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ORIGINAL ARTICLE

Do drug transporter (ABCB1) SNPs and P-glycoprotein function influence cyclosporine and macrolides exposure in renal transplant patients? Results of the pharmacogenomic substudy within the symphony study

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Authors declare that they have had no involvements that might raise the question of bias in the work reported or in the conclusions, implications, or opinions stated.

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Summary

The function of the efflux pump P-glycoprotein (Pgp) and ABCB1 single nucleotide polymorphisms (SNPs) should be considered as important tools to deepen knowledge of drug nephrotoxicity and disposition mechanisms. The aim of this study is to investigate the association of C3435T, G2677T, C1236T, and T129C ABCB1 SNPs with Pgp activity and exposure to different immunosuppressive drugs in renal transplant patients. Patients included in the Symphony Pharmacogenomic substudy were genotyped for ABCB1 SNPs. According to the design, patients were randomized into four immunosuppressive regimens: low and standard dose of cyclosporine (n = 30), tacrolimus (n = 13), and sirolimus (n = 23) concomitantly with mycophenolate and steroids. Pgp activity was evaluated in PBMC using the Rhodamine 123 efflux assay. TT carrier patients on C3435T, G2677T, and C1236T SNPs (Pgp-low pumpers) showed lower Pgp activity than noncarriers. Pgp-high pumpers treated with cyclosporine showed lower values of Pgp function than macrolides. There was a negative correlation between cyclosporine AUC and Pgp activity at 3 months. Results did not show any correlation between tacrolimus and sirolimus AUC and Pgp activity at 3 months. We found an important role of the ABCB1 SNPs Pgp function in $CD3^+$ peripheral blood lymphocytes from renal transplant recipients. Pgp activity was influenced by cyclosporine but not macrolides exposure.

Introduction

The human multidrug-resistance MDR1 gene, also known as ABCB1, encodes for P-glycoprotein (Pgp), a 170 kDa membrane protein first described in drug-resistant cells. Pgp has been extensively studied both for its role in normal physiology and for its potential role in clinical drug resistance [1]. This protein acts as a primary active transporter, playing

an important role in protecting tissues from toxic xenobiotics and endogenous metabolites. Pgp effectively modulates the absorption, cellular metabolism, and toxicity of pharmacological agents [2,3]. As a drug-efflux pump, it extrudes a range of hydrophobic drugs from cells contributing to drug disposition in humans and reducing the bioavailability of many oral medications [4–6]. Pgp expression could be an important component of a complex detoxifying system in kidney against xenobiotics or in regulating the traffic of metabolites responsible for the nephrotoxicity against different drugs [7]. Pgp also modulates cells of the immune system, particularly interfering with dendritic cell maturation and T-cell activation [8–10]. This multifaceted involvement in drug disposition, cancer drug resistance, and regulation of the immune response makes Pgp an attractive target for further investigation to increase understanding of its function in drug pharmacokinetic strategies in cells and tissues.

The key proteins involved in biotransformation and transport of tacrolimus (Tac) and cyclosporine (CsA) include cytochrome P450IIIA (CYP3A) and ABCB1. There are several clinical agents broadly used as substrates, inhibitors, or inducers of the Pgp, including immunosuppressive drugs [11]. Therefore, the degree of expression and function of ABCB1 could directly influence the therapeutic effectiveness of such agents, affecting their bioavailability. Single nucleotide polymorphisms (SNPs) in ABC drug-efflux pumps may play a role in response to drug therapy and disease susceptibility. The effect of various genotypes and haplotypes on the expression and function of these proteins is not yet clear, and their real impact remains controversial [12,13].

In the past ten years, more than 50 SNPs have been identified in the gene encoding for ABCB1. The SNPs that have been most widely studied are the C-T transition at position 3435 within exon 26, the C-T transition at position 1236 within exon 12, and the G-T/A transition at position 2677 within exon 21 [14–16]. These SNPs have also been the ones most frequently described as associated with Pgp function on Tac and CsA absorption in renal transplant recipients. Anglicheau *et al.* [17] demonstrated that SNPs in ABCB1 gene were associated with the Tac requirements, and therefore transplant population with Tac treatment should be genotyped for ABCB1 SNP. Moreover, ABCB1 SNPs are also associated with CsA exposure in the first post-transplant week [18]. Furthermore, authors suggest that the nephrotoxicity related to Tac may also be enhanced by donor and recipient ABCB1 polymorphisms [19]. The inter-individual differences in the pharmacokinetics of calcineurin inhibitors have been related to inter-individual heterogenicity in enzymatic activity of Pgp. There are also several clinical studies on ABCB1 genotype related to Pgp expression and function but the results are quite controversial [20–26].

