

The *SLCO1A2* -189_-188InsA polymorphism reduces clearance of rocuronium in patients submitted to elective surgeries

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Abstract

Purpose Rocuronium (ROC) is a neuromuscular blocker mainly eliminated by biliary excretion dependent on organic anion transporting polypeptide 1A2 (OATP1A2) hepatocellular uptake. However, the influence of *SLCO1A2* (gene encoding OATP1A2) genetic polymorphism on ROC pharmacokinetics was never described before. The objective of this work was to evaluate the influence of genetic polymorphisms of *SLCO1A2* on the pharmacokinetics of rocuronium (ROC). **Methods** Patients undergoing elective surgeries under general anesthesia using rocuronium as a neuromuscular blocker were genotyped for *SLCO1A2* polymorphisms in the coding region (41A>G, 382A>T, 404A>T, 502C>T, 516A>C, 559G>A, 830C>A, and 833delA) and in the promoter region (-1105G>A, -1032G>A, -715T>C, -361G>A, and -189_-188insA). Rocuronium pharmacokinetic parameters were estimated by non-compartmental analysis.

Results None of the patients had heterozygous or homozygous variant of 404A>T, 382A>T, 502C>T, 833delA, 830C>A, 41A>G, and -715T>C. A linkage disequilibrium was found between -1105G>A and -1032G>A genotypes. Patients genotyped as -A or AA ($n = 17$) for *SLCO1A2* -

189_-188InsA showed reduced total clearance of ROC compared to patients genotyped as -/- ($n = 13$) (151.6 vs 207.1 mL/min, $p \leq 0.05$). The pharmacokinetics parameters of ROC were not significantly different between other *SLCO1A2* genotypes.

Conclusion *SLCO1A2* -189_-188InsA polymorphism is related to the reduced clearance of rocuronium in patients submitted to elective surgeries under general anesthesia.

Trial registration NCT 02399397 (ClinicalTrials.gov)

Keywords Pharmacogenetics · Rocuronium · Neuromuscular blockade · SNPs · Pharmacokinetics

Introduction

The organic anion transporting polypeptide 1A2 (OATP1A2), also known as human OATP-A or OATP1, mediates the cellular uptake of endogenous compounds and a variety of drugs. OATP1A2, encoded by *SLCO1A2* gene, is expressed in blood-brain barrier, distal nephrons, and in the apical membrane of bile duct cells (cholangiocytes) in the liver. The expression of OATP1A2 in these different tissues suggests its relevance to different processes of drug kinetic disposition such as drug distribution to the central nervous system, renal excretion, metabolism, and biliary excretion [1–4]. The list of OATP1A2 substrates includes endogenous substances, for instance bile acids, thyroid and steroid hormones, and drugs, e.g., statins, methotrexate, fexofenadine, imatinib, and HIV protease inhibitors. In vitro studies with cell transfected with *SLCO1A2* have contributed significantly to the identification of substrates and to characterize the functional relevance of this transporter in pharmacokinetics [5, 6].

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Rocuronium (ROC) is a neuromuscular blocker often used during general anesthesia to facilitate endotracheal intubation, artificial ventilation, and surgical procedures. It is mainly eliminated as the unchanged drug by biliary excretion depending on the hepatocellular uptake by OATP1A2, but also by renal excretion, while the metabolism of ROC is practically absent [5–7]. The distribution of ROC to the liver is considered a determining factor for the duration of neuromuscular blockade. The concentration of ROC in bile ranged from 2 to 1217 $\mu\text{g/mL}$ (median = 377 mg/mL), while bile/plasma concentration ratio ranged from 130 to 2800 (median = 400) in ASA (American Society of Anesthesiology) classification I–III patients undergoing surgery under general anesthesia [7]. Rocuronium fraction unbound in plasma determined by ultrafiltration was 0.54 [8].

A large inter-individual variability in drug disposition and response results from the complex interaction between demographic, clinical, genetic, and environmental factors. Age, renal function, liver function, and race were already described as covariates to the pharmacokinetics of ROC [9]. Patients with renal impairment (ASA II–III, creatinine clearance <30 mL/min) showed a fourfold reduction in the total clearance of ROC when compared to healthy patients (ASA I–II) [10]. Reduction in ROC clearance was also observed in cirrhotic patients when compared to healthy patients [11]. However, studies investigating the influence of pharmacogenetics on ROC kinetic disposition are absent. The objective of this work was to evaluate the influence of genetic polymorphisms of *SLCO1A2* on the in vivo activity of OATP1A2 and the resulting alterations on the pharmacokinetics of ROC. This work was addressed to identify the potential covariates to the kinetic disposition of ROC. This clinical investigation included only ASA I–III patients undergoing elective surgery under general anesthesia using the ROC as neuromuscular blocker.

