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ORIGINAL ARTICLE

# Pharmacogenetic analysis of the absorption kinetics of cyclosporine in a population of Spanish cardiac transplant patients

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**KEYWORDS**
Pharmacogenetics;
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**Abstract**
*Objective:* To determine how single nucleotide polymorphisms located on genes *MDR1, CYP3A4,* and *CYP3A5* affect the absorption kinetics of cyclosporine in cardiac transplant patients.
*Method:* We selected a sample of 30 adult patients having previously undergone a primary cardiac transplant and who had received cyclosporine as an immunosuppressant. During the first month after the transplant, we performed a pharmacokinetic study of each patient to determine values in the cyclosporine concentration area under the 12-hour curve, steady-state cyclosporine concentration, maximum cyclosporine concentration, and time to reach that concentration. Single nucleotide polymorphisms were genotyped in all patients: *MDR1 3435C > T, CYP3A4-390A > G*, and *CYP3A5 6986A > G*.
*Results:* Being a carrier of the T-allele for polymorphism *MDR1 3435C > T* is associated with higher values in the cyclosporine concentration area under the 12-hour curve ($P$=.01) and in steady-state cyclosporine concentration ($P$=.05), compared with those from patients who do not carry that allele.
*Discussion:* Our results show that genotype differences in *MDR1 3435C > T* can explain part of the variability in cyclosporine absorption among individuals in the population of Spanish cardiac transplant recipients.



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**PALABRAS CLAVE**
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Farmacocinética

**Análisis farmacogenético de la cinética de absorción de ciclosporina en una población española de pacientes trasplantados cardíacos**

**Resumen**
*Objetivo:* Determinar el papel de polimorfismos de nucleótido único localizados en los genes *MDR1, CYP3A4* y *CYP3A5* sobre la cinética de absorción de ciclosporina en pacientes trasplantados cardíacos.
*Método:* Se seleccionó una muestra de 30 pacientes adultos sometidos a un primer trasplante de corazón que habían recibido ciclosporina como tratamiento inmunosupresor. En el primer mes después del trasplante se realizó un estudio farmacocinético a cada paciente para determinar los valores del área de concentración de ciclosporina bajo la curva de 12 h, concentración de ciclosporina en estado de equilibrio, concentración de ciclosporina máxima y el tiempo en alcanzar dicha concentración. En todos los pacientes se genotipificaron los polimorfismos de nucleótido único: *MDR1 3435C > T, CYP3A4-390A > G* y *CYP3A5 6986A > G*.
*Resultados:* Ser portador del alelo T para el polimorfismo MDR1 3435C > T se asoció a valores mayores de área de concentración de ciclosporina bajo la curva de 12 h ($p = 0,01$) y de concentración de ciclosporina en estado de equilibrio ($p = 0,05$), en comparación con los pacientes no portadores de dicho alelo.
*Discusión:* Nuestros resultados muestran que las diferencias genotípicas de *MDR1 3435C > T* podrían explicar parte de la variabilidad interindividual en la absorción de la ciclosporina en la población española de trasplantados cardíacos.



## Introduction

Cardiac transplantation continues to be the definitive treatment in patients suffering from terminal cardiac failure. Cyclosporine A (CsA) is one of the most frequently used drugs to avoid rejection in this type of patient. As is the case with other calcineurin inhibitors, its pharmacokinetics is characterised by the presence of a generally poor, highly variable, and unpredictable oral bioavailability and great interindividual variability during first-pass metabolism and systemic clearance.[1] For some years now it has been known that a great deal of such variability is due to differences of activity in the glycoprotein P-gp, CYP3A4 and CYP3A5.[2]

The product of the gen *MDR1 (ABCB1)* is the glycoprotein P-gp which belongs to the family of ABC membrane transporters (subfamily B). Its ATP dependent activity consists of acting as a pump for a wide range of both endogenous and exogenous substances including immunosuppressant drugs used in cardiac transplant patients such as calcineurin inhibitors, sirolimus and corticosteroids, in such a way that it eliminates these drugs from the cytoplasm and moves them to extracellular space.[3]

The CYP enzymatic family is made up of more than 50 casual isoenzymes of the oxidative metabolism of many endogenous and exogenous compounds.[4] The CYP3A subfamily, which represents the majority of CYP proteins in the human liver, metabolises more than 50% of all drugs used today.[5] It is made up by the isoenzymes CYP3A4, CYP3A5, CYP3A7, and CYP3A43. CYP3A4 and CYP3A5 have a very similar substrate specificity profile and are, quantitatively, the most important forms of this subfamily.

