

# Association Between Genetic Polymorphisms and Steady-State Plasma Concentration of Warfarin Optical Isomers

**Yiwei Liu**

First Affiliated Hospital of Fujian Medical University

**Quanyao Chen**

Women and Children's Hospital, School of Medicine, Xiamen University <https://orcid.org/0000-0003-4408-2565>

**Wai-Kit Ming**

First Affiliated Hospital of Sun Yat-sen University

**Feiyu Wang**

First Affiliated Hospital of Fujian Medical University

**Rongfang Lin**

First Affiliated Hospital of Fujian Medical University

**Cuihong Lin**

First Affiliated Hospital of Fujian Medical University

**Pinfang Huang** (✉ [youdai2002@sina.com](mailto:youdai2002@sina.com))

The First Affiliated Hospital of Fujian Medical University <https://orcid.org/0000-0002-4588-6285>

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## Research article

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# Abstract

**Background** We evaluated the effects of CYP2C19\*2, CYP2C9\*3, VKORC1 A1639G, CYP4F2, and MDR1 C3435T gene polymorphisms on the plasma concentrations of R- and S-warfarin enantiomers at the same dose.

**Methods** The plasma concentrations of R- and S-warfarin were determined by ultra performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) in 136 patients. The PCR-RFLP genotyping results were verified by pyrosequencing. The Hardy–Weinberg equilibrium of genotype frequencies was assessed using the Chi-square analysis. Relationships between genotype and plasma concentrations of the enantiomers were analyzed by Kruskal–Wallis test.

**Results** There was no significant difference in the dosage between groups ( $P > 0.05$ ). The CYP2C19\*2, CYP2C9\*3, CYP4F2, MDR1 C3435T, and VKORC1 A1639G mutation frequencies were 37.5%, 6.25%, 19.12%, 31.25%, and 3.31%, respectively. The plasma concentrations were non-normally distributed. The S-warfarin plasma concentration was significantly higher in CYP2C9\*3 carriers than in non-carriers ( $P = 0.018$ ) and in patients carrying the T allele of CYP4F2 than those carrying the C allele ( $P = 0.03$ ). The VKORC1 A1639G polymorphism did not affect the steady-state plasma concentrations of R- and S-warfarin. The enantiomer ratio in homozygous patients (GG) was significantly lower than that in heterozygous patients (GA) and those lacking the mutation (AA) ( $P = 0.039$ ). Enantiomer plasma levels were not significantly different between MDR1 C3435T and CYP2C19\*2 ( $P > 0.052$ ).

**Conclusions** The CYP2C9\*3 and CYP4F2 mutations are associated with increased plasma concentrations of S-warfarin. The VKORC1 A1639G polymorphism might affect the plasma ratio of R- and S-warfarin. Maintenance dose reduction during warfarin administration can be considered for patients with the CYP2C9\*3 and CYP4F2 mutations.

## 1 Background

Warfarin, a coumarin drug, is widely used in anticoagulant therapy in its racemic form. Studies have shown that the pharmacological activity of the S-enantiomer in vivo is 3–5-fold higher than that of the R-enantiomer [1], both of which are metabolized by different epoxidases but can still compete with each other for enzyme-binding sites [2]. Individual patient-related pharmacogenomic factors and drug–drug interactions resulting from clinical combinations can significantly influence the pharmacokinetics and pharmacodynamics of warfarin [3], thereby amplifying the adverse effects associated with this drug, including internal bleeding, thereby limiting its clinical use. Recent studies have focused on the effects of the CYP2C19 polymorphism on the in vivo mechanisms of warfarin enantiomers [4]; however, the effect of steady-state plasma concentrations on different enantiomers remains unclear. In this study, we performed a genetic analysis to evaluate individualized warfarin treatment in the clinical setting.

## 2 Methods

## **2.1 Patients**

We enrolled patients who were regularly administered warfarin (Orion, Espoo, Finland) anticoagulant therapy for more than 1 month in the First Affiliated Hospital of Fujian Medical University as a part of their atrial fibrillation treatment (paroxysmal atrial fibrillation, persistent atrial fibrillation, and permanent atrial fibrillation), deep vein thrombosis treatment, or during heart valve replacement. An international normalized ratio (INR) between 1.5 and 3.0 measured three times in succession was considered as the maintenance dose (Figure 1). The liver and kidney function indicators of all enrolled patients were normal. Demographic characteristics, genetic factors, and concomitant medication data were collected from the medical records of each enrolled patient in this study (Table 1).