Methods

Patients

Eligible subjects ($n = 35$) were adult patients scheduled to undergo small–medium sized elective surgeries under general anesthesia and classified as class I–III according to the ASA classification. All patients were recruited at the Surgical Centre of the Clinical Hospital of the School of Medicine of Ribeirão Preto, USP. Estimated glomerular filtration rate (eGFR) was estimated from serum creatinine values using CKD-EPI equation [12]. All patients taking fluoxetine, carbamazepine, aminoglycoside antibiotics (gentamicin, amikacin, neomycin, streptomycin, tobramycin), or OATP1A2 inhibitors such as rifampicin and verapamil were excluded due to potential pharmacokinetic interaction with ROC [10, 13, 14].

Patients with impaired liver function or eGFR <30 mL/min were excluded from this study.

Clinical protocol

All patients scheduled for elective surgery attended the surgical center after 12-h fasting. On arrival in the operating room, two 20-gauge cannulas were inserted into peripheral veins in opposite limbs, one for drug administration and other for blood collection. All patients included in this study received intravenous (i.v.) midazolam as pre-anesthetic medication and were slowly induced with 1 to 3 mg/kg intravenous (i.v.) propofol. Fentanyl (2–3 $\mu\text{g/kg}$ i.v.) was then administered. Patients received a single intravenous dose 0.3–0.9 mg/kg of ROC (Esmeron®, Organon, Schering-Plough, São Paulo, SP, Brazil) for muscle relaxation. Patients were monitored during surgery including continuous blood pressure measure, pulse oximetry, central and peripheral temperature, and electrocardiography. The neuromuscular blockade was monitored using the train of four (TOF) response of the adductor pollicis muscle resulting from stimulation of the ulnar nerve (2 Hz for 2 s, TOF-Watch® SX Monitor, Organon Ireland Ltd., Swords, Ireland).

Before the surgical procedure, whole blood samples (4 mL) were collected using EDTA as anticoagulant for DNA extraction. For the pharmacokinetics analysis, blood samples (4 mL) were collected at times 0, 2, 5, 10, 15, 20, 30, 60, 120, 180, 240, and 360 min after ROC administration. Blood samples were collected into heparin-containing tubes and then centrifuged at $3220\times g$ for 10 min at 4 °C. All plasma samples were added to 0.2 mL of 1-M monobasic sodium phosphate solution per each 1 mL of plasma to avoid hydrolysis of ROC [15, 16]. Plasma samples were stored at -20 °C until analysis.

Analysis of ROC in plasma by LC-MS/MS

ROC was analyzed in human plasma by LC-MS/MS according to the method developed and validated by this research group [17]. ROC and verapamil, used as internal standard, were resolved in a reverse phase LiChrospher® 100 RP-18e Sorbent (125 \times 4 mm, 5 μm ; Merck, Darmstadt, Germany) and mobile phase constituted by mixture of water:acetonitrile:trifluoroacetic acid (50:50:0.1, v/v) at 1.0 mL/min. The protonated molecular ions $[M + H]^+$ and their product ions were monitored at m/z transitions 529 $>$ 487 for ROC and 455 $>$ 165 for the internal standard (verapamil).

Samples of 120 μL of the mixture plasma: 1 M monobasic sodium phosphate buffer 5:1 v/v (corresponding to 100 μL plasma) were added to 1 M phosphate buffer (50 μL), 25 μL of internal standard solution (verapamil 1 $\mu\text{g/mL}$), 100 μL of a saturated solution of potassium iodide, and 2 mL of dichloromethane. After 30 min of reciprocating mechanical shaking,

samples were centrifuged for 10 min at 5 °C (1800×g). The lower organic layer was transferred to conical tubes and evaporated to dryness. The residue was reconstituted in 100 µL of mobile phase and vortexed for 10 s. An aliquot of 50 µL was injected into the chromatographic system. The lower limit of quantification was 5 ng/mL, and the method was linear in the interval of 5–2000 ng/mL of plasma. The relative standard deviation and the relative error (inaccuracy) did not exceed 15%.

Genotyping assays

Genomic DNA was extracted from whole blood samples by salting out procedure [18]. The single nucleotide polymorphisms (SNPs) in the coding region of *SLCO1A2* 404A>T, 382A>T, 559G>A, 502C>T, 516A>C, 833delA, 830C>A, and 41A>G and SNPs in the promoter region *SLCO1A2* -1105G>A, -1032G>A, -715T>C, -361G>A, and -189_-188insA were evaluated using direct nucleotide sequencing.

