ABCB1* genotype in kidney transplantation.

In conclusion, findings that mutant alleles for exons 21 or 26 of the *ABCB1* gene predict increased susceptibility to CsA-related adverse effects can be taken to suggest that pretransplant genetic evaluation to identify subjects at risk might serve to modulate CsA therapy in this population. Whether our present findings might apply also to other calcineurin inhibitors is worth investigating.

CONCISE METHODS

Study Design

This prespecified analysis included renal transplant recipients randomized into the MYSS study who had provided a written informed consent to the evaluation of *ABCB1* gene polymorphisms in exons 12, 21, and 26. MYSS was a prospective, randomized, clinical trial aimed to evaluate whether mycophenolate mofetil offers advantages over azathioprine in the opportunity to reduce or stop steroids in patients receiving maintenance immunosuppressive therapy with the CsA microemulsion Neoral.²⁰ At completion of the first 6-mo treatment period, steroid dosage was progressively tapered and eventually discontinued and patients were then followed for up to month 72 post-transplantation, with clinical and laboratory evaluations every 6 mo.^{20,33} Genetic data and any other relevant information were retrieved from the study population without interfering with patient management and were handled according to standard regulations for data registration, use, and preservation of patient anonymity and privacy. The study protocol was approved by the Ethical Committee of the Coordinating Center and of all of the Transplant Centers involved in the study.

Outcomes

The main outcome variable was the incidence of DGF, defined as the need for dialysis therapy within the first week post-transplantation in patients without evidence of renal artery or vein thrombosis, urinary tract obstruction, or acute/hyperacute biopsy-proven graft rejection. Additional outcomes were the rate of renal function recovery over the first week post transplant; achieved GFR on follow-up; new-onset proteinuria or diabetes; incidence of cardiovascular events, infections, CMV reactivations, or neoplasms; and freedom from acute allograft rejection before and after steroid withdrawal.

The GFR was estimated by using the abbreviated Modification of Diet in Renal Disease formula based on gender, age, and serum cre-

atinine values.³⁴ Diabetes was diagnosed by World Health Organization criteria.

Previous exposure of recipients and corresponding donors to CMV infection was assessed by evaluating CMV serology at the time of transplantation. Primary CMV infection was diagnosed in recipients with evidence of viral antigens in their peripheral blood leukocytes who had no evidence (negative serology) for previous CMV exposure. CMV reactivation was diagnosed when viral antigenemia was detected in recipients with evidence (positive serology) of previous exposure to the virus. CMV disease was diagnosed when CMV antigenemia was associated with at least one clinical or laboratory sign such as neutropenia, leukopenia, or pneumonia-like symptoms (*i.e.*, fever, cough, or dyspnea).³⁵

Acute rejection episodes were diagnosed when patients presented with three or more of the following: temperature 38 °C or higher without obvious signs of infection, graft swelling or tenderness, a rise of 0.30 mg/dl or more in serum creatinine concentration in the presence of low or therapeutic CsA trough concentration; oliguria, increased resistive index on Doppler ultrasonography, and clinical response to steroid treatment consistent with rejection.

Genotyping

Genomic DNA was collected from EDTA-anticoagulated whole blood using the Nucleon BACC2 kit (Amersham, Biosciences, Buckinghamshire, UK). The primer sequences (Sigma-Aldrich, UK) used for PCR are shown in Table 7 (together with detailed information on the analysis). Subsequently, the PCR product was subjected to denaturing HPLC (dHPLC) analysis or to direct sequencing. Moreover, according to currently common practice in pharmacogenomics research, we have confirmed 20% of the genotyping results obtained with dHPLC with direct sequencing.

Haplotypes analyses were based on exons 12, 21, and 26 genotypes, as described previously.^{37,38} Of the different possible haplotype combinations derived from the homozygous genotypes of *ABCB1*, the most frequent ones were CC-GG-CC ($n = 38$) and TT-TT-TT ($n = 19$), the two haplotypes previously associated with highest and lowest P-gp activity, respectively.^{37,38} Therefore, comparative analyses of the main study outcomes (DGF and CMV infection) were focused on these two haplotypes.

Whole-Blood CsA Measurements

CsA levels were measured in whole-blood samples collected immediately before CsA administration (C_0) or 2 h later (C_2). C_0 and C_2 were measured daily for the first 15 d after transplantation, monthly from month 1 to month 21, and then as per clinical practice in each Transplant Center until study end.

Statistical Analyses

Genotype frequencies for the various SNPs were assessed for deviation from Hardy-Weinberg equilibrium using chi-square test. Pairwise D' and r^2 values for linkage disequilibrium (LD) were calculated using LD plotter (<http://innateimmunity.net/IIPAG2/Bioinformatics>). Statistical analyses were performed by comparing wild-type patients (CC for exon 12, GG for exon 21, and CC for exon 26) with those carrying at least one allelic "T" variant of *ABCB1* (CT+TT, GT+TT, and CT+TT for exons

12, 21, and 26, respectively). Two out of the 147 patients who carried the rare GA and TA allelic variants in the exon 21 were categorized as heterozygous and included in the GT group.

Dichotomous and polychotomous baseline characteristics of the patients were compared with the use of the chi-square test or Fisher's exact test, and continuous characteristics were compared with the use of unpaired *t* test or Wilcoxon rank-sum as appropriate. Because of a skewed distribution, a nonparametric Wilcoxon rank-sum test was used to compare GFR levels at various time points (e.g., for the first 8 d and every months since transplantation, between the *ABCB1* SNPs). An "acute" GFR slope (Δ GFR) was calculated on the basis of available eGFRs from day 0 to day 8 to describe the rate of renal function recovery after kidney transplant. Univariate and multivariable logistic regression models were used to investigate the effects of polymorphisms on the safety and efficacy outcomes. Multiple logistic regressions included the independent variables that were significantly ($P < 0.05$) associated with the outcome in the univariate approach.

Kaplan-Meier analysis was used to investigate whether cumulative incidence of new episodes of CMV antigenemia after transplantation differed between *ABCB1* SNPs. The difference between the survival curves was assessed by means of the log-rank test. Cox proportional hazards models, with adjustment for main demographic and clinical covariates that were significantly associated with the outcome in the univariate approach, were used to detect the effect of polymorphisms on CMV episodes.

The data were presented as numbers and percentages, means \pm SD, or medians and interquartile ranges, as appropriate. All of the statistical analyses were performed using SAS version 9.1 (SAS Institute Inc, Cary, NC). A *p* value of less than 0.05 was considered as statistically significant.

APPENDIX

The Mycophenolate Steroids Sparing (MYSS) Genetics Study Group Organization*

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DISCLOSURES

None.

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