Considering that ABCB1 polymorphisms may partly explain the large inter-individual variations in the pharmacokinetics of the immunosuppressors, we investigated the effect of the four SNPs in the ABCB1 gene (exon 1b T-129C, exon 12 1236C>T, exon 21 2677G>T, and the exon 26 3435C>T).

Materials and methods

This pharmacogenomic study is within the framework of the Symphony trial. It was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization-Good Clinical Practice Guidelines (ICH-GCP), and with local ethical committee or institutional review board approval at each center. All patients provided written informed consent before inclusion in the pharmacogenomic substudy. The main objective of the Symphony trial was to establish the differences on MPA and its metabolites exposure among the four immunosuppressive groups: MMF in combination with full or reduced doses of CsA, Tac, or sirolimus. A secondary objective was a pharmacokinetic population analysis and the pharmacogenetic evaluation of genes involved in immunosuppressors exposure.

Reagents

Annexin-V PE (AnnV) (BD Pharmingen, San José, CA, USA), 7-aminoactinomycin D (7-AAD) (Sigma-Aldrich, Madrid, Spain), binding buffer (BB) (Medical and Biological Laboratories, Nagoya, Japan), phosphate buffered saline (PBS) (PAA, Cambridge, UK), Biotarget-1 (Biological Industries, Israel), Rhodamine 123 (Molecular Probes Inc., Eugene, OR, USA). CD3-APC conjugated Mouse anti-Human monoclonal antibody (BD Pharmingen). PSC833 (kindly provided by Novartis, Basel, Switzerland).

Subjects

Seventy renal transplant recipients (58.6% men; mean age: 47.9 $\pm$ 11.8 years) from eight Spanish centers of the Symphony study were included in the Pharmacogenomic substudy. Four patients were withdrawn because of logistic pitfalls. Patients were originally randomized to four branches of immunosuppressive regimen, all of them consisting of daclizumab induction, mycophenolate mofetil and corticosteroids potentiated by either low dose of CsA, standard dose of CsA, Tac, or sirolimus (SRL). Low and standard dose of CsA were combined into one group (CsA) for the statistical analyses, yielding three treatment groups according to immunosuppressive regimen: CsA (n = 30), Tac (n = 13), and sirolimus (n = 23).

Immunosuppressive regimes

The MMF daily dose range across the branches of immunosuppressive regimen was 1680–1946 mg over the first 3 months of treatment. CsA mean daily dose on Day 7 was 425 mg decreased to 222 mg (±81 mg) and 183 mg (±79 mg), respectively, by Month 3. These doses corresponded to median trough levels by Day 7, Month 1 and 3 of 292, 218, and 164 ng/ml for the standard-dose group, and 75.5, 109, and 80.5 ng/ml for the low-dose group, respectively. During the first 3 months, the Tac and SRL range daily doses were 4.3–5.8 mg and 2.9–3.5 mg, respectively (median trough levels by Day 7, Month 1, and Month 3: 8.1, 7.7 and 7.1 ng/ml, and 4.6, 7.5 and 7.8 ng/ml, respectively). The mean exposure (AUC_{0-12}) to CsA, Tac, or SRL as appropriate during the first 3 months in the high-dose CsA, low-dose CsA, Tac, and SRL groups were 4842.3–9230.7 ng h/ml, 2796.2–3601.6 ng h/ml, 129.3–152.3 μg h/ml, and 134.1–160.9 μg h/ml, respectively.

Pharmacokinetic analysis

The AUC_{0-12} of MPA and its metabolites between treatment groups was compared at each time along the follow-up. Pharmacokinetic data were collected on Day 7 and on Months 1 and 3 post-transplant. For this purpose, at each visit, 11 blood samples were collected: before the first MMF administration of the day [predose (Time 0)] and up to 12 h postdose (at 20, 40, 75 min and 2, 3, 4, 6, 8, 10, and 12 h postdose). Pharmacokinetic analysis of MPA was carried out with a standard no compartmental model using WinNonlin. All AUC results were dose-corrected at 2 g/day to obtain the right correlation in the pharmacokinetic dates.

