Research Article

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Pharmacogenomics

Association of SNPs with the efficacy and safety of immunosuppressant therapy after heart transplantation

Aim: Studying the possible influence of SNPs on efficacy and safety of calcineurin inhibitors upon heart transplantation. **Materials & methods:** In 60 heart transplant patients treated with tacrolimus or cyclosporine, we studied a panel of 36 SNPs correlated with a series of clinical parameters during the first post-transplantation year. **Results:** The presence of serious infections was correlated to *ABCB1* rs1128503 (p = 0.012), CC genotype reduced the probability of infections being also associated with lower blood cyclosporine concentrations. Lower renal function levels were found in patients with rs9282564 AG (p = 0.003), related to higher blood cyclosporine blood levels. A tendency toward increased graft rejection (p = 0.05) was correlated to rs2066844 CC in *NOD2/CARD15*, a gene related to lymphocyte activation. **Conclusion:** Pharmacogenetics can help identify patients at increased risk of clinical complications.

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Keywords: cyclosporine • graft rejection • infection • polymorphism • renal function • tacrolimus

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Background

The heart transplantation (HT) survival rate is over 80% in the first year and about 50% after 10 years. Survival among adult recipients has increased over the years, but still needs to be improved. New advances are therefore required in order to continue improving the success rates. The leading causes of mortality during the first year after HT are primary allograft failure, infections and allograft rejection. Renal dysfunction is also a matter of concern [1,2]. The standard post-HT therapy comprises a calcineurin inhibitor (CNI), mycophenolate mofetil (MMF) and corticosteroids [3].

Both tacrolimus (TAC) and cyclosporine (CsA) show highly variable pharmacokinetics and pharmacodynamics and have narrow therapeutic indexes [4]. Therapeutic drug monitoring is an essential and indispensable instrument for CNI dosing but even with this procedure targeting blood levels is sometimes difficult. Poor control can lead to toxicity or complications such as rejection or infection [5–7]. Changes in the expression or function of proteins and enzymes involved in drug transport, metabolism or mechanism of action can lead to changes in the treatment response and toxicity. Pharmacogenetics deals with characterization of the underlying genetic variants, with a view to establishing effective doses and minimizing adverse reactions. In this context, many studies have been made of the most common genetic variants, in other words, SNPs, especially in *ABCB1*, the gene which encodes for the most important CNI transporter, P-glycoprotein, and in the gene coding for the most important metabolizing enzyme, *CYP3A5* [4,8].

However, there is still little information on the clinical efficacy and safety implications of these genetic variants. The present work therefore aims to determine whether the presence of different SNPs can influence the occurrence of clinical complications in the first year after HT.

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Materials & methods

Study design & patients

All the adult patients that underwent HT in our hospital from 2008 to 2010, survived at least 1 month after HT, and received at least one CNI dose, were included in the study. Retransplanted patients, combined organ-transplanted patients, and all those who did not give their written informed consent were excluded from the study. The final number of included patients was 60, of which 36 received CsA and 24 TAC. None of the patients were treated with mTOR inhibitors. We retrospectively analyzed the evolution of the patients during the first year post-HT based on the electronic medical records, and focusing on presence/absence of allograft rejection, presence/absence of infection and renal function at 1 year calculated with the modification diet in renal disease (MDRD) formula. We considered 'presence of allograft rejection (cellular or humoral)' only in those cases in which corticosteroid bolus treatment or substantial modification of previous immunosuppressant therapy was required. In turn, we considered 'presence of infections' only in those cases in which the recipient needed hospital readmission or prolongation of hospital stay. Renal function (glomerular filtration rate [GFR]) was calculated with MDRD formula. Renal dysfunction was established when the GFR was <60 ml/min, according to international consensus [9,10].

The study was approved by the local Clinical Research Ethics Committee.

Genomic DNA was collected from EDTA-anticoagulated whole blood of the HT recipients. The DNA was extracted from 200 µl of blood using the UltraCleanBloodSpin DNA Isolation Kit (MoBio Laboratories, Inc., CA, USA). DNA was stored at -20°C until use, after quantification using a spectrophotometer (NanoDrop Technologies, Inc., DE, USA) to determine the concentration and purity.