The exclusion criteria were as follows: a diagnosis of hematological or coagulopathy disorder; heart function grade III or above; clear hemorrhagic disease or bleeding tendency; liver and kidney dysfunction; severe malnutrition; pregnancy; long-term administration of drugs that might interfere with warfarin function and metabolism (such as aspirin, clopidogrel, heparin and vitamin K, amiodarone, rifampicin, and barbitol); bleeding or thromboembolism during observation; and missing clinical and laboratory-related examination data.

## **2.2 Blood withdrawal and sample treatment**

Two milliliters of blood was collected from each patient in the steady state at 12–15 h post warfarin administration into an EDTA anticoagulation tube. Whole blood (200  $\mu$ L) was used for genotyping (see Section 2.5), and the remaining volume of the suspended plasma was stored at  $-80^{\circ}\text{C}$ . The concentrations of *R*- and *S*-warfarin were measured by UPLC-MS/MS within 5 days of blood collection.

For each calibration curve standard, quality control sample, and patient sample, 300  $\mu$ L of plasma was pipetted into a 2-mL test tube containing 10  $\mu$ L of internal standard solution (carbamazepine; China National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) and 100  $\mu$ L of HCl (1 M) solution. Proteins were denatured by adding 2 mL of dichloromethane and vortexed for 3 min, followed by centrifugation for 5 min at  $2550 \times g$ . The organic phase was transferred into a vial and dried with nitrogen. The residue was dissolved in 200  $\mu$ L of mobile phase (see Section 2.4) and filtered through a 0.22- $\mu$ m microporous membrane. Ten microliters of this solution was injected into the UPLC-MS/MS system (see Section 2.4) for analysis.

## **2.3 Analysis**

Chromatography was performed using the Waters Acquity UPLC system (Milford, MA, USA) with an Astec Chirobiotic V column (250 mm  $\times$  2.1 mm, 5  $\mu$ m) (Supelco, USA) at  $25^{\circ}\text{C}$ . The mobile phase comprised 55% mobile phase A (0.02% formic acid in water) and 45% mobile phase B (methanol). The flow rate was 0.2 mL/min.

MS detection was performed on a Waters TQD tandem mass spectrometer equipped with an electrospray ionization source. The mass spectrometer was set at the multiple reaction monitoring mode and

quantification was performed at  $m/z$  307 >161.18 for warfarin (cone voltage: 50 V) and  $m/z$  237.31 >194 for carbamazepine (cone voltage: 40 V). The collision energy was 20 V.

## ***2.4 Validation procedures***

Calibration standards were prepared by spiking blank plasma samples with standard solutions. The final concentrations of the calibration samples were 4.3, 8.6, 26.0, 65.0, 130.0, 260.0, 520.0, and 1040.0  $\mu\text{g/L}$  for *R*-warfarin (Toronto Research Chemicals, North York, Canada) and 4.8, 9.6, 28.6, 71.5, 143.0, 286.0, 572.0, and 1144.0  $\mu\text{g/L}$  for *S*-warfarin (Toronto Research Chemicals).

Accuracy and intra- and inter-day precisions were determined using pooled plasma ( $n = 5$ ) at three different concentrations: lower limit of quantitation, high QC (approximately 80% upper limit of quantitation), and upper limit of quantitation. A signal-to-noise ratio of  $\geq 3$  was used as the lower limit of detection.

## ***2.5 Genotyping***

DNA was extracted from blood samples using the adsorption column method with the Blood Genomic DNA Extraction Kit (Beijing Tiangen Biotech, Beijing, China) within 24 h of blood collection. Genotyping was carried out by PCR-RFLP, and primers were designed by Shanghai Shenggong Bioengineering [5-9]. The restriction enzymes *Sau3aI*, *SmaI*, *PvuII*, *NsiI*, and *MspI* were provided by Promega (Madison, WI, USA). The results were verified by multiplex PCR and sequencing (HiSeq XTen sequencers, Illumina, San Diego, CA, USA). A panel containing 25 target SNP sites was designed. Library preparation was performed by two-step PCR. The plate was sealed and the PCR was carried out in a thermal cycler (T100TM, Bio-Rad, Hercules, CA, USA) using the following program: one cycle of denaturation at 95°C for 3 min, five cycles of denaturation at 94°C for 30 s, annealing at 55°C for 20 s, and elongation at 72°C for 30 s, with a final extension at 72°C for 5 min. AMPure XP beads were used to purify the amplicon product. The libraries were then quantified and pooled. Paired-end sequencing of the library was performed on the HiSeq XTen sequencer (Illumina).