For the analysis of pharmacokinetic interactions between drugs, C_{max} and AUC_{0-12} values for MPA and its metabolites, CsA, Tac, and SRL were normalized by the dosage of the medication taken prior to blood sampling [27].

Isolation of peripheral blood lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples by Ficoll-Paque Plus density gradient centrifugation according to the manufacturer's instructions. Cells were then washed and resuspended in Biotarget-1 medium supplemented with 1% L-glutamine, 1% sodium pyruvate, and 1% pen-streptomycin. Cell viability is always greater than 90%. Lymphocytes were frozen in lymphocyte freezing media as described previously [28].

Genotyping of ABCB1 polymorphisms

For genotyping we obtained DNA from 66 renal transplant patients. Patients were genotyped for SNPs in ABCB1 gene; exon 1b:T-129C (rs3213619), exon 12:1236C>T (rs1128503), exon 21:2677 G>T (rs2032582), and the exon 26:3435 C>T (rs1045642). DNA was extracted from a peripheral whole blood sample using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Sydney, Australia) and was stored at −80 °C. Genotyping procedures were performed with the MassARRAY™ SNP genotyping system (Sequenom Inc., San Diego, CA, USA). The method involves multiplex PCR and single base extension assays, designed by the AssayDesigner software (Sequenom Inc.), and followed by mass spectrometry analysis with the Bruker Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA). Spectral output was analyzed and checked using MassARRAY™ Typer 3.4 software (Sequenom Inc.). The primers and the iPlex assay are listed in Table 1.

Measurement of Pgp function in PBMCs

Intracellular Rho123 uptake

Human PBMCs (1×10^6 cells) were incubated with Rho123 at a final concentration of 200 ng/ml for 30 min at 37 °C 5% CO_2, avoiding light exposure, in the presence or absence of the specific inhibitor PSC833 (10 μM). At the end of the Rho123 uptake, cells were kept on ice to stop Pgp activity and then washed twice with ice-cold PBS without Ca^{2+} and Mg^{2+} to remove extra cellular Rho123. PSC833 was also maintained during the washes to avoid periods of noninhibition. Cells were diluted in 50 μl of BB containing 2 μl of CD3-APC and incubated on ice for 20 min. After washing, 250 μl of BB containing 2 μl AnnV (to detect apoptotic cells) was added to all samples and incubated for 15 min on ice, avoiding light exposure. A total of 2 μl of 7-AAD (to detect cells with membrane damage) was added prior to flow cytometry analysis.

Table 1. Primer properties in a 4-SNP iPlex assay for sequenom SNP genotyping.

SNP	Exon	rs	2nd-PCRP	1st-PCRP	UEP_MASS
C3435T	26	1045642	ACGTTGGATGACTGCAGCATTGCTGAGAAC	ACGTTGGATGTATGTTGGCCTCCTTTGCTG	5048.3
G2677T	21	2032582	ACGTTGGATGTGCAATAGCAGGAGTTGTTG	ACGTTGGATGCATATTTAGTTTGACTCACC	6027.9
C1236T	12	1128503	ACGTTGGATGGTTTTTTTCTCACTCGTCCTG	ACGTTGGATGCACAGCCACTGTTTCCAACC	6646.3
T129C	1b	3213619	ACGTTGGATGCTTCGCTCTCTTTGCCACAG	ACGTTGGATGCCTCTGCTTCTTTGAGCTTG	7070.6

Flow cytometry analysis

Flow cytometry was performed on a FACSCalibur flow cytometer (Becton, Dickinson and Company, BD, Franklin Lakes, NJ, USA) with four-color analysis. Gating was based on forward scatter and side scatter dot plots by encircling populations with amorphous regions and then excluding dead cells (life gate) by 7-ADD counterstaining and Annexin-V. We monitored the linearity of the flow cytometer measurements daily using calibration standard beads (K0110; Dako, Glostrup, Denmark). These quantitative measurements included mean fluorescence intensity in linear values, which were transformed into calibrated values of molecules of equivalent soluble fluorochrome (MESF). A calibration curve (log = a × log + b) was constructed to convert fluorescence measurements to MESF units. Data were analyzed using Cell Quest software. Each experiment was performed in duplicate.