A genetic analysis platform (MassARRAY; Sequenom, Inc., CA, USA) was used to genotype each sample according to the instructions of the manufacturer, at the SCSIE-UCIM (Facultad de Medicina, Universidad de Valencia, Valencia, Spain). The study was previously designed and validated by our group, meaning that the correct functioning of the technique (correct genotyping of the SNPs included in the study) has already been tested and published. We have genotyped more than 600 samples, run in triplicates, with appropriate controls and assessing that the obtained and expected frequencies of each variant are consistent [11,12].

The panel of SNPs comprised 36 SNPs in 14 different genes encoding for metabolizing enzymes, transporters and molecular targets of TAC, CsA and other immunosuppressive agents (Table 1). It was not designed especially for this study, but with the aim of being useful for many different studies involving the use of immunosuppressants, mainly in adult solid organ transplantation, but not exclusively for it. The most important relationships between the immunosuppressant drugs and the genes, some of them included in the study, are summarized in Figure 1.

Table 1. SNPs tested.

Gene	SNP	Alternative nomenclature
ABCB1	rs1045642	3435C>T
	rs2032582	2677G>T/A
	rs1128503	1236C>T
	rs2229109	1199G>A
	rs2235013	1725 + 38G>A
	rs2235033	1554 + 24T>C
	rs3213619	-129T>C
	rs9282564	61A>G
ABCC2	rs2273697	1249G>A
	rs3740066	3972C>T
	rs717620	-24C>T
ABCG2	rs2231137	34G>A
	rs2231142	421C>A
CYP2B6	rs2279343	*CYP2B6*4*
	rs3745274	*CYP2B6*6*
CYP2C19	rs4244285	*CYP2C19*2*
CYP2C9	rs1057910	*CYP2C9*3*
CYP3A4	rs2740574	*CYP3A4*1B*
CYP3A5	rs776746	*CYP3A5*3*
	rs10264272	*CYP3A5*6*
	rs41303343	*CYP3A5*7*
MTHFR	rs1801131	1298A>C
	rs1801133	677C>T
NOD2/CARD15	rs2066844	R702W
	rs2066845	Gly908Arg
SLCO1A2	rs11568564	R168C
	rs72559749	Asn277Del
	rs11568563	E172D
SLCO1B1	rs2306283	*SLCO1B1*1B*
	rs4149056	*SLCO1B1*5*
TPMT	rs1142345	*TPMT*3C*
	rs1800460	*TPMT*3B*
	rs1800462	*TPMT*2*
UGT1A9	rs6714486	-276T>A
	rs72551330	98T>C
	rs17868320	855 + 32405C>T

The immunosuppressive regimen administered consisted of a CNI + MMF + corticosteroids during the first year post-HT. The MMF dose was of 1000 mg/12 h unless infection (500 mg/8 h) or rejection occurred (1500 mg/12 h). The CsA target levels were 200–300 ng/ml during the first 6 months and 100–200 ng/ml thereafter. The TAC target levels were 10–15 ng/ml the first 6 months and 5–10 ng/ml thereafter. Corticosteroids were progressively reduced to 6 mg/day by month 6 post-HT. We analyzed the trough levels (C_0, C_{min}) of TAC and CsA using a clinical chemistry system (Dimension; Siemens Healthcare, IL, USA). The average blood levels of TAC or CsA during the first year post-HT were assessed separately for each recipient measuring the concentration in the routine blood extraction performed every 2 months in the first year after transplantation. Levels were expressed as the 'trough concentration/dose corrected for weight' ratio, C_0/Dc, ([ng/ml]/[mg/kg/24 h]).

Statistical analyses

Statistical analysis and calculations were performed using the SPSS statistical package (SPSS® version 19, IBM®, NY, USA) and Prism 4 (GraphPad Software, Inc., CA, USA). Quantitative continuous variables were described as the median (interquartile range) or mean and its 95% CIs, according to the results of the Kolmogorov–Smirnoff or Shapiro–Wilk normality tests. Categorical variables in turn were expressed as percentages. The Fischer exact test and Kruskal–Wallis or Mann–Whitney tests were employed for the univariate analysis. After that, multivariate logistic or linear regression models were performed. Each model was controlled for recipient age, donor age, recipient sex, donor sex, kind of CNI and ischemia time. Statistical significance was considered for $p < 0.05$ in all cases.