In recent years various studies have been published on the effect that the genotype of single nucleotide polymorphisms (SNP) of the genes *MDR1, CYP3A4,* and *CYP3A5* may have in the function of the proteins which they codify and therefore in the pharmacokinetics of the CsA. In spite of the evidence accumulated in this time, there are still discrepancies in the clinical interpretation of the results of such studies.[6] One of the factors which may help to explain such discrepancies is the highly diverse ethnical origin of the populations in which the different studies have taken place. The genetic context of each population is an element of confusion which introduces important differences into the population frequencies of the allele of these SNP, which makes it difficult to extrapolate to the Spanish population results obtained in other populations genetically distant.

As no such study has been carried out to date amongst patients from our area, in this study we proposed determining the role of *MDR1 3435C > T, CYP3A4-390A > G, and CYP3A5 6986A > G* in the absorption kinetics of CsA in a Spanish population of adult cardiac transplant patients.

## Methods

### Population

The study involved a total of 30 patients of Caucasian origin (23 males and 7 females) who had an average (standard deviation) in age of 43 (14) (18-67) years when they underwent their first orthopaedic cardiac transplant in the Hospital Universitario Reina Sofia (Córdoba). All patients received treatment with CsA together with corticosteroids, mycophenolate mofetil or azathioprine, or sirolimus, associated or not to induction treatment using basiliximab or ATG. The treatment with CsA began within the first 24 hours following the transplant, the dosage was 4 mg/kg/day divided into 2 doses. Then the dosage was modified to

achieve a recommended therapeutic range for the pre-dose concentration of CsA (C0) of 200-400 ng/mL during the first month after the transplant.

## Genotyping

The genotyping of each SNP was carried out by means of *polymerase chain reaction-restriction fragment length polymorphism* (PCR-RFLP). For each PCR reaction we used 300 ng of genomic DNA, 0.2 µL of Taq polymerase 5 U/µL, 2 µL of 10x PCR Buffer without $Mg^{2}$+, 1.6 µL of $MgCl_2$ 25 mmol, 1.6 µL of dNTPs 2 µmol (Dominion MBL, Cordoba, Spain) and 1 µL (15 pmol/µL) of each primer (Isogen Life Bioscience BV, Maarssen, the Netherlands), all in a final volume of 20 µL of $H_2O$. The sequences of the primers used for each SNP can be seen in Table 1. The products obtained in each PCR were subjected to digestion with a restriction enzyme, separated by means of non-denaturalising electrophoresis in an agarose gel (2%) and finally, visualised in the Gel Printer Plus ultraviolet transilluminator (Tecnología para Diagnóstico e Investigación S.A., Madrid, Spain).

***MDR1 3435C > T***
The DNA was denaturalised at 94ºC for 5 minutes, followed by 40 cycles each of which consisting of a first denaturalisation phase (94ºC, 35 s), another of alignment (56ºC, 35 s) and finally one of elongation (72ºC, 40 s), with an final extension of 7 min at 72ºC. Next, 10 µL of the product obtained from the PCR were subjected to digestion with 10 U of the Mbol restriction enzyme and 2 µL 10x Buffer R (Fermentas, Madison, United States) in a final volume of 30 mL for 16 h at 37°C. The digestion fragments corresponded to genotypes TT (390pb), CT (390pb + 230pb + 160pb), and CC (230pb + 160pb).

***CYP3A4-290A > G***
The DNA of the sample was denaturalised at 94ºC for 5 minutes, followed by 40 cycles each of which consisting of a first denaturalisation phase (94ºC, 35 s), another of alignment (59ºC, 35 s) and finally one of elongation (72ºC, 40 s), with a final extension of 7 min at 72ºC. Next, 10 µL of the product obtained from the PCR was subjected to digestion with 5 U of the Mbol restriction enzyme and 2 µL 10x Buffer B (Fermentas, Madison, United States) in a final volume of 30 µL for 16 h at 37°C. The products obtained were separated by means of non-denaturalising agarose electrophoresis, at 4% in this case. The digestion fragments corresponded to the genotypes GG (210pb + 175pb), AG (210pb + 175pb + 169pb + 41pb), and AA (175pb + 169pb + 41pb).