## ***2.6 Statistics***

The probability of deviation from the Hardy–Weinberg equilibrium was calculated for each SNP. Skewness and kurtosis were used to test whether the blood concentration values were normally distributed (SPSS 17.0, IBM, Armonk, NY, USA). One-way analysis of variance was used to evaluate data showing a normal distribution, and Student–Newman–Keuls  $q$ -test was used for pairwise comparison between groups. Kruskal–Wallis test was used to compare the steady-state plasma concentrations in patients among different genotype groups. A  $P$  value of  $< 0.05$  was considered to indicate statistical significance.

# **3 Results**

## ***3.1 Patient demographics and characteristics***

A total of 136 patients were enrolled in this study, including 66 males and 70 females, with an average age of  $57.9 \pm 11.7$  years, bodyweight of  $63.4 \pm 10.4$  kg, and height of  $164.4 \pm 7.1$  cm; all enrolled patients had normal liver and kidney functions (Table 1).

### **3.2 Method development and validation**

The retention times of carbamazepine, *R*-warfarin, and *S*-warfarin were approximately 6.2, 7.2, and 9.4 min, respectively. Blood impurities did not interfere with measurements in the two ion channels.

The calibration curves showed good, reliable linearity over the concentration range of 4.3–1040.0 µg/L for *R*-warfarin ( $r = 0.9988$ ) and 4.8–1144.0 µg/L for *S*-warfarin ( $r = 0.9962$ ). The extraction recovery rate of *R*-warfarin was 77.86%–80.87% (RSD < 5.43%), whereas that of *S*-warfarin was 77.62%–81.88% (RSD < 6.56%). The lower detection limits of *R*- and *S*-warfarin were 1.0 and 1.5 µg/L ( $S/N \geq 3$ ), respectively (Table 2).

### **3.3 Plasma concentrations and gene polymorphisms**

The total plasma concentrations of *R*- and *S*-warfarin in 136 patients were  $601.01 \pm 514.12$  and  $278.19 \pm 242.94$  µg/L, respectively. The normality test by skewness and kurtosis suggested a non-normal distribution ( $P > 0.208$ ). Patients were classified based on the different genotypes of *CYP2C19\*2*, *CYP2C9\*3*, *VKORC1 A1639G*, *CYP4F2*, and *MDR1 C3435T*. There was no significant difference in warfarin dose among the genotyping groups ( $P > 0.05$ ) (Figure 2). The differences in the plasma concentrations among the groups are shown in Table 3. There were no significant differences in the plasma concentrations of the two enantiomers for *CYP2C19\*2*, *MDR1 C3435T*, and *VKORC1 A1639G* ( $P > 0.05$ ). The plasma concentration of *S*-warfarin in the *CYP2C9\*3* mutant carriers was higher than that in the *CYP2C9* carriers ( $P = 0.018$ ). The plasma concentration of *S*-warfarin in patients with the T allele of *CYP4F2* was higher than that in patients with the C allele ( $P = 0.03$ ) (Table 3 and Figure 3).

## **4 Discussion**

Genetic polymorphism is an important factor influencing individual differences in the in vivo drug mechanism and metabolism. It has been reported that the *CYP2C19\*17* polymorphism affects the different enantiomeric clearance and maintenance dose of warfarin [4]. The plasma concentration excludes the influence of pharmaceutical preparation and patient compliance and establishes a more direct link with physiological effects than drug dosage. We first evaluated the relationship between the steady-state plasma concentration of warfarin enantiomers and polymorphisms of *CYP2C19\*2*, *CYP2C9\*3*, *CYP4F2*, *MDR1C3435T*, and *VKORC1 A1639G* in settings where the dose is not different.

*CYP2C19\*2* (681 G > A) is located on exon 5 and the mutation of this gene might decrease the enzymatic activity of the encoded protein. We found that the effect of *CYP2C19\*2* polymorphism on the steady-state plasma concentration of *R*- and *S*-warfarin was not significant, but the p value was close to 0.05 (for *R*-warfarin, this value was 0.052). Further studies of a larger sample size are needed to confirm this result.