Results

On applying the corresponding statistical tests, no associations between the patient's baseline characteristics and the studied SNPs were found. No differences were observed between the patients who received CsA or TAC (Table 2). After the first year of post-HT follow-up, a total of 62.7% patients had experienced at least one rejection episode, 46.8% had suffered at least one infection and 21.8% had renal dysfunction (GFR < 60 ml/min) at the end of the period.

Serious infection of any kind in the first year after transplantation

Results from univariate analysis including clinical covariates (recipient age, donor age, recipient sex, donor sex, kind of immunosupressant and isquemia time) did not yield any statistically significant correlation with the serious infections recorded.

After multivariate logistic regression analysis including the SNPs and clinical covariates (recipient age, donor age, recipient sex, donor sex, kind of immunosuppresant and ischemia time), *ABCB1* rs1128503 was the only independent variable that remained in the model. Infections were more frequent in patients carrying the T allele (backward method, results from last step in Table 3).

Renal function (MDRD) 1 year after transplantation

ABCB1 rs9282564 was found to be associated with renal function as assessed by MDRD formula 1 year after transplantation. Specifically, the GFR was 84.1 ml/min (median, interquartile range = 71.0–98.3) for AA carriers and 58.1 ml/min (median, interquartile range 47.8–70.5) for AG/GG carriers (Figure 2, Mann–Whitney U-test, $p = 0.001$). After multivariate linear regression including recipient age, donor age, recipient sex, donor sex, immunosuppressant and GFR (MDRD) before transplantation, the only variables remaining in the model were recipient age ($p = 0.009$) and *ABCB1* rs9282564 ($p = 0.003$). The parameters of the regression line are 125.4 (94.6; 156.3) as the constant, -25.1 (-41.2; -9.1) as the dependent value for *ABCB1* rs9282564 and -0.8 (-1.4; -0.2) for age as independent term (values given as coefficient and 95% CI).

On the other hand, we wanted to determine whether the above findings showed any consistent correlation to blood CNI levels, as a secondary observation after identifying significant correlations between the two SNPs and the described clinical parameters. In the case of *ABCB1* rs1128503, the CC patients, who presented fewer infections, also showed lower blood CNI levels. This difference in blood drug levels proved statistically significant in months 2, 10 and 12 after transplantation in the patients administered CsA ($p = 0.012$, 0.025 and 0.033, respectively) (Figure 3A). The same trend was observed in patients treated with TAC: the patients that experienced fewer infections (CC at rs1128503) also had lower TAC blood C_0/D_c, although in this case the difference with the other group (CT/TT at rs1128503) was not statistically significant, probably due to the smaller sample size (data not shown).

Regarding *ABCB1* rs9282564, the G allele carriers showed lower GFR, and in these patients the blood CNI levels were higher. AG patients in turn showed higher CsA levels, reaching statistical significance in month 2 after transplantation ($p = 0.002$, Figure 3B). None of the patients receiving CsA were GG carriers. As in the previous case, the results corresponding to the