Statistical analysis

Demographic variables, baseline characteristics, and transplantation-related data were described by frequencies and mean. Nonparametric statistics (Kruskal–Wallis, and chi-squared test) were applied to study differences in baseline data according to immunosuppressive regimens. Allele and genotype frequencies for the various SNP were assessed for deviation from Hardy–Weinberg equilibrium. Frequencies of genotypes and alleles were given with their 95% confidence intervals (95% CI). Pgp activity was confirmed to be normally distributed by the Kolmogorov-Smirnov method. Differences between the groups were assessed using an analysis of variance, followed by *post hoc* Fisher's test. P values were corrected for the number of variables compared according to the Bonferroni method. Low and standard dose of CsA, were combined into one group, and also Tac and SRL group were gathered as macrolides in some analysis. Linear regression analysis was used to analyze the impact of immunosuppressors pharmacokinetic parameters in Pgp activity. All statistical studies were performed with SPSS 12.0 K for Windows (12.0.1; SPSS Inc; Chicago, IL, USA).

Results

Distribution of ABCB1 SNPs and Pgp activity related to age and sex

Data from renal transplant recipients were collected. Descriptive analysis of the sample is shown in Table 2. Demographic variables (sex and age) did not reveal differences among groups of patients according to treatment. No significant differences for biochemical parameters (creatinine or albumin) at baseline were found.

We found a positive correlation among the three SNPs of ABCB1 gene: C3435T-G2677T ($r^2 = 0.91$, $P = 0.001$), C3435T-C1236T ($r^2 = 0.96$, $P = 0.0001$), and G2677T-C1236T ($r^2 = 1.0$, $P = 0.0001$). The genotype distribution was in Hardy-Weinberg equilibrium. The analysis of haplotypes in the additive model was not significant ($P = 0.09$) for TTT but was significant for the recessive model ($P = 0.026$), with similar results to those in the genotype analysis. The Pgp activity value in individuals with TTT haplotype (39% frequency) was 16 units less than Pgp activity in wild-type individuals (Table 3).

Influence of the ABCB1 SNP on Pgp activity

The prevalence of the ABCB1 SNP C3435T (rs1045642): CC, CT, and TT genotypes was 24.07%, 59.26%, and 16.67% of the recipients, respectively. ABCB1 G2677T (rs2032582): GG, GT, and TT genotypes were found in 35.9%, 52.8%, and 11.3%, respectively. For ABCB1 C1236T (rs1128503) SNP prevalence, CC, CT, and TT genotypes were detected in 34%, 50%, and 16% of recipients, respectively, and T129C (rs3213619) SNP with CT, and TT genotypes were identified in 10% and 90% of the recipients.

Pgp activity was influenced by the different ABCB1 polymorphisms analyzed. Pgp activity was higher in the carriers of 3435 CC genotype and 3435 CT heterozygous patients,

Table 2. Sample description at baseline ($n = 66$).

	CsA* ($n = 30$)	Tac† ($n = 13$)	SRL‡ ($n = 23$)	*P*-value
Age, mean, (SD)§	48.18 (10.18)	52.36 (13.43)	47.41 (12.03)	$P = 0.444$
Gender male, (%)¶	65.5	72.7	41.2	$P = 0.165$
Creatinine (mg/dl), mean (SD)§	8.52 (2.81)	8.17 (2.58)	6.89 (2.93)	$P = 0.289$
Albumin (g/dl), mean (SD)§	4.25 (0.45)	4.01 (0.55)	4.18 (0.80)	$P = 0.633$

SD, standard deviation.

*Standard immunosuppression with normal and lose dose of cyclosporine, mycophenolate mofetil (MMF), and corticosteroids (CS).

†Low dose of tacrolimus with daclizumab induction, MMF and CS.

‡Low dose of sirolimus with daclizumab induction, MMF and CS.

§Kruskal–Wallis test.

¶Chi-squared test.

Table 3. SNPStats, haplotypes analysis. Analysis of haplotypes in the recessive model ($P = 0.026$).