CYP2C9 is responsible for the metabolism of warfarin, phenytoin, ibuprofen, and losartan, among others. The CYP2C9 polymorphisms contribute to differences in the metabolic drug capacity among individuals [10]. The CYP2C9 polymorphisms, such as the \*3 mutation, have been reported to reduce the dose requirement of warfarin to achieve the same therapeutic effect by 37% [11]. However, the effect of CYP2C9 polymorphism on the steady-state plasma concentration of warfarin enantiomers has not been reported. The steady-state plasma concentration of R-warfarin in \*3 carriers was not significantly different from that in non-carriers ( $P = 0.063$ ), whereas the steady-state plasma concentration of S-warfarin in \*3 carriers was higher than that in non-carriers ( $P = 0.018$ ), suggesting that CYP2C9\*3 significantly affects only S-warfarin metabolism. Because the anticoagulant activity of S-warfarin is considerably higher than that of R-warfarin, the administration of the racemic form will have a significant effect on the therapeutic outcome in patients with such altered genetic backgrounds.

The CYP4F2 enzyme is involved in vitamin K1 metabolism; its mutation leads to an increased concentration of vitamin K1 in the steady-state phase, necessitating stable doses of warfarin [12]. We found that the plasma concentration of S-warfarin in the mutated CYP4F2 population was higher than that in the wild-type CYP4F2 population ( $P = 0.03$ ). There was no significant increase in the plasma concentration of R-warfarin ( $P = 0.052$ ).

P-Glycoprotein (P-gp), encoded by a multi-drug resistance gene (MDR1), acts as a drug efflux transporter, which can actively reduce drug absorption. Whether P-glycoprotein acts on warfarin transport remains controversial. Studies have suggested that the C3435T polymorphism in the MDR1 gene does not affect the steady-state concentration of warfarin alone [13], whereas Tavares et al. [14] reported different conclusions. Our data suggested that differences in the MDR1 genotype did not significantly affect warfarin plasma concentrations.

The VKORC1 mutations result in increased levels of VKORC1 mRNA and protein expression, and an increase in warfarin dose might be required to inhibit the VKORC1 protein. The VKORC1 C1173T polymorphism affects the potency relationship of S/R warfarin in vivo [3]. Our results suggest that the VKORC1 A1639G polymorphism does not affect the steady-state plasma concentrations of R- and S-warfarin. However, the S/R warfarin concentration ratio in mutant homozygous (GG) patients was significantly lower than that in mutant heterozygous (GA) and wild-type homozygous (AA) populations ( $P = 0.039$ ), suggesting that these mutations affect the potency of S/R warfarin in vivo.

We assessed the influence of genetics to provide individualized warfarin treatment by evaluating the total plasma concentrations of R- and S-warfarin in patients with different gene polymorphisms. Our UPLC-MS/MS method is 20-fold more sensitive than the method reported for determining the chiral isomers of warfarin in biological samples [15, 16], and thus is more effective for studying pharmacokinetics and monitoring routine plasma concentrations.

We used a chiral column with a macrocyclic glycopeptide to resolve the R and S optical isomers of warfarin, which were bonded to high-purity silica microparticles by covalent bonding for better enantioselectivity. Good enantiomeric separation can be achieved by a simple solvent mixture of formic acid in

water and methanol. Compared with supercritical fluid chromatography separation reported previously [17], our method is more suitable for use by other researchers for separation because of the triethylamine on the  $\beta$ -cyclodextrin column, and provides a better resolution [18]. Using this method, the inhibitory effect of triethylamine on analyte ionization in MS was avoided.

## 5 Conclusions

Polymorphisms in *CYP2C19\*2* and *MDR1 C3435T* had no significant effect on the plasma concentrations of *R*- and *S*-warfarin, whereas the *CYP2C9\*3* mutation significantly increased the concentration of *S*-warfarin. The *CYP4F2* mutation increased the plasma concentrations of both *R*- and *S*-warfarin, with a greater increase observed for the *S*-enantiomer. The *VKORC1 A1639G* polymorphism might affect the potency relationship of *S/R* warfarin *in vivo*. Individualized treatment with warfarin based on different genotypes can significantly reduce the risk of bleeding (particularly major bleeding) and elevated INR [19]. These results suggest that maintenance doses could be reduced as appropriate during warfarin administration for patients with the *CYP2C9\*3* and *CYP4F2* mutations.