***CYP3A5-6986A > G***
When designing the primers for the PCR reaction of this SNP, a series of modifications were made to the sequences obtained with Primer3, in such a way that in the oligonucleotide in Fw a nucleotide was changed to thereby eliminate the Sspl natural recognition site and in the Rev oligonucleotide a site was generated which gave rise to the splice by said enzyme in the case of R=A. With each sample, the DNA was denaturalised at 94°C for 5 min, followed by 40 cycles, each of which consisting of a first denaturalisation phase (94ºC, 35 s), another of alignment (52ºC, 35 s) and finally one of elongation (72ºC, 40 s), followed by a final extension of 7 min at 72ºC. Next, 10 µL of the product obtained from the PCR were subjected to digestion with 10 U of Sspl 2 µL 10x Buffer G (Fermentas, Madison, United States) in a final volume of 30 µL for 16 h at 37°C. The digestion fragments corresponded to the genotypes AA (167pb + 50pb), AG (217pb + 167pb + 50pb), and GG (217pb).

## Pharmacokinetic analysis

*Sample extraction* The determination of the concentrations of CsA was made with total blood samples, using EDTA as anticoagulant. Extractions were made once the drug had reached a state of stationary balance (that is, at least after 3 days without requiring dosage modification). To calculate the $AUC_{0\text{-}12\ h}$, the times of extraction were 0.5, 1, 2, 3, 4, 6, 8, and 12 hours after the morning dose of CsA.

*Method of determination.* The concentrations of CsA in the blood were determined by FPIA-Axsym fluorescence polarization immunoanalysis (Abbott Diagnostics, Abbott Park, United States).

*Pharmacokinetic parameters.* Derived from non-compartmentalised methods, the pharmacokinetic parameters were the maximum observed concentration ($C_{max}$, ng/mL), the time needed to achieve the maximum observed concentration ($T_{max}$, h) and the area of concentration under the curve between 0 and 12 hours after the dose ($AUC_{0\text{-}12}$,

**Table 1** Sequence of the primers used for genotyping the polymorphisms analysed

| SNP (dbSNP) | Primer sequences |
|---|---|
| *MDR1 3435C > T* | Fw: 5'-TGT TTT CAG CTG CTT GAT GGC AAA-3' |
| (rs1045642) | Rev: 5'-GGT AAC AAC TAA CCC AAA CAG GA-3' |
| *CYP3A4-290A > G* | Fw: 5'-GGA ATG AGG ACA GCC ATA GAG ACA AGG GGA-3' |
| (rs11539969) | Rev: 5'-CCT TTC AGC TCT GTG TTG CTC TTT GCT G-3' |
| *CYP3A5 6986A > G* | Fw: 5'-CAA TTT TTC ACT GAC CTC ATA TTC T-3' |
| (rs776746) | Rev: 5'-TGC GTT CCG AAG TAT ACT ACT ACC CAT TAC ACC AGG TTT GTC CCT TCT TTA T-3' |

dbSNP *indicates single nucleotide polymorphism database reference number;* SNP. *single nucleotide polymorphism.*

ng/h/mL), calculated by the trapezoidal method and the plasmatic concentration in the stationary state (Css, ng/mL), calculated as Css = $AUC_{0\text{-}12\,h}$/ dosage interval. The $AUC_{0\text{-}12}$ values were also adjusted according to the dose (D, mg/kg) and weight (kg) of each patient, thereby obtaining the variables $AUC_{0\text{-}12}$/D, and $AUC_{0\text{-}12}$/D/weight.

## Ethical aspects

The analysis was carried out adhering to the fundamental principles established in the Declaration of Helsinki (1964), the Council of Europe Convention on Human Rights and Biomedicine (1997) the UNESCO Universal Declaration on the Human Genome and Human Rights (1997) and also in line with the requirements established by Spanish legislation in the areas of biomedical investigation, the protection of personal data and bioethics. The project underwent evaluation by the Ethics and Clinical Investigation Committee of the Reina Sofia University Hospital. All patients received information about the analysis and written informed consent was requested before taking part therein.

## Data analysis

To examine population homogeneity, the genotypic frequencies of the different SNPs were analysed against the expected frequencies according to the Hardy-Weinberg equilibrium, using the $\chi^2$ test with Yates' correction for continuity. The Shapiro-Wilk test was used to check the level of conformance to a normal distribution model of the values of the different quantitative variables. The results are expressed as a median and their variability is presented, in brackets, as an interval between the first and the third quartile. For hypothetical contrasts the Kruskal-Wallis test was used. The values of the different variables were processed and analysed in an R free computing environment (GNU). Values of <.05 were considered statistically significant.

# Results

The allele frequency distribution of the genotyped SNPs in our population was in accordance with the Hardy-Weinberg equilibrium. *MDR1 3435C > T* (CC, 8; CT, 16; TT, 6; $\chi^2$=0.386; *P*=.534; D=0.827), *CYP3A4-390A > G* (GG, 26; GA, 4; AA, 0; $\chi^2$=1.028; *P*=.310; D=0.134) and *CYP3A5 6986A > G* (TT, 27; TG, 3; GG, 0; $\chi^2$=2.457; *P*=.116; D=0.075).