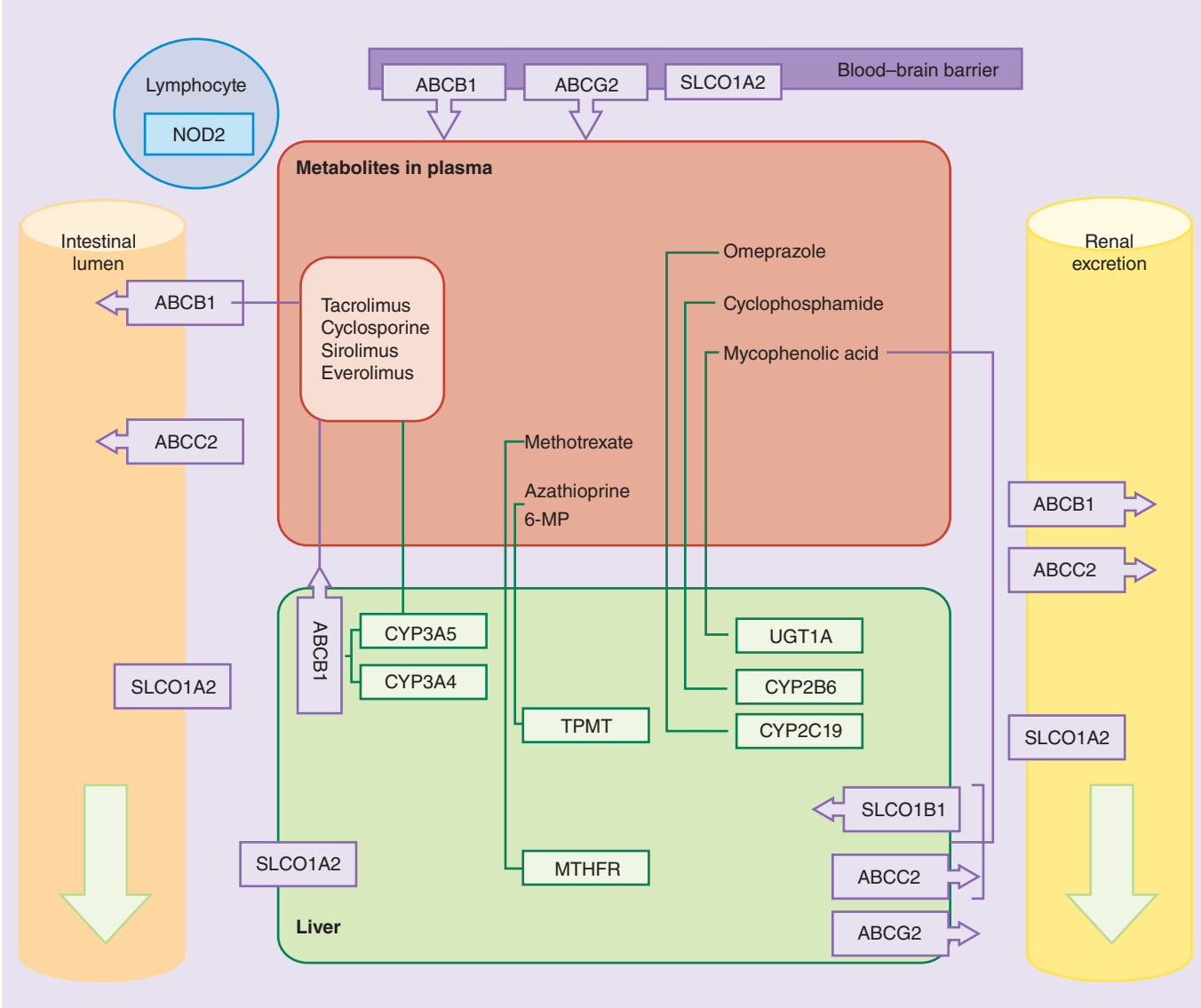

Figure 1. Most relevant relationships among immunosuppressants and the genes included in the study. SNPs included for each gene are listed in Table 1. Omeprazole is not an immunosuppressant, but a drug usually administered concomitantly to these patients. 6-MP: 6-mercaptopurine.

patients treated with TAC showed the same tendency, though without statistically significant differences.

Allograft rejection in the first year after transplantation

No statistically significant association was found between the studied SNPs and allograft rejection in the first year after transplantation, although a relevant tendency was observed for *NOD2/CARD15* rs2066844: 76.1% of the CC carriers suffered allograft rejection versus 33.3% of the CT/TT carriers (p = 0.050, Fischer exact test). On performing the multivariate logistic regression analysis including recipient age, donor age, recipient sex, donor sex, kind of CNI and ischemia time, this SNP did not remain in the model. The univariate tests of clinical covariates yielded a significant p-value (0.046, t-test) with recipient's age, marking more rejection in younger recipients, and also significant p-value (0.006, bilateral Fischer's test) regarding the kind of CNI employed, which points more rejection in patients treated with CsA.

Discussion

A panel of 36 different SNPs was designed and 60 HT recipients were genotyped, employing Sequenom MassARRAY® technology, in order to study some of the most relevant polymorphisms in genes related to treatment with CNI. The results of our study show that some SNP could be involved in certain clinical events such as the presence of serious infections, alterations in

Table 2. Baseline characteristics of the study population.