## Abbreviations

UPLC-MS/MS, ultra performance liquid chromatography/tandem mass spectrometry;

INR, international normalized ratio;

RSD, relative standard deviation

P-gp, P-Glycoprotein;

HWE, Hardy–Weinberg equilibrium

## Declarations

### Acknowledgments

Not applicable.

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### Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

### **Authors' contributions**

Yiwei Liu and Pinfang huang conceived and designed the study. Yiwei Liu and Rongfang Lin performed research. Wai-Kit Ming, Feiyu Wang and Cuihong Lin contributed to patient recruitment and data collection. Yiwei Liu and Quanyao Chen analyzed the data and drafted the manuscript. All authors reviewed and approved the final manuscript.

### **Ethics approval and consent to participate**

The study was approved by the Ethics Committee of the First Affiliated Hospital of Fujian Medical University. Informed consent was obtained from each patient.

### **Consent for publication**

Not applicable.

### **Competing interests**

The authors declare that they have no competing interests.

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## Tables

**Table 1.** Demographic characteristics of the patients

Demographic characteristics	Value, mean $\pm$ SD
Sex (male/female)	66/70
Age (years)	57.9 $\pm$ 11.7
Weight (kg)	63.4 $\pm$ 10.4
Height (cm)	164.4 $\pm$ 7.1
ALT (U/L)	24.7 $\pm$ 19.9
AST (U/L)	29.1 $\pm$ 13.4
Scr (mmol/L)	80.1 $\pm$ 22.1

**Table 2.** Results of accuracy and precision for R- and S-warfarin (% , n = 5)

Drug	Concentration ( $\mu\text{g/L}$ )	Accuracy	RSD	Intra-day	Inter-day
R-warfarin	5.0	101.1	4.63	1.09	6.96
	260.0	98.64	3.37	2.26	4.61
	1040.0	97.70	2.15	2.15	5.33
S-warfarin	5.0	101.4	3.99	2.13	6.84
	290.0	98.24	2.55	3.15	4.17
	1144.0	98.63	3.22	2.19	4.74

**Table 3.** Analysis of the effect of genes on plasma concentration.

Gene/Statistics	Genotype	n	R-warfarin (µg/L)	S-warfarin (µg/L)	S/R	INR	DOSE
<b><i>CYP2C19*2</i></b>	*1*1	54	563.55 ± 308.87	281.17 ± 254.11	0.466 ± 0.194	2.10±0.43	2.84±0.90
-	*1*2	62	571.99 ± 651.41	248.21 ± 218.88	0.457 ± 0.196	2.01±0.35	2.73±1.12
-	*2*2	20	792.08 ± 452.15	363.10 ± 273.63	0.438 ± 0.120	2.23±0.38	2.46±0.87
$X^2$	-	-	5.927	5.162	0.124	4.291	2.7992
$p$	-	-	0.052	0.076	0.940	0.117	0.2467
<b><i>CYP2C9*3</i></b>	*1*1	119	545.84 ± 329.00	256.03 ± 223.96	0.447 ± 0.175	2.07±0.40	2.70±0.99
-	*1*3	17	987.16 ± 1118.41	433.34 ± 314.38	0.535 ± 0.241	2.15±0.38	2.99±1.11
-	*3*3	0	/	/	/		
$X^2$	-	-	3.455	5.565	1.102	1.1844	1.1436
$p$	-	-	0.063	0.018	0.294	0.2765	0.2849
<b><i>CYP4F2</i></b>	CC	86	574.27 ± 571.00	260.61 ± 241.47	0.451 ± 0.199	2.08±0.39	2.71±0.98
-	CT	48	613.29 ± 364.95	276.40 ± 185.10	0.456 ± 0.155	2.10±0.42	2.81±1.06
-	TT	2	1455.72 ± 423.49	1077.16 ± 312.97	0.74 ± 0.000	1.69±0.85	2.25±0
$X^2$	-	-	5.894	7.045	3.097	2.3306	0.4145
$p$	-	-	0.052	0.03	0.213	0.3118	0.8128
<b><i>MDR1 C3435T</i></b>	CC	57	648.95 ± 675.03	287.92 ± 209.39	0.489 ± 0.190	2.04±0.40	2.91±1.20
-	CT	73	577.72 ± 365.41	278.52 ± 274.06	0.437 ± 0.186	2.11±0.39	2.057±0.81
-	TT	6	428.90 ± 201.72	181.76 ± 93.20	0.411 ± 0.068	2.08±0.47	3.21±0.75
$X^2$	-	-	0.476	2.187	2.606	1.0319	3.6902
$p$	-	-	0.788	0.335	0.272	0.5969	0.1580
<b><i>VKORC1</i></b>	AA	128	562.42 ± 346.15	268.54 ± 237.34	0.454 ± 0.186	2.09±0.40	2.75±1.01
-	AG	7	678.38 ± 386.92	395.43 ± 298.33	0.560 ± 0.096	1.84±0.25	2.62±0.94
-	GG	1	4998.18	692.18	0.138	2.16±0	2.25±0