Due to the small size of the sample, we proceeded to regroup the genotypes of the patients according to the presence or the lack of one of the 2 alleles, thereby gaining statistical power. As a result, Table 2 shows the results of the analysis carried out to find differences in the pharmacokinetic parameters on account of the presence or the lack of the distinct majority alleles of *MDR1 3435C > T*, *CYP3A4-390A > G*, and *CYP3A5 6986A > G* in our population. Allele T carriers for the polymorphism *MDR1 3435C > T* presented greater values of $AUC_{0\text{-}12}$ (*P*=.01) and of Css (*P*=.05) in comparison to patients who were not carriers of said allele, without there being statistically significant differences in the other pharmacokinetic parameters analysed.

**Table 2** Differences in the pharmacokinetic parameters according to the genotypes of *MDR1 3435C > T, CYP3A4-390A > G* and *CYP3A5 6986A > G*

| SNP (dbSNP) | Genotypes | AUC0-12 (ng/h/mL) | AUC0-12/D (ng/h/mL/mg) | AUC0-12/D/weight (ng/h/mL)/(mg/kg) | $T_{max}$, h | $C_{max}$N, ng/mL | Css, ng/mL |
|---|---|---|---|---|---|---|---|
| *MDR1 3435C > T* | TT/TC | 6686 | 40.1 | 2508 | 2 | 1137 | 554 |
| *rs10456422* | (n=22) | (5583-7050) | (27.7-47.2) | (2111-2927) | (1-2) | (1030-1380) | (481-587) |
| | CC (n=8) | 4569 | 31.6 | 2479 | 2 | 1326 | 340 |
| | | (4165-4811) | (23.7-38.7) | (1960-3059) | (1-2) | (865-1511) | (301-435) |
| | *P* | 0.010 | 0.241 | 0.815 | 0.794 | 0.959 | 0.05 |
| *CYP3A4-390A > G* | AA | 6188 | 34.5 | 2333 | 2 | 1107 | 554 |
| rs2740574 | (n=26) | (4701-6977) | (27.0-45.2) | (1931-2917) | (1-2) | (975-1380) | (460-593) |
| | GA/GG | 5420 | 50.8 | 3104 | 2 | 1.340 | 562 |
| | (n=4) | (5084-6522) | (41.2-52.5) | (3015-3432) | (1.50-2) | (1306-1364) | (507-598) |
| | p | 0.833 | 0.202 | 0.239 | 0.771 | 0.372 | 0.762 |
| *CYP3A5 6986A > G* | GG | 5970 | 32.4 | 2333 | 2 | 1137 | 554 |
| rs776746 | (n=27) | (4694-6913) | (27.0-46.1) | (1931-2929) | (1-2) | (1000-1380) | (459-593) |
| | GA/AA | 7052 | 43.8 | 2927 | 2 | 1188 | 587 |
| | (n=3) | (6236-7364) | (42.1-49.0) | (2874-3021) | (1.50-2) | (1078-1288) | (519-613) |
| | *P* | 0.285 | 0.175 | 0.516 | 0.770 | 0.893 | 0.656 |

$AUC0_{\text{-}12}$ indicates area of concentration beneath the curve between 0 and 12 hours after the dose; $AUC0_{\text{-}12}$/D, area of concentration beneath the curve between 0 and 12 hours after the dose; $AUC0_{\text{-}12}$/D/weight, area of concentration beneath the curve between 0 and 12 hours after the dose, dose and weight-normalised; $C_{max}$, maximum concentration observed; Css, average concentration in a state of equilibrium; $T_{max}$, time taken to achieve the maximum observed concentration.
The values are expressed as a median (first quartile -third quartile). For hypothesis contrasts, the Kruskal-Wallis test was used and differences were considered statistically significant when the P value was <.05.