	Baseline patient characteristics		
	CsA (n = 36)	TAC (n = 23)	p-value applying χ^2 test
Recipient sex (male, %)	69.4	78.3	0.458
Recipient age (years)	53 ± 11	52 ± 10	0.691
Donor age (years)	42 ± 12	43 ± 9	0.830
Etiology (%)			0.523
– Ischemic	-36.1	-21.7	
– iDMC	-51.9	-48.1	
– Other	-12	-30.2	
AHT (%)	45.7	34.8	0.408
Insulin-dependent diabetes (%)	13.9	8.7	0.547
Smoker (%)			0.038
– Current	-14.3	-43.5	
– Former	-37.1	-30.4	
– Never	-48.6	-26.1	
Dyslipidemia (%)			
– PVR	2.22 ± 1.23	2.26 ± 1.25	0.911
– Previous cardiac surgery (%)	13.9	13	0.926
– Creatinine pre-HT (mg/dl)	1.17 ± 0.58	1.12 ± 0.41	0.750
– Creatinine 1 month after HT (mg/dl)	0.99 ± 0.32	0.91 ± 0.37	0.441
– GFR <60 ml/min (MDRD, %)	30.2	27.1	0.680
– Ischemia time (min)	164 ± 56	164 ± 44	0.678
– Urgent HT	27.8	47.8	0.117
– Primary graft failure (%)	17.1	21.7	0.662

AHT: Arterial hypertension; CsA: Cyclosporine A; GFR: Glomerular filtration rate; HT: Heart transplantation; iDMC: Idiopathic dilated myocardiopathy; MDRD: Modification of diet in renal disease; PVR: Pulmonary vasculature resistance; TAC: Tacrolimus.

renal function and graft rejection during the first year post-HT. Analyzing together TAC and CsA side effects can be controversial, but the three outcomes that we are measuring are related to both drugs, as it has been extensively published [13–15]: infections, renal damage and allograft rejection are possible consequences of both tacrolimus and cyclosporine treatment. In general, it can be said that the main side effects of CNIs are coincident, there is only a little variation in diabetes incidence, being higher with TAC, higher dislipemia with CsA and higher arterial hypertension also related to CsA. It is also said that TAC is more powerful in avoiding allograft rejection (and this is consistent with our results), but the meta-analyses performed so far are not conclusive [16,17].

Infections are a common problem following HT, though with important inter-individual differences in incidence. Many factors are involved in this complication (immunosuppressant use, prophylaxis, exposure…), though our results show a clear relationship (without differences in the baseline characteristics) between SNP rs1128503 in the *ABCB1* gene and serious infections (i.e., cases requiring hospitalization). This genetic variant is one of the three most studied SNPs in *ABCB1* gene (rs1045642, 1128503 and 2032582), in strong linkage disequilibrium. It is pur-

Table 3. Association between the presence of serious infection of any kind and *ABCB1* rs1128503 genotype.

SNP/genotype		n	Infection of any kind		Odds ratio (95%CI)	p-value
			Yes, n (%)	No, n (%)		
ABCB1 rs1128503	CC	12	2 (16.7)	10 (83.3)	1	
	CT/TT	35	20 (57.1)	25 (42.9)	6.67 (1.3–35.0)	0.012

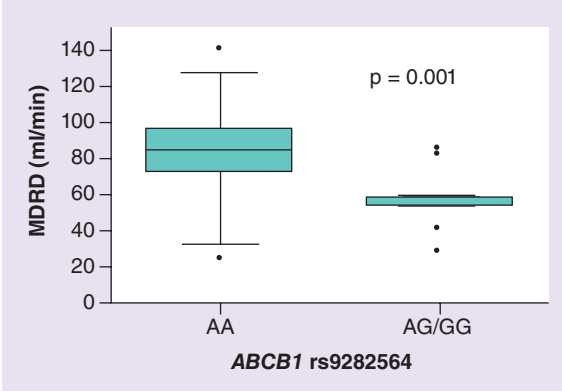

Figure 2. Renal function (glomerular filtration rate) measured by modification diet in renal disease formula, 1 year after heart transplantation, in relation to the *ABCB1* rs9282564 genotype. One year after heart transplant, MDRD (median, interquartile range) was calculated in all the available patients (n = 55; n = 46 AA, n = 9 AG/GG). Mann–Whitney test was applied, reaching a statistically significant difference between the two groups (p = 0.001). A dot represents outlier values.
MDRD: Modification of diet in renal disease.