Coefficients	Coef	se	t.stat	*P*-value
(Intercept)	46.647	2.317	20.130	0.000
hap. TTT	−16.162	7.079	−2.283	0.026
hap. rare	−14.630	NA	NA	NA
Haplotypes	**loc.1**	**loc.2**	**loc.3**	**hap.freq**
haplo.base	C	G	C	0.50893
hap. TTT	T	T	T	0.39286
hap. rare	–	–	–	0.09821

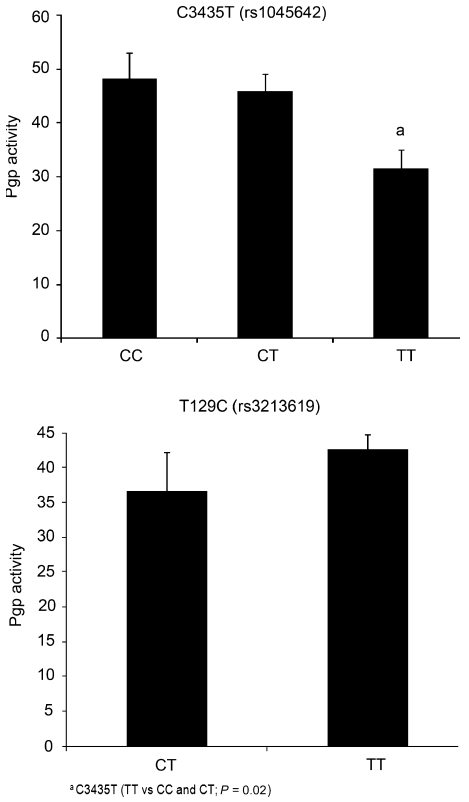

Figure 1 Pgp activity by flow cytometry analysis measured with MESF units of Rho123 fluorescence in $CD3^+$ T-cell population and the influence of two ABCB1 polymorphisms: C3435T (rs1045642) and T129C (rs3213619). Values are means (MESF) ± SD; $n = 54$ patients for C3435T (CC = 13; CT = 32 and TT = 9, $P = 0.02$), and $n = 51$ patients for T129C (CT = 5 and TT = 46, NS).

whereas the lowest activity was found in the carriers of 3435 TT genotype (CC: 48.33 ± 4.69; CT: 45.90 ± 3.18; TT: 31.62 ± 3.37; $P = 0.02$) (Fig. 1).

Similar results were found in the G2677T and C1236T SNPs. The ABCB1 G2677T genotype showed higher Pgp activity in the GG and GT subjects compared with homozygous T carriers patients (GG: 45.96 ± 3.79; GT: 45.71 ± 3.38; TT: 30.47 ± 4.52; $P = 0.04$). Carriers of 1236 CC genotype showed higher Pgp activity than CT heterozygous, which showed intermediate values, and TT homozygous which displayed the lowest activity (CC: 46.11 ± 4.11; CT: 43.71 ± 2.68; TT: 30.90 ± 3,33; $P = 0.02$). Pgp activity was not influenced by the T129C SNP. No significant differences were observed between genotypes (Fig. 1). All values are represented as means (MESF) ± SD.

Variations of Pgp activity depending on immunosuppressor therapy

Pgp activity was evaluated in isolated PBMCs obtained from patients in the Cmin of the pharmacokinetic study, at the steady state of immunosuppression treatment at 3 months after renal transplantation.

Results showed lower Pgp activity in patients with CsA ($n = 28$) compared with macrolides ($n = 26$) ($P = 0.04$). Considering that the two macrolides showed different effects on Pgp function, we further analyzed this group. CsA patients showed lower Pgp activity than Tac patients (39.31 ± 2.72 vs. 55.06 ± 8.57 $P = 0.02$, respectively) and SRL patients (39.31 ± 2.72 vs. 46.19 NS) (Fig. 2). In addition, Tac was also higher than SRL patients but this difference did not reach statistically significant levels. All patients received CsA, Tac, or SRL with steroids and MMF at fixed daily doses. Considering that the Pgp activity values in healthy volunteers in our area was 45 ± 4.98 [29], transplant patients with macrolides showed similar Pgp activity. Patients treated with CsA showed a significant decrease in Pgp activity ($P < 0.05$).