$X^2$			3.869	4.516	6.467	2.6775	0.1433
$p$			0.144	0.105	0.039	0.2622	0.9309

## Figures

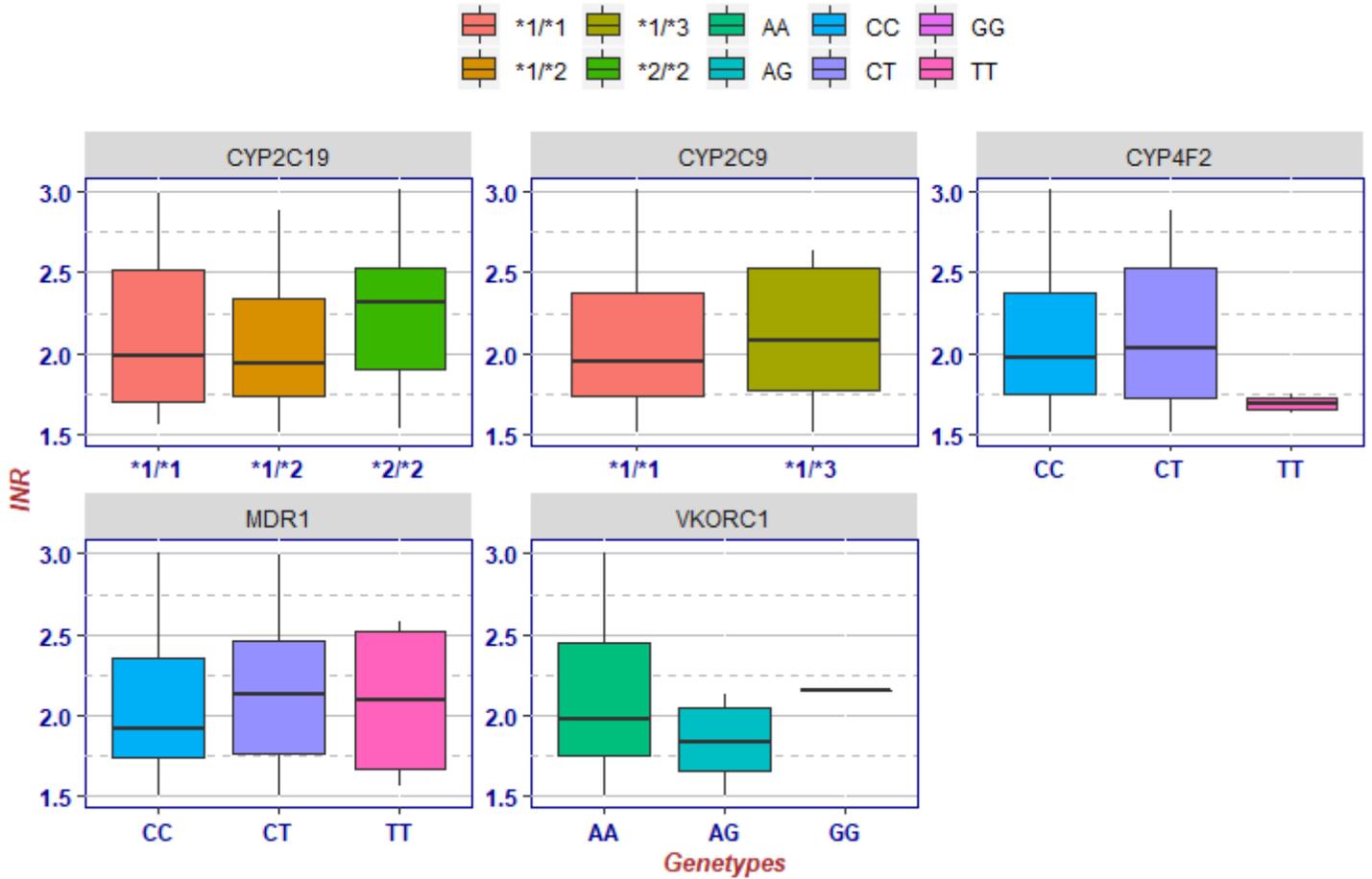


Figure 1