posed that the three of them impair the gene expression, resulting in a lower activity of the efflux pump glycoprotein P, encoded by the gene. Accordingly, the variants (T, T and T/A, respectively) of these SNPs are expected to cause a lower activity of expulsion of the drug out of the enterocyte, and therefore elevation of the drug blood levels [18,19]. In our results, patients with the T variant, presented higher frequency of serious infections and this finding was also correlated to higher CsA levels, supporting the idea that higher CNI levels, related to the SNP variant, could be the cause of excessive immunosuppression and therefore increased susceptibility to infections.

Renal function is one of the most important predictors of survival in HT [20]. Some studies have suggested that certain SNPs could be implicated in the development of acute nephrotoxicity after renal transplantation [21]. We found that patients with changes in SNP rs9282564 from *ABCB1* had poorer renal function 1 year after HT, thus suggesting a predisposition in these individuals. Once again, considering the blood CNI levels in relation to this SNP, we found consistent results: those patients who carried the variant associated with poorer renal function (AG) also had higher CNI levels, statistical significance being reached for CsA group, 2 months after HT, thereby suggesting that the higher drug levels could contribute to renal damage. The impact of this SNP in *ABCB1* gene function is much less known. It makes a change in the protein sequence, Asn21Asp, and *in vitro* studies conclude that G carriers have decreased ability to efflux drugs, which is in agreement with our findings [22].

Rejection is the second most frequent cause of death during the first year after HT. We found higher rejection rates in relation to CC versus CT/TT in SNP rs2066844 of *NOD2/CARD15*, a gene involved in lymphocyte activation. The patients with the variant CC would have higher lymphocyte activation, and therefore greater release of cytokines and more intense inflammation. As previous studies have shown, the proinflammatory state is related to increased rejection rates [23].

It is essential to optimize the use of the currently available immunosuppressant regimens. It is here where pharmacogenetics could play a key role in the immediate future.

The DNA of the recipient cells involved in the transport and metabolism of immunosuppressants is not dis-

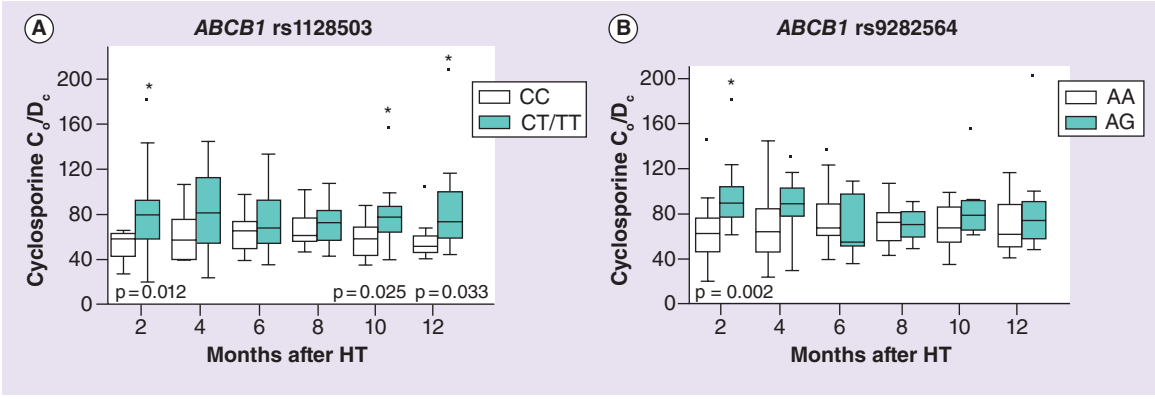

Figure 3. Cyclosporine blood levels during the first year post-transplantation. Cyclosporine corrected trough concentrations C_0/D_c ([ng/ml]/[mg/kg/24 h]) in heart transplant recipients (median, interquartile range) in relation to the *ABCB1* rs1128503 **(A)** and rs9282564 **(B)** genotype. Blood levels were measured every 2 months during 1 year after transplantation.
A dot represents outlier values.
*Statistically significant p-values applying the Kruskal–Wallis and Dunn's multiple comparison tests.
C_0: Trough concentration; D_c: Corrected dose; HT: Heart transplantation.

turbed after HT. It is therefore possible to analyze the SNPs before HT, and thus identify specific problems of each patient before they become clinically visible.