## Discussion

To date, studies aimed at analyzing pharmacogenetic aspects of CsA have not found a clear relation between the different *MDR1, CYP3A4* and *CYP3A5* polymorphisms and their pharmacokinetic parameters.[7-9] Min et al carried out an analysis (14 healthy volunteers of which 11 were of African-American origin and 3 Caucasian) of the effect of *MDR1 3435C > T* and *CYP3A4-290A > G* in the kinetic of absorption of the CsA ($AUC_{0\text{-}24\ h}$, $T_{max}$, $C_{max}$, $t_{1/2}$, and CL/F).[10] They found that carriers of the A allele of *CYP3A-290A > G* presented greater values of $AUC_{0\text{-}24\ h}$/D and lesser values of CL/F. With regard to *MDR1 3435C > T*, although the $C_{max}$ and the el $AUC_{0\text{-}24\ h}$ in the CT and TT Group was 15% and 22 % greater than in the CC, neither of these differences were statistically significant. Anglicheau et al[11] studied in 100 renal transplant patients of Caucasian origin, in a stable condition, the influence of the polymorphisms *MDR1 [−129T > C, 1236C > T, 2677G > (T/A), 3435C > T],* and *CYP3A5 6986A > G* in concentration values of the dose of CsA ($C_0$), $C_{max}$, $AUC_{0\text{-}4\ h}$, $AUC_{0\text{-}12\ h}$, in absolute values and normalised by the dose. They only found a weak association between *MDR1 1236C > T* and the values of $C_{max}$/D and $AUC_{0\text{-}4}$/D which they considered insufficient for dose optimisation in clinical practice. None of the pharmacokinetic parameters were associated with *CYP3A5 6986A > G*. Mai et al[12] carried out a retrospective analysis of the effect of different MDR1 haplotypes in the pharmacokinetics of CsA on 98 renal transplant patients of Caucasian origin in a stable condition. They observed no differences in their analysis of haplotypes in the $C_0$ values and of CsA concentration 2 hours after the dose, ($C_2$) nor $AUC_{0\text{-}12\ h}$. Kuzuya et al[13] carried out a study of 97 renal transplant patients of Asian origin amongst whom they analysed the effect of *MDR1 [−129T > C, 1236C > (T/A), 3435C > T].* The interval since the transplant was of 2-17 (average, 7) years. The pharmacokinetic parameters analysed were $AUC_{0\text{-}2\ h}$, $C_{max}$ and $C_{min.}$ These authors did not observe significant differences between the required does of CsA and the *MDR1* polymorphisms studied.

On the other hand, other studies do exist which do find, in a manner similar to ours, a relation between some *MDR1, CYP3A4,* and CYP3A5 polymorphisms and the pharmacokinetic parameters of the CsA. As a result, the study carried out by Balram et al[14] on a subgroup of Asian cardiac transplant patients demonstrated that patients with the CC genotype presented an $AUC_{0\text{-}4\ h}$ 11% less than carriers of the TT genotype. In our study we observed an even greater difference as TT genotype carriers presented an $AUC_{0\text{-}12\ h}$ 5.3% and 38% greater in comparison to CT and CC genotype carriers, respectively. Chowbay et al[15] analysed, in 275 healthy volunteers and 14 Asian cardiac transplant patients, the influence of *MDR1 [1236C > T, 2677G > (T/A), 3435C > T]* and *CYP3A-290A > G* in the values of $AUC_{0\text{-}12\ h}$, $AUC_{0\text{-}4\ h}$, $C_{max}$ and $C_{min}$ in stable condition after the transplant. These authors found that the values of $AUC_{0\text{-}12\ h}$, $AUC_{0\text{-}4\ h}$ and $C_{max}$ were greater in those patients presenting the *MDR1* haplotype T-T-T. In line with the above, Barnard et al[16] recently observed that greater doses of CsA were necessary for the *MDR1* genotype CC carriers, in comparison to CT and TT carriers, for 3 and 12 months after the transplant.

Unlike other authors, we could not observe significant differences in the kinetic parameters analysed for the SNP of the *CYP3A4 and CYP3A5* genes, probably due to insufficient statistical power due to the low allele frequency of the SNP analysed and our study's limited sample size.

The determining factors for the different results observed were the following: genotypic variability associated to the race/ethnic group of the chosen populations, the type of transplant, the variables chosen for the pharmacokinetic analysis as well as the point in time following surgery in which the study was carried out. These would justify, in part, some of the discrepancies in the results of the studies published to date. For example, with regard to the distribution of the frequencies of the *MDR1 3435C > T* alleles, great differences according to the race of the population studied have been observed.[17] Thus, in African-American population the C allele is much more predominant that in Caucasian population, which implies that the majority of people of African-American origin present a CC genotype, associated to a greater expression of P-gp. However, there is a low frequency of the C allele in Asians. Therefore, these variations have an influence on the studies results on the bioavailability of many drugs, including the CsA. We consider that it is important to carry out a study on the pharmacogenetic factors which may influence the kinetic of CsA absorption based on the genotype frequencies of said SNP in the Spanish population.[18]

To conclude, our results are in accordance with the data published by certain authors in respect of the *MDR1* polymorphism *3435C > T* because the genotype differences of said SNP may explain part of the interindividual variability in the absorption of CsA observed in the population in our context.

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