INR of patients in different genotypes.

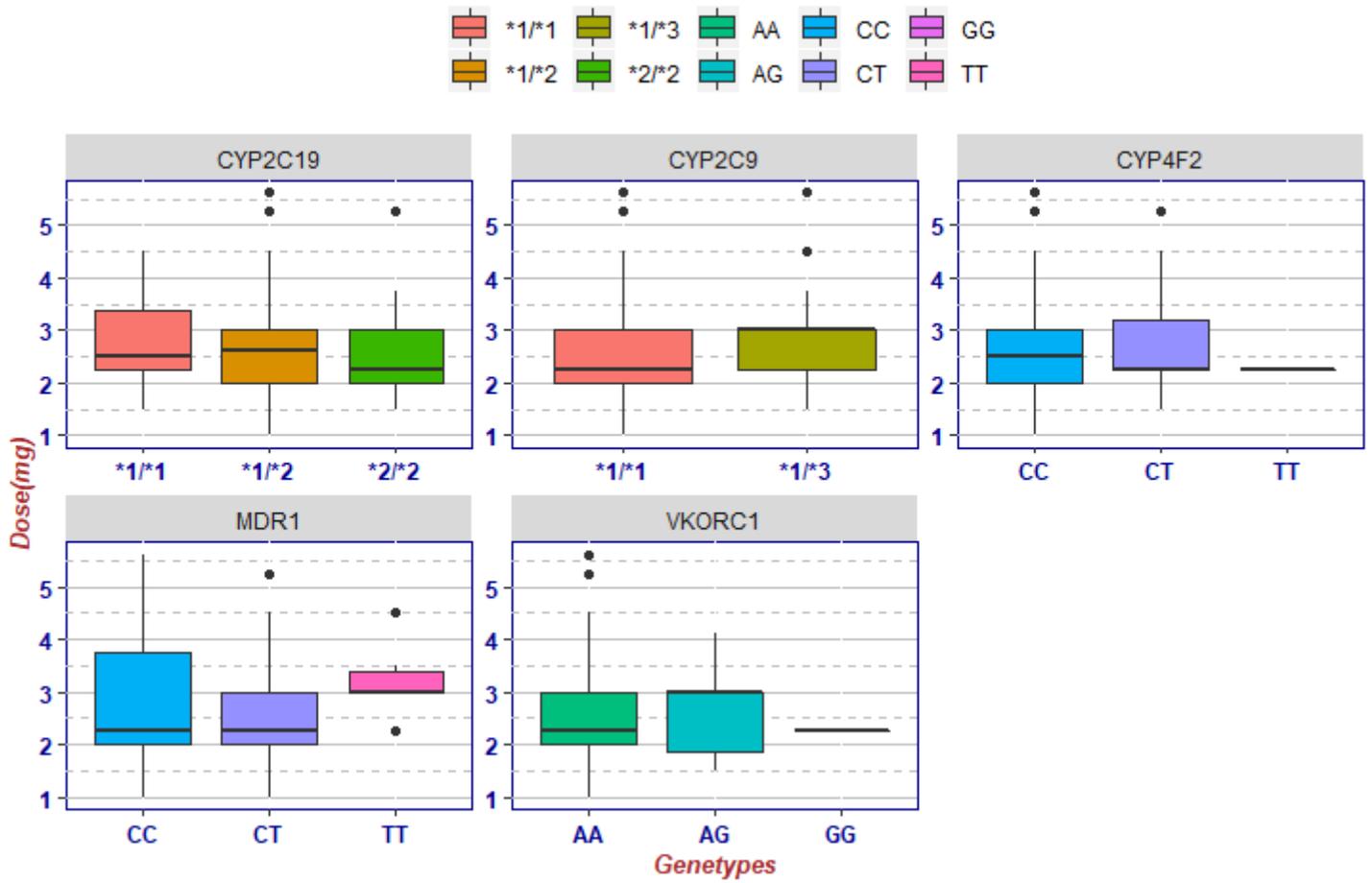


Figure 2

Dose of warfarin in different genotypes.