Knowledge of increased patient susceptibility to complications could help prescribe immunosupressants within the correct dosing range, to extend or increase the prophylaxis of infections, or may even allow switching the CNI to some other less nephrotoxic immunosuppressants (everolimus, sirolimus). The aim of this study was to determine whether pharmacogenetics may help in the management of HT patients in the daily clinical setting. The idea was to maintain the real follow-up protocol after HT used in our hospital, which includes controls every 2 months after HT, in the absence of serious events. The purpose was to establish whether pharmacogenetic data may be useful when included in the daily practice, without changing any clinical follow-up routine. It is needless to say that taking only one sample every 2 months to measure immunosuppressant drug levels could mask intrapatient variability. However, our study does not aim to underscore this fact but rather to determine whether despite such variability the drug levels measured every 2 months effectively indicate that something is happening in patient response in relation to the SNPs.

Although an economic study of the costs of these complications has not been made, it is obvious that all these events imply very high costs, both economically and in relation to the patient health and quality of life. Pharmacogenetic tests are becoming increasingly cheaper and are thus more assumable. The incorporation of pharmacogenetic studies to real life clinical practice will depend on the creation of well-designed sets of SNPs which, in a cost–effective manner, can correlate clinical complications to concrete genotypes, taking into consideration the global and complicated treatment of polymedicated patients [12]. Our results contribute to underscore the need for prospective controlled studies with pharmacogenetic analysis prior to transplantation though this is a preliminary study, and its results must be confirmed by larger trials. This is a retrospective pilot study, and the findings therefore must be interpreted within the limitations imposed by these characteristics. Also, the main limitation of the present study is the low number of patients: given the high number of SNPs considered and their relatively low frequency, a much higher number of subjects are required to provide robust evidence of a clinical impact of the study findings. Increasing the number of patients will be the next step before designing new prospective controlled studies.

Conclusion & future perspective

A great deal of work has already been performed in the field of organ transplantation pharmacogenetics. Despite of this, only a few relevant prospective studies in this area, have used SNPs information to tailor immunosuppressant therapy, regarding CNI initial dose. The contribution of SNPs information regarding toxicity and efficacy of these drugs will also play a major role in the near future in order to reach a cor-

Executive summary

- Survival among adult recipients of heart transplantation has increased over the years, but still new advances are required in order to continue improving the success rates. The leading causes of mortality during the first year after heart transplantation are primary allograft failure, infections and allograft rejection. Renal dysfunction is also a matter of concern. All these problems can be related direct or indirectly to the calcineurin inhibitor therapy.
- Both tacrolimus and cyclosporine (CsA) show highly variable pharmacokinetics and pharmacodynamics and have narrow therapeutic indexes. Therapeutic drug monitoring is essential but not enough for predicting drug efficacy and/or related adverse events. Pharmacogenetics can play a relevant role filling this gap.
- Infections were more frequent in patients carrying the T allele in *ABCB1* rs1128503 (57.1% of T carriers had serious infection vs 16.7% in the noncarriers, p = 0.012) and accordingly, this was supported by statistically significant higher levels of CsA at months 2, 10 and 12 post-transplantation, suggesting excessive immunosuppression.
- In *ABCB1* rs9282564, the G allele carriers showed lower glomerular filtration rate (calculated by modification diet in renal disease formula), and accordingly in these patients the blood CsA levels were higher, suggesting toxicity, reaching statistical significance in month 2 after transplantation.
- A relevant tendency was observed for *NOD2/CARD15* rs2066844: 76.1% of the CC carriers suffered allograft rejection versus 33.3% of the CT/TT carriers (p = 0.050). Although statistical significance was not reached, the severity of the outcome makes the data worthy of being noted and followed in future studies.
- Although this study contains a small number of patients, and they are split into tacrolimus or CsA, treatment, both drugs share most of the transporters, metabolizers and targets. For this reason, we think that the results are relevant to be considered in both settings. Increasing the sample size will confirm the interest of the presented findings.

rect personalized treatment for such a relevant chronic therapy.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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