The polymerase chain reaction (PCR) was developed using primers 5'-CATCCACAGGCAGATTATTGAA-3' (forward) and 5'-CGGCCCTTTGACTCATT-3' (reverse) for 404A>T, 5'-ACTAGGGGTGCCCTGAGAAG-3' (forward) and 5'-ACCTCCAGGGGCACTAGACT-3' (reverse) for 559G>A; 5'-TAGTTGGTTGGGACCCGATA-3' (forward) and 5'-AGTACCATAGGAAGAATCGGACT-3' (reverse) for 833delA, 5'-GGCACGTA CTGGCTTTCTT-3' (forward) and 5'-TGGAAAATAATCCCACGATTG-3' (reverse) for -1105G>A, -1032G>A, and -715T>C; and 5'-GATTTTTGAGCCTTGGTAGGG-3' (forward) and 5'-CTCTTCAGGGTGTCCAAG-3' (reverse) for -361G>A and -189_-188insA.

PCR reactions were performed in a final volume of 25 µL with 200 pg of DNA, 0.2 µmol/L of each primer, 2.5 µL of 10 × PCR buffer, 250 µmol/L dNTPs, 2 mmol/L MgCl₂, and 0.2 units/µL Taq DNA polymerase (LGC, Cotia, SP, Brazil). PCR amplification consisted of an initial denaturation for 3 min at 95 °C followed by 35 cycles of denaturation at 95 °C for 30 s, 30 s of annealing at 60 °C, and 45 s of extension at 72 °C. After purification by digestion EXO/SAP according to the manufacturer instructions (Fermentas, Burlington, ON, Canada), direct nucleotide sequencing was performed by ABI PRISM ABI3130 using the Big dye terminator protocol (Applied Biosystems, Foster City, CA, USA).

Pharmacokinetic and statistical analysis

Pharmacokinetic parameters were calculated based on individual curves of plasma concentration versus time. Considering that patients were treated with different doses of ROC, AUC values were normalized by dose (AUC/D) for

comparison between groups. Total drug clearance from plasma (CL) was calculated by the equation: $CL = \text{dose} / AUC$.

The distribution of genotypes for each polymorphism was evaluated for deviation from Hardy-Weinberg equilibrium using chi-squared test. The pharmacokinetic data are expressed as median and interquartile range. Genotypes were compared using non-parametric tests for unpaired data (Mann-Whitney test). Total clearance of rocuronium was regressed against the potential covariates age, sex, creatinine clearance, weight, and *SLCO1A2* genotypes using SPSS for Mac (v. 24; SPSS Inc., Chicago, IL, USA). A *p* value ≤0.05 was considered to indicate statistical significance.

Results

The demographic and clinical characteristics are presented in Table 1. Among the 35 eligible patients, 5 patients did not complete the study for the following reasons: genotype not determined (*n* = 2) and pharmacokinetic data collection not completed (*n* = 3). All polymorphisms genotyped were in Hardy-Weinberg equilibrium (Table 2). There were no patients with heterozygous or homozygous variants of 404A>T, 382A>T, 502C>T, 833delA, 830C>A, 41A>G, and -715T>C. Linkage disequilibrium was found between -1105G>A and -1032G>A genotypes (*D'* = 1, *R*² = 1, <http://analysisstools.nci.nih.gov/Ldlink/>). All patients genotyped as -/A to *SLCO1A2* -189_-188insA were also genotyped as AG for *SLCO1A2* -1105G>A and -1032G>A polymorphisms. One patient genotyped as homozygous mutant (AA) to -189_-188insA was also genotyped as homozygous mutant (AA) to *SLCO1A2* -1105G>A and -1032G>A. However, two patients

Table 1 Demographic and clinical characteristics of patients submitted to surgery using rocuronium as neuromuscular blocker

Parameters	Median (interquartile range) or number (%)
Age (years)	50 (39.5–75)
Sex (male/female)	13 (43)/17 (56)
ASA classification	I (<i>n</i> = 17) II (<i>n</i> = 12) III (<i>n</i> = 1)
Body weight (kg)	71.5 (59.1–79.5)
Body mass index (kg/m ²)	25.9 (24.4–28.4)
eGFR (mL/min/1.73 m ²)	82 (62–91)
Rocuronium dose (mg/kg)	0.6 (0.5–0.6)

ASA classification: American Society of Anesthesiology classification for physical status: ASA I—healthy patient, ASA II—patient with mild systemic disease, ASA III—patients with severe systemic disease, ASA IV—patient with severe disabling systemic disease, and ASA V—patient at risk for life-limiting. eGFR: glomerular filtration rate estimated using CKD-EPI equation. Data are expressed as median and the interquartile range or absolute numbers and percentiles

Table 2 Genotype and allelic frequency of OAT1A2 polymorphisms

SNP	Genotype frequency (%)	