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High-Resolution Melting Assay for Genotyping Variants of the CYP2C19 Enzyme and Predicting Voriconazole Effectiveness

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ABSTRACT Voriconazole is a triazole antifungal agent recommended as primary treatment for invasive aspergillosis, as well as some other mold infections. However, it presents some pharmacokinetic singularities that lead to a great variability intra- and interindividually, nonlinear pharmacokinetics, and a narrow therapeutic range. Most experts have recommended tracing the levels of voriconazole in patients when receiving treatment. This azole is metabolized through the hepatic enzyme complex cytochrome P450 (CYPP450), with the isoenzyme CYP2C19 being principally involved. Allelic variations (polymorphisms) of the gene that encodes this enzyme are known to contribute to variability in voriconazole exposure. Three different allelic variants, CYP2C19*17, CYP2C19*2, and CYP2C19*3, could explain most of the phenotypes related to the voriconazole metabolism and some of its pharmacokinetic singularities. We designed a rapid molecular method based on high-resolution melting to characterize these polymorphisms in a total of 142 samples, avoiding sequencing. Three PCRs were designed with similar cycling conditions to run simultaneously. The results showed that our method represents a fast, accurate, and inexpensive means to study these variants related to voriconazole metabolism. In clinical practice, this could offer a useful tool to individually optimize therapy and reduce expenses in patients with fungal infections.

KEYWORDS CYP2C19, high-resolution melting, voriconazole, pharmacogenomics, polymorphisms

Invasive fungal disease (IFD) is one the most threatening complications of prolonged and profound neutropenia and is a major cause of morbidity and mortality in transplant and hematological patients, with mortality rates ranging from 40 to 90% (1). Therefore, early diagnosis and a proper antifungal prescription have a direct impact on patient outcomes (2).

Triazoles are currently the preferred agents for the treatment and prevention of IFDs in most patients (3, 4). One of the new-generation triazole antifungal agents with broad-spectrum activity is voriconazole (VCZ). This azole is approved for the treatment of invasive aspergillosis, candidemia in nonneutropenic patients, disseminated *Candida* infections, and esophageal candidiasis, as well as infections caused by *Scedosporium* spp. and *Fusarium* spp. (5). However, despite its widespread use, optimizing the dose and efficacy is still a challenge due to significant interpatient variability in blood exposure. As a result, experts around the world recommend VCZ monitoring by

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measuring the trough levels (i.e., the drug level at the moment just prior to the following dose administration).

VCZ is mainly metabolized in the liver by the cytochrome P450 (CYP) enzymes, mainly by CYP2C19 isoenzyme and to a lesser extent by CYP3A4 and CYP2C9 (6). Allelic variations of the genes that encode these enzymes are known to contribute to variability in VCZ exposure (7, 8). Concerning the CYP2C19 gene, 35 variant alleles have been identified (https://www.pharmvar.org/) (9). Most of the variant alleles CYP2C19*2 and CYP2C19*3 are frequently associated with decreased enzyme activity, while the *17 variant, located at the gene promoter, is related to increased CYP2C19 transcription, most probably due to the modulation of GATA4 binding (10). Individuals can be classified in the following categories (depending on the genotype and the functionality of this isoenzyme to metabolize its substrate): ultrarapid metabolizer (UM; CYP2C19*17/*17), rapid metabolizer (RM; CYP2C19*1/*17), extensive metabolizer or normal metabolizer (EM or NM; CYP2C19*1/*1), intermediate metabolizer (IM; CYP2C19*1/*2, CYP2C19*1/*3, and CYP2C19*2/*17), and poor metabolizer (PM; CYP2C19*2/*2, CYP2C19*2/*3, and CYP2C19*3/*3) (11, 12). The CYP2C19*17 allele is a gain-of-function variant arising from a single nucleotide polymorphism (SNP) in the gene promoter region. The UM and RM phenotypes carrying this allelic variant present increased enzyme activity compared to the normal or extensive metabolizer (13). Conversely, the *2 and *3 alleles are loss-of-function variants. The corresponding IM category involves a single loss-of-function variant and significant reduction in enzyme activity compared to the EM, whereas PMs with two loss-of-function variants have no enzyme activity (14).