R-warfarin S-warfarin

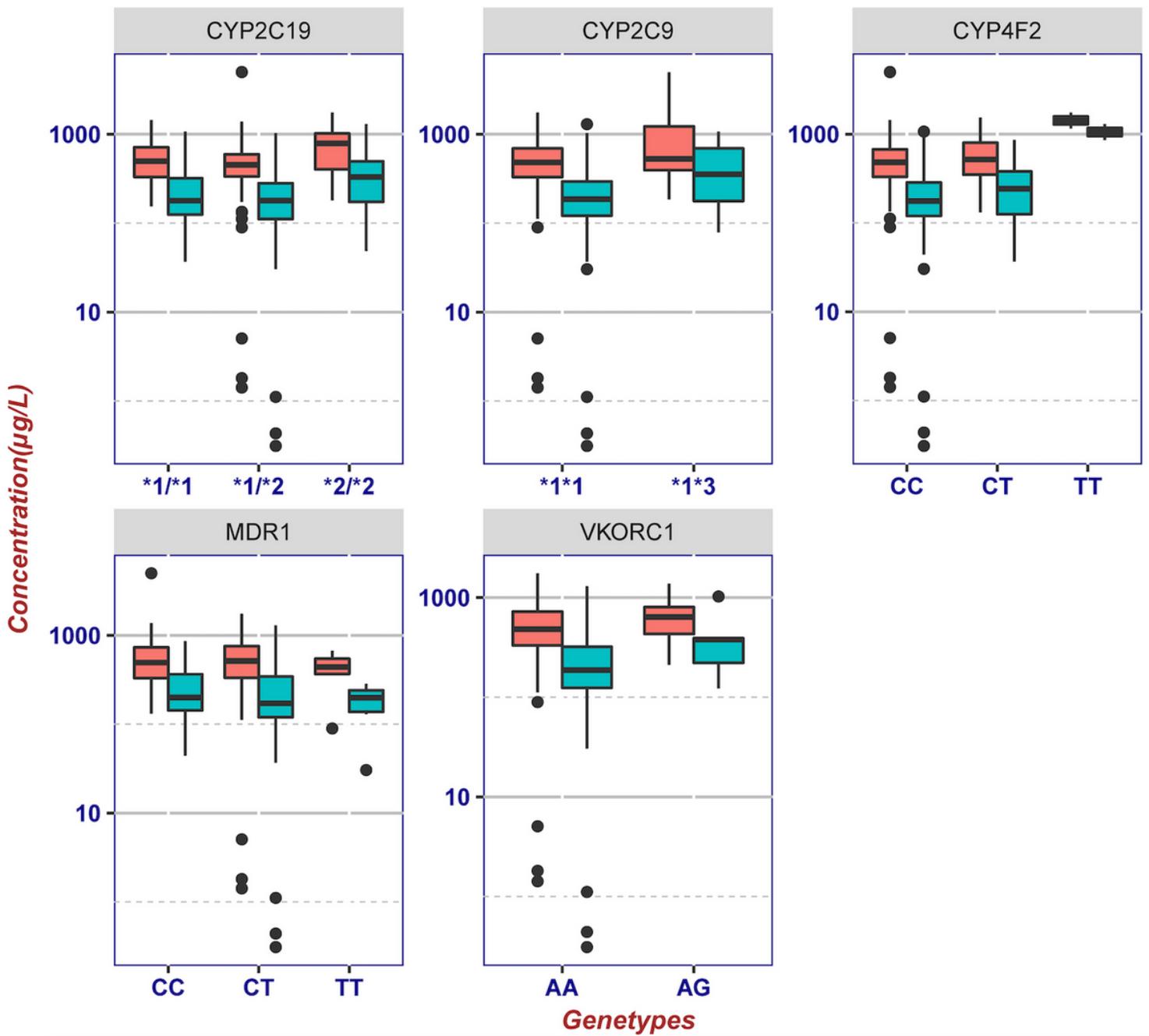


Figure 3

Plasma concentration of R- and S-warfarin in different genotypes.