Influence of ABCB1 SNPs and immunosuppressor therapy on Pgp activity

Considering the immunosupressor treatment, the individual contribution of each SNP on Pgp activity increase was analyzed. Genotypes were sorted into high pumpers (CT and CC) and low pumpers (TT) correlating with Pgp function. High pumper patients showed more Pgp activity than low pumpers, independently of immunosuppressor treatment.

Pgp activity in high pumper patients treated with CsA in the C3435T SNP showed lower values of Pgp activity than the macrolides group ($P < 0.05$). However, in CsA low pumper Pgp showed a similar trend but there were no significant differences. Both CsA and macrolides high pumpers showed higher Pgp function than low pumpers ($P < 0.05$) (Fig. 3). The same results in Pgp activity profile were observed for G2677T and C1236T SNPs. In contrast, the high pumpers patients with T129 SNP were the TT heterozygote and displayed the opposite behavior in Pgp expression compared with the other three SNPs and any statistical difference was observed (data not shown).

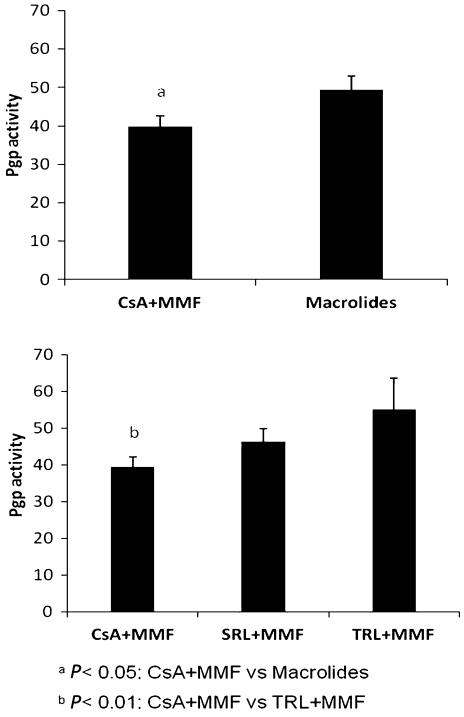

Figure 2 Variations of Pgp activity depending on immunosuppressor therapy; $n = 54$ patients for each SNP: gathered in CsA, $n = 28$ and macrolides, $n = 26$ ($n = 17$ for SRL and $n = 9$ for Tac).

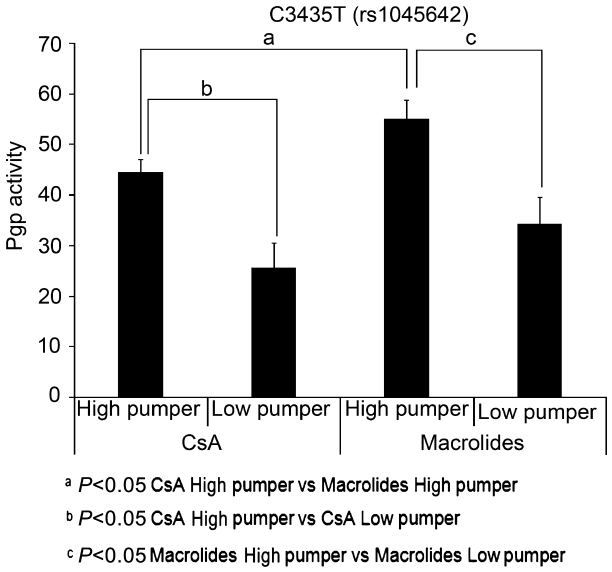

Figure 3 Influence of ABCB1 SNPs and immunosuppressor therapy on Pgp activity; $n = 54$ patients for the C3435T SNP (CsA, $n = 28$ and macrolides, $n = 26$).

Influence of Pgp activity and ABCB1 SNP in the CsA AUC and Cmin

We analyzed the correlation between different immunosuppressor pharmacokinetic parameters and Pgp activity. We focused on the sub-group of high pumpers and low CsA doses because these patients should have less cellular CsA concentration because of increased drug extrusion and lesser lymphocyte exposure. A negative correlation between CsA AUC and Cmin and Pgp activity was seen at 1 month (Fig. 4) with regression analysis between Pgp activity and AUC (R sq linear = 0.365; $P = 0.05$) and Pgp activity and Cmin (R sq linear = 0.44; $P = 0.01$). When low pumpers exposed to high doses of CsA were evaluated, no correlation was seen, probably because of the low number of patients. In addition, we did not find any correlation between the macrolides (Tac and SRL) AUC or Cmin and Pgp activity at 1 month.