Numerous factors have been shown to influence VCZ exposure, such as age and patient weight, comorbidities, renal and hepatic function, comedications, and also the individual´s CYP2C19 genetic background (15, 16). In general terms, it is estimated that 15 to 30% of the drug response variability is caused by genetic polymorphisms (17). Recent reports have tried to clarify the influence of genetic characteristics on an individual´s response to VCZ treatment. While several studies strongly suggest that genetic variants may significantly affect VCZ trough plasma concentrations, other authors consider that nongenetic factors such as the route of administration, liver function, C-reactive protein level, and patient age might contribute more than the genetic context. For instance, Andes et al. identified the CYP2C19 enzyme as primarily responsible for the VCZ metabolism (18). Other studies demonstrated the consequences of VCZ rapid metabolism and the potential impact of CYP2C19 genetic variants (19–21), and Gautier-Veyret and other authors have shown that VCZ variability could be partially explained by the combination of the genetic score that takes into account both CYP2C19 and 3A genotypes, the VCZ route of administration, and any comedication (22–25). Recently, Veringa et al. showed that VCZ metabolism is decreased during inflammation, resulting in higher VCZ trough concentrations, reinforcing the need of frequent monitoring of VCZ serum concentrations during and following severe inflammation (26). Also, You et al. suggested that age, CYP2C19 genetic status, and liver function status are the strongest predictors of VCZ concentration outside the therapeutic range (16).

According to these reported results, understanding how genetic variability affects efficacy, safety, or drug interaction for this antifungal would be useful for optimally dosing this azole, avoiding therapeutic failure, or mitigating drug interactions and even suggesting a change of treatment.

The aim of this study was to establish a new molecular tool that improves the management of the patients treated with VCZ. A simple, rapid, and cost-effective method based on HRM methodology for screening three genetic variants in the CYP2C19 enzyme (*17, *2, and *3) was standardized and validated. CYP2C19*17 is the most frequent variant in individuals from the European ancestry associated with VCZ subtherapeutic levels. Rapid knowledge of the allelic profile of these variants and the VCZ level would allow optimizing the treatment of the patient and improving outcome. This tool,
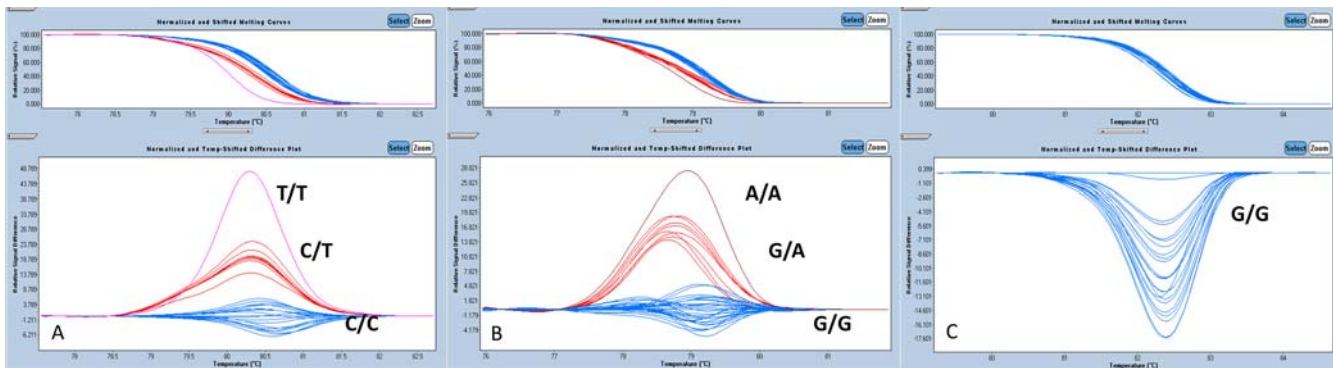
FIG 1 Representative HRM profiles obtained for each of the targets amplified. The upper graphs show normalized melting curves, and the lower graphs show different plot representations (samples with the similar melting curves are indicated by the same color). (A) CYP2C19*17 and CYP2C19*17/*17 (T/T, pink cluster, control genotype); CYP2C19*1/*17 (C/T; red cluster); CYP2C19*1/*1 (C/C; blue cluster). (B) CYP2C19*2 and CYP2C19*2/*2 (A/A; brown cluster, control genotype); CYP2C19*1/*2 (G/A; red cluster); CYP2C19*1/*1 (G/G; blue cluster). (C) CYP2C19*3 and CYP2C19*1/*1 (G/G; blue cluster).

together with the therapeutic drug monitoring, might guide clinicians to customize treatments by dosage modification in order to maximize efficacy and reduce toxicity.

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RESULTS

Genotyping of different CYP2C19 variants. HRM for CYP2C19*17 was first standardized by using the 78 samples belonging to the Biobank. The results were verified by sequencing. The allele and genotype frequencies of this SNP were in line with those reported previously (9) and consisted of 49 samples (62.8%) that presented the wild-type genotype CYP2C19*1/*1, C/C, 27 samples (34.6%) that presented the mutated CYP2C19*1/*17 allele (corresponding to an SNP in the −806 position, C/T genotype), and 2 samples (2.5%) that presented the double mutated CYP2C19*17/*17 allele T/T. One of each genotype was selected as a control for subsequent assays.

Second, using the standardized method, the analysis was replicated in the 53 samples from patient at-risk of invasive aspergillosis and extended to the genotyping of CYP2C19*2 and CYP2C19*3 variants. These 53 subjects were from European ancestry (20 male and 33 female) and a heterogeneous group in terms of age (18 to 67 years), treatment outcome, or liver toxicity. The quantitative PCR (qPCR)-amplified fragments of 174, 199, and 148 bp for CYP2C19*17, CYP2C19*2, and CYP2C19*3, respectively, the cycle thresholds of the amplification being lower than 30 for all the samples. After the HRM analysis, melting curve clusters for different genotypes were obtained for each variant (Fig. 1). For the variant CYP2C19*17, three melting curves clusters allowed C/C, C/T, and T/T allelic differentiation (Fig. 1A). This also happened in the case of CYP2C19*2 (G/G and G/A) and CYP2C19*3 (G/G) (Fig. 1B and C).

The frequency of CYP2C19*1/*1 (C/C) and CYP2C19*1/*17 (C/T) genotypes among the 53 samples studied were 64.15 and 35.65%, respectively. The CYP2C19*17/*17 (T/T) genotype was not detected (Fig. 2, included as a positive control). In relation to CYP2C19*2, 18.87% of samples showed A/G genotype, and 81.13% were wild type (G/G). The CYP2C19*2/*2 (A/A) genotype was not detected. Regarding CYP2C19*3, all patients were G/G for this allele. CYP2C19*3/*3 (A/A) and CYP2C19*3/*1 (A/G) were not detected.

In this study, the SNPs were successfully genotyped by HRM. In addition, the results were 100% concordant with those obtained by the specific TaqMan assay and Sanger sequencing (data not shown).