Discussion

The bioavailability and metabolism of CsA and Tac are primarily controlled by efflux pumps belonging to the ABC transporter family and members of the cytochrome P-450 isoenzyme system. Several *in vitro* studies have identified immunosuppressors as substrates and/or inhibitors of Pgp [30–34]. As such they could alter the bioavailability of many concomitant drugs, causing potentially important drug interactions. So far, several SNPs have been identified in the ABCB1/MDR1 gene that might alter Pgp expression and function in humans, leading to clinical interest in the pharmacokinetics of immunosuppressors to improve their dosing in individuals [35]. Despite the association of SNPs with Pgp protein expression and function, indicating that Pgp is a major determinant in the absorption and disposition of drugs, there is still controversy as to whether the pharmacokinetics and pharmacodynamics of drugs could be modified by MDR1 genotypes/haplotypes [17,36–41].

Our results confirm a significant correlation between ABCB1 genotyping and Pgp activity in PBMCs from renal transplant recipients. Homozygous 3435 TT carrier subjects showed the lowest Pgp activity compared with 3435 CT and CC carriers. Although our results on CC $\geq$ CT > TT in relation to Pgp function corroborate the data reported by others [13,42–45], there are some discrepancies among authors, and the functionality of 3435 CT ABCB1 SNP remains a matter of controversy [7,46–50]. In our renal transplant population, we described the influence of ABCB1 SNPs on Pgp function in PBMCs, supporting results reported in other tissues where the functional effect of Pgp polymorphism was also demonstrated [35,42]. Blood samples were obtained from patients from different centers and all measurements were performed with frozen cells. In a previous study [28] we demonstrated that different working conditions did not affect Pgp activity. Measurement of rhodamine efflux from peripheral blood cells

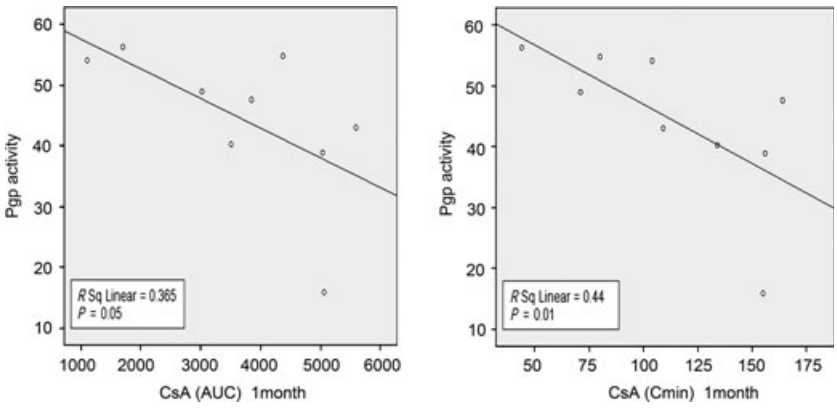

Figure 4 Influence of the Pgp activity and ABCB1 SNP in the CsA AUC and Cmin in high pumper low-dose renal transplant patients at 1 Month. Regression analysis between Pgp activity and AUC (R sq linear = 0.365; $P = 0.05$ and coef: -, 650) and Pgp activity and Cmin (R sq linear = 0.44; $P = 0.01$, and coef: -, 767); $n = 9$ patients high pumper and low doses of CsA with Pgp function data.

allows assessment of the polymorphisms effect not only on Pgp expression but also on Pgp activity.

Current immunosuppressive drugs are characterized by a narrow therapeutic index and large intra- and inter-individual variability both in pharmacokinetics and in pharmacodynamics. Pgp is an important component of a detoxifying system, and ABC transporters affect the bioavailability of their substrate drugs [7,51]. CsA was the first immunosuppressor shown to modulate Pgp activity in laboratory models and clinical trials, and intestinal Pgp has been shown to determine oral clearance of CsA [52]. Tac is both a substrate and an inhibitor for Pgp, less active than CsA. SRL is a Pgp substrate which may limit its intestinal absorption, and Pgp is also probably involved in its excretion [51]. Our group has shown an *in vitro* inhibitory effect of Pgp activity in T-cell subpopulations incubated with immunosuppressors, particularly CsA either alone or associated with SRL [29]. In the present study, patients with CsA showed lower Pgp activity compared with macrolides, both Tac and SRL. Interestingly, macrolides did not affect Pgp function, achieving similar values as those found in healthy volunteers. Thus, the results observed in patients replicated data observed in *in vitro* T-cell studies.

In view of the pharmacokinetic variability of immunosuppressors, pharmacogenomic research could help to improve drug dosage. Numerous studies have addressed the relevance of the MDR1 polymorphism in dose requirement, blood concentrations, chronic rejection, and nephrotoxicity in renal transplant patients treated with CsA and Tac. Studies supported that the dose requirements are influenced by ABCB1 polymorphism, although the use of ABCB1 genotype as a helpful marker in clinical practice remains unclear [17,37,45,53,54].

Some ABCB1 SNPs have been correlated with the *in vivo* activity of Pgp and should therefore be considered in renal transplant recipients treated with Tac [55,56]. So far, no authors have described an association of Pgp activity, MDR1 polymorphisms, and exposure to three immunosuppressors at 7 days, and 3, 6, and 12 months in renal transplantation. The present results demonstrate for the first time the existence of this correlation in a multicenter clinical study in which CsA, Rapa, and Tac exposure were well defined and co-medication drugs were specified. Our results show that patients on CsA and macrolides therapy display the same correlation pattern between Pgp activity depending on the genotypes of the three ABCB1 SNPs studied as observed in the general population ($CC \geq CT > TT$). The values of Pgp activity in patients with CsA were lower than those for macrolides in the SNPs studied. Our results also confirm the inhibitory effect of CsA described by other authors [57–59].

The CsA is a Pgp modulator, and the variability of its absorption and disposition has been attributed to intestinal Pgp expression and activity. The movement of a drug through membranes is an essential step in absorption. Delays or loss of drug during absorption can introduce large variability in drug response. In this study, we found a negative correlation between Pgp activity in lymphocytes and CsA AUC and Cmin at steady state conditions in those patients with high activity of Pgp and low CsA doses. Regarding the correlation between exposure of CsA and Pgp activity we should consider that CsA is a potent Pgp inhibitor, so an increase in CsA exposure should lead to a decrease in Pgp activation. However, we must not overlook the intestinal effect. In the enterocyte, CsA is absorbed and excreted back, in part, to the intestinal lumen through specific proteins belonging to the ABC family. Therefore, the small intestine emerges as the first bottleneck in the entry of CsA into the organism. In addition, metabolism in the liver cannot be underestimated before the distribution to tissues, where ABC proteins are also present. All these processes influence the distribution and subsequent elimination of the drug from the organism. As a result of this complex interplay between enzyme activities and efflux transporters, the concentrations of CsA in plasma could also be affected. C3435T SNP correlates with lower intestinal

Pgp expression, and this in turn does directly affect the oral bioavailability of Pgp substrates. Thus, in individuals with lower intestinal Pgp concentrations the extent of drug absorption from the gastrointestinal tract should be higher. Consequently, an increase in the plasma levels in comparison to the remainder of the population would be observed.

We may conclude that in patients with a CT or CC nucleotide exchange in exon 26 (C3435T) with high Pgp activity on the apical surface of intestinal enterocytes, more CsA is removed from the cells, resulting in decreased bioavailability. In this context, high pumpers treated with low doses of CsA would show less drug exposure and this could affect the therapeutic response. In our study, we found a negative correlation between CsA exposure (AUC and Cmin) in plasma and Pgp activity, which might reflect the increasing CsA bioavailability because of enhanced Pgp blocking in the intestinal enterocytes. However, these results should be interpreted with caution because of the small sample size. Additional studies with larger sample sizes will be required to confirm the results. Genotyping research in this field may yield more refined immunosuppressive drug therapy while further exploring the role of ABC transporters as biomarkers in the measurement of immunosuppressive drug concentration.

Authorship

NL and JMG: designed research/study. NL and IL: performed research/study. HC, PGB, AC, MS, JMC, FO, JSP, MÁG and HE: contributed new reagents or analytic tools. NL, IL and JT: analyzed data. NL, IL and JT: wrote or contributed to the writing of the manuscript.

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