Correlation of genotype with observed phenotype. A total of 11 patients were recruited to study the correlation between genotype and VCZ exposure. Four of eleven patients (36.36%) presented the mutated CYP2C19*1/*17 allele (Table 1). The remaining
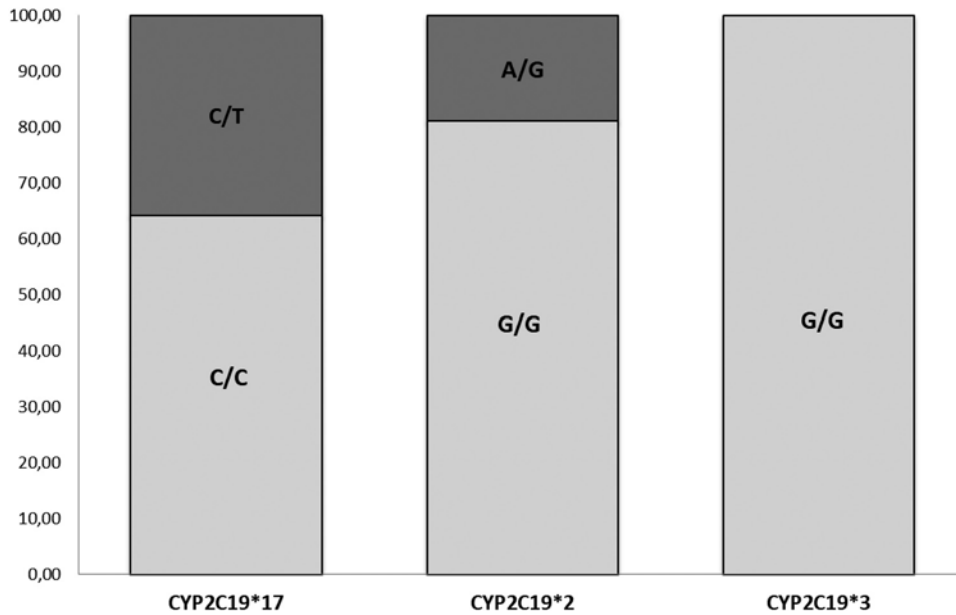
FIG 2 Variant allele frequency results for 53 subjects determined by HRM.

patients (7/11, 63.63%) showed the wild-type genotype (CYP2C19*1/*1). The same proportion could be observed for the CYP2C19*2 variant, accounting for 36.36% of the patients with the mutated genotype (CYP2C19*1/*2), while the remaining 63.63% presented the wild-type genotype. All samples (100%) presented the wild-type genotype for the CYP2C19*3 variant. The predicted phenotype resulted from the combination of the three genotypes studied (Table 1).

VCZ and VCZ N-oxide concentration were measured by HPLC/UV from each patient sample as described above. The metabolic rate (*mr*), established as the ratio between VCZ N-oxide/VCZ, was calculated in order to estimate the metabolic capacity of the enzyme CYP2C19 and to relate it to the predicted phenotype (Table 1). Among the patients classified as extensive metabolizers (EM, CYP2C19*1/*1, nonmutated alleles), the *mr* values ranged between 0.73 and 0.93. From patient number 2, the *mr* was not calculated as no VCZ N-oxide was detected. Three additional patients were considered rapid metabolizers or RM, with a mutated allele in the gene promoter region (CYP2C19*1/*17), which may confer on them an increased enzyme activity. The calculated *mr* from these patients was ≥2 in two patients (2.8 and 4.3, respectively). Patient 5 showed an *mr* value (0.72) that cannot be explained by the genotype

TABLE 1 Correlation between genotype/phenotype and VCZ *mr*

| Patient | Sample type | CYP2C19*17 | CYP2C19*2 | CYP2C19*3 | Genotype | Predicted phenotype[a] | Concn (mg/liter) | | *mr*[b] |
							VCZ	VCZ N-oxide	
1	Serum	*1/*1	*1/*2	*1/*1	*1/*2	IM	2.30	1.76	0.76
2	Plasma	*1/*1	*1/*1	*1/*1	*1/*1	EM	1.32	ND[c]	ND
3	Plasma	*1/*17	*1/*1	*1/*1	*1/*17	RM	0.56	1.57	2.80
4	Serum	*1/*17	*1/*1	*1/*1	*1/*17	RM	0.61	2.61	4.30
5	Serum	*1/*17	*1/*1	*1/*1	*1/*17	RM	3.45	2.48	0.72
6	Plasma	*1/*1	*1/*1	*1/*1	*1/*1	EM	2.77	2.20	0.79
7	Plasma	*1/*1	*1/*2	*1/*1	*1/*2	IM	3.86	2.53	0.65
8	Plasma	*1/*1	*1/*1	*1/*1	*1/*1	EM	2.67	1.96	0.73
9	Plasma	*1/*17	*1/*2	*1/*1	*2/*17	IM	1.96	3.19	1.63
10	Plasma	*1/*1	*1/*2	*1/*1	*1/*2	IM	3.91	3.25	0.83
11	Serum	*1/*1	*1/*1	*1/*1	*1/*1	EM	2.90	2.69	0.93

[a]RM, rapid metabolizer; EM, extensive metabolizer; IM, intermediate metabolizer.
[b]*mr*, metabolic rate (i.e., the VCZ N-oxide/VCZ ratio).
[c]ND, not determined.

found. The rest of the patients were classified as intermediate metabolizers (IM), with an *mr* ranging between 0.65 and 1.63. None of the patients were classified as poor metabolizers (PM).

DISCUSSION

As the CYP2C19 genotype influences VCZ metabolism, the development of easy and well-standardized tools to characterize it appears to be very useful for predicting patient response to treatment.

Currently, U.S. Food and Drug Administration has approved different methods of CYP2C19 genotyping (https://www.fda.gov/MedicalDevices/ProductsandMedical Procedures/InVitroDiagnostics/ucm330711.htm). However, these methods have high operating and maintenance costs and require high-precision equipment, making them unavailable in most laboratories (27). Thus, CYP2C19 genotyping has generally been performed using directed sequencing (the gold standard for mutation detection) or the PCR-restriction fragment length polymorphism method. In addition, there are other methodologies such as the real-time PCR (qPCR) that use specific probes or intercalating agents to provide high specificity, flexibility, efficiency, and lower cost (19).

The objective of this research was to develop a simple and accurate method for simultaneous genotyping of three CYP2C19 variants (commonly named as CYP2C19*2, CYP2C19*3, and CYP2C19*17), based on a high-resolution melting curve (HRM) analysis. This method is expected to be easily applied in clinical laboratories in order to guide individual VCZ dosage (28), instead of using different and expensive commercial kits. On the other hand, some of the kits require array platforms only available in specialized laboratories, whereas the HRM method could be performed using a qPCR machine, which is common equipment of most clinical laboratories. This method allowed the characterization of three genetic variants in a collection of 142 samples, reducing the time for analysis and the cost per sample in comparison to other genotyping methods (TaqMan probes or sequencing analysis) (29). The results obtained were 100% concordant between the three techniques. Also, by using the HRM approach, the detection of these variants allowed us to establish the frequency of the different SNPs in our study population.

Approximately 35% of the studied samples presented the CYP2C19*1/*17 variant (C/T polymorphism). This polymorphism (rs12248560, PhamVar PV00097), located at position −806 of the gene (C>T) (27), is able to specifically bond nuclear proteins in its 5′ end and consequently improves the transcription of CYP2C19 (20). The presence of this variant and the double mutated CYP2C19*17/*17 genotypes (C/T and T/T, respectively) have been classified as both rapid and ultrarapid metabolizers (RM and URM). This genetic background involves a higher activity of the metabolic enzyme and subtherapeutic VCZ concentrations after regular dosage and also therapeutic failure (30). The frequency of these genotypes (C/T or T/T) has been estimated to be around 25% (31), although recently published papers suggest that the percentage could be even higher and that CYP2C19 genotype is an important contributor of VCZ underexposure (32). In a recent meta-analysis, CYP2C19 harbors the highest frequency of increased activity alleles (CYP2C19*17), ranging from 1.5% in East Asians to 22.4 and 23.5% in Europeans and Africans, respectively (33). In these patients VCZ concentration is expected to be low, and VCZ N-oxide is expected to be high (the *mr* value is also expected to be high). In our study, the low number of patients included to study the genotype-phenotype association (11 samples) does not allow us to establish any strong conclusion. The calculated *mr* from patients who presented a predicted RM phenotype was >2, in contrast to patients classified as extensive and intermediate metabolizers (IM) who showed *mr* values lower than 2. Our group also found significant differences between *mr* in the groups of samples from treated patients classified by VCZ concentration (therapeutic, subtherapeutic, or uptherapeutic) (34). In the therapeutic group the median *mr* was 0.88. In the subtherapeutic and uptherapeutic groups the median *mr* values were 2.17 and 0.34, respectively. Higher *mr* values were observed between subtherapeutic samples, the most probable cause being comedications with enzyme inducer drugs or a rapid/

ultrarapid genetic profile. In addition, Bennis et al. suggested that a patient with a high VCZ N-oxide/VCZ ratio ($>$10) should receive an alternative drug rather than increased the VCZ dosage of VCZ (35).

We found around 20% of samples presenting with a mutated CYP2C19*2 polymorphism (A/G genotype). This is an SNP at position 681 of exon 5 (G$>$A), which leads to an aberrant splicing resulting in the alteration in the reading framework and generating a nonfunctional protein (23). The presence of this mutated allele alters the efficiency of the enzyme and leads to an intermediate-poor phenotype (IM and PM). The data found in the literature for this genotype in the Caucasian population reports a frequency ranging from 15 to 23% (15, 36). These patients with an impaired VCZ metabolism (poor or intermediate metabolizers) would present high VCZ concentration but low VCZ N-oxide, and the *mr* value would expected to be low. Three out of 11 patients were classified as intermediate metabolizers (IM) in our study, with a range of metabolic rates between 0.65 and 0.83. Patient 9 was classified also as an IM but showed a therapeutic VCZ concentration (and an *mr* concordant with an EM, i.e., 1.63) and a double mutated genotype (CYP2C19*2/*17). Recently published works indicate that the CYP2C19*17 increased-function allele is unable to completely compensate for the nonfunctioning CYP2C19*2 (37). Thus, the predicted metabolic phenotype of the CYP2C19*2/*17 genotypic combination is not clear yet (38).

None of the analyzed samples showed the CYP2C19*3 variant. This genotype has very little representation in the Caucasian population (0.4%).

Four patients presented wild-type genotypes for all the three analyzed variants and then were classified as EM. VCZ concentrations were found within the therapeutic range, and the *mr* ranged between 0.79 and 0.93.

Despite the small sample size, it was possible to observe a tendency that would explain the VCZ metabolic profile according to the presence or not of the different SNPs (15). The method developed here could help in the clinical management of patients being treated with VCZ. The availability of a fast and easy molecular method to characterize CYP2C19 variants and to correlate with their corresponding *mr* can offer an useful tool to individually optimize therapy and to reduce time and costs in comparison with other genotyping methods such as specific TaqMan probes or sequencing analysis. Information on the CYP2C19 genotype can predict metabolic activity before initiating VCZ therapy and thus help determining dosage.

MATERIALS AND METHODS

DNA samples. Seventy-eight samples of DNA (ISCIII Biobank) isolated from 78 different patients were used to standardize the method. Three of these samples were included as controls for later analysis after complete characterization (control genotypes).

In addition, 53 clinical samples from patients at high risk of invasive aspergillosis enrolled under the scope of the Invasive Fungal Infection and Genetics (IFIGEN) Study Group were also included in the study. Approval was obtained from the Ethics Subcommittee for Life and Health Sciences of the University of Minho, Minho, Portugal (125/014), and the National Commission for the Protection of Data, Lisbon, Portugal (1950/015).

DNA was extracted from blood samples using a phenol chloroform protocol and quantified by using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Finally, samples from 11 treated patients included in a research project (PI13/01718 AES 2013) aiming to correlate CYP2C19 genetic polymorphisms with VCZ exposure were also evaluated. A total of 11 samples (whole blood or saliva) were studied from the 11 Spanish patients who expressed their consent to participate in the study (IEC Report PI 112013-v2). DNA extraction was performed using a QIAamp DNA minikit (Qiagen, Izasa, Madrid, Spain) according to the manufacturer's instructions. All samples were quantified and stored at −20°C until analysis.

Genotyping methods. Genotyping of the CYP2C19 polymorphism was performed by using a new high-resolution melting analysis method developed and validated in our laboratory, as described below. The analysis results obtained with this method were compared to those obtained by an specific TaqMan assay (Applied Biosystems/Life Technologies, Pleasanton, CA) and also confirmed by Sanger sequencing. The following three alleles commonly named as CYP2C19*17, CYP2C19*2, and CYP2C19*3 were studied. Details of the methods are described below.

High-resolution melting analysis. Genotypes for the CYP2C19*2 (concretely CYP2C19*2A, c.G681A; rs4244285, PhamVar ID PV00111), CYP2C19*3 (concretely CYP2C19*3A, c.G636A; rs4986893, PhamVar ID PV00085), and CYP2C19*17 (c.C806T; rs12248560, PhamVar PV00097) polymorphisms were detected using a qPCR, followed by an HRM analysis of the amplicons obtained (LC480; Roche, Madrid, Spain)

TABLE 2 Primers used in this study[a]

SNP [PhamVar ID]	Technique	Primer	Sequence (5′–3′)	Amplicon size (bp)	T_m (°C)
rs12248560 (*17) [PV0009]	PCR	Cyp6001	TTGTGCTGTGGGTCCATTTA	808	58
		PER3	GTCCTTTATAATGAAATCCTCTA		
	Seq	Var17HRMF	GAACAGGATGAATGTGGTAT		
		PER3	GTCCTTTATAATGAAATCCTCTA		
	qPCR-HRM	Var17HRMF	GAACAGGATGAATGTGGTAT	174	60
		Var17HRMR	GAGCTGAGGTCTTCTGAT		
rs4244285 (*2A) [PV00111]	PCR	Cyp400F	CAACCAGAGCTTGGCATATTG	409	60
		Cyp400R	TGATGCTTACTGGATATTCATGC		
	Seq	Var2HRMF	CCAGAGCTTGGCATATTGTATC		
		Cyp400R	TGATGCTTACTGGATATTCATGC		
	qPCR-HRM	Var2HRMF	CCAGAGCTTGGCATATTGTATC	199	60
		Var2HRMR	TGTCCATCGATTCTTGGTGT		
rs4986893 (*3A) [PV00085]	PCR	Var3extF	ATGTGATCTGCTCCATTA	265	56
		Var3extR	CATCTTTTCCAGATATTCACC		
	Seq	Var3extF	ATGTGATCTGCTCCATTA		
		Var3extR	CATCTTTTCCAGATATTCACC		
	qPCR-HRM	Var3HRMF	AACATCAGGATTGTAAGC	148	60
		Var3HRMR	GGCAAGACTGTAGTATTC		

[a]PharmVar ID is as defined in https://www.pharmvar.org/gene/CYP2C19. Seq, sequencing; T_m, melting temperature.

using the primer sequences listed in Table 2. The primers were designed using the Beacon Designer 7.0 software (Premier Biosoft, Palo Alto, CA) and synthesized by Sigma-Aldrich (Madrid, Spain). For primers design, the sequences of two closely related genes (CYP2C9 and CYP2C18, GenBank accession numbers KF248045.1 and AK313403.1) were taken into account.

Reactions were performed in a 20-μl final reaction volume containing 1 μl of LightCycler 480 Resolight dye (Roche Diagnostics, Mannheim, Germany), 10 μl of SensiMix DNA (Quantace; Ecogen, Madrid, Spain), and 1 μl of each primer (20 μM), 2 μl of water (PCR-grade; Roche Diagnostics, Madrid, Spain), and 5 μl of DNA (25 ng). Although the conditions of HRM analysis were established independently for each variant, all of the PCRs were performed using the same cycling conditions: an initial denaturation step for 5 min at 95°C, followed by 50 cycles of denaturation (10 s at 95°C), annealing (10 s at 60°C), and extension (10 s at 72°C). Each run included samples tested in duplicate, using three samples of known genotypes as positive controls and one sample as a negative control.

Following amplification, specific HRM curves were obtained after 1 cycle of 1 min at 95°C and 1 min at 40°C, followed by a 10-min ramp from 65 to 95°C at a rate of 0.02°C/s, 25 acquisitions per grade, and a final cooling step of 30 s at 40°C. HRM analysis was performed using the LightCycler 480 gene scanning software (Roche) in a LightCycler 480 Instrument II (Roche). The melting curve data were manually adjusted, and the fluorescence was normalized, fixing the pre- and post-melt slider settings at between 76 and 80°C and between 86 and 89°C, respectively, with the threshold at 0 or 1 specific amplification and a sensitivity range between 0.20 and 0.30. The samples were clustered according to their melting curves by taking positive-control samples as a reference (sequence previously known) of the different genotypes for each variant (CYP2C19*2, CYP2C19*3, and CYP2C19*17).

TaqMan probe assay. A comparative study using an available commercial assay was carried out. For each of the SNPs, TaqMan SNP genotyping assays (Applied Biosystems/Life Technologies) were used. Generally, 20 μl of reaction mixture contained 10 μl of TaqMan genotyping master mix (Applied Biosystems/Life Technologies), 4 μl of water (PCR-grade, Roche Diagnostics), 1 μl of TaqMan probe (*2, C_25986767_70; *3, C_27861809_10; or *17, C_469857_10), and 5 μl of DNA (5 ng/μl). PCR conditions were the same for the three variants, including a preincubation of 10 min at 95°C, followed by 50 cycles of 95°C for 15 s and annealing at 60°C for 90 s. The assignment of genotypes was done by analyzing the results with LightCycler 480 software 1.5.0 sp3 with an endpoint genotyping tool (Roche Diagnostics).

Sanger sequencing. For all samples, targeted SNPs were subsequently sequenced to verify results of HRM and TaqMan. The reaction mix for the different amplifications contained 5 μl of 10× PCR-Buffer II ([Applied Biosystems/Life Technologies], 4 μl of MgCl₂ [Applied Biosystems/Life Technologies], 36 μl of PCR-grade water [Roche Diagnostics], 1 μl of deoxynucleoside triphosphates [2.5 μM; Applied Biosystems/Life Technologies], 1 μl of each primer [50 μM, as indicated in Table 2], 0.5 μl of AmpliTaq DNA polymerase [Applied Biosystems/Life Technologies]) and 2 μl of DNA. The PCR conditions used for each of the three variants were denaturation for 5 min at 94°C, followed by 30 cycles that included 30 s at 94°C and 45 s at a different annealing temperatures for each of the variants (58°C for *17, 56°C for *3, and 60°C for *2) and a 1-min elongation at 72°C. PCR products were visualized on a 0.8% agarose gel using BenchTop 1-kb DNA ladder (Promega Corporation, Madison, WI) as the molecular weight marker. Samples were purified using a column with an UltraClean PCR Clean-Up kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's instructions and then stored at −20°C.

Amplicons corresponding to the different variants were sequenced. The primers used varied in some cases from those employed in amplification, as can be seen in Table 2. A final volume of 10 μl consisting of 6.4 μl of water, 0.6 μl of primer (50 μM), and 3 μl of the purified PCR product was prepared for this

purpose. Sequencing was carried out by the Sanger method on an ABI 3730XL (Applied Biosystems). The sequences of all the processed samples were edited and analyzed using the DNASTAR software package (DNAStar, Inc.; Lasergene), using as reference several CYP2C19 gene sequences (GenBank accession numbers EU369458, EU369463, EU639468, EU369470, and EU369472 to EU369484).

Correlation between CYP2C19 genotyping and VCZ/metabolite exposure. Serum and/or plasma trough samples were obtained from 11 treated patients, as detailed previously. Most of them received the standard voriconazole dosage regimen (the maintenance dose was 200 mg given twice a day orally). These blood samples were processed to quantify the concentration of VCZ and its N-oxide metabolite (VCZ N-oxide). A reversed phase chromatographic method with UV detection was used using Alliance 2695/PDA 2696 (Waters Cromatografía, Cerdanyola del Vallés, Spain) equipment. The proposed method enables the simultaneous quantification of azoles and azole metabolites (34).

Each compound was characterized by its specific retention time (tr) and UV absorption spectrum. Standard solutions of known concentrations were used to establish a calibration standard curve to calculate the concentration of the studied samples. The limit of quantification for the method was set at 0.25 mg/liter for the two azoles. The concentrations in the patient samples were classified as therapeutic when they ranged between 1 and 5.5 mg/liter (39). Concentrations were calculated by relating the area of the chromatographic peak obtained at that wavelength to the theoretical concentration. The method has been developed and validated in our laboratory following the guidelines for the validation of bioanalytical methods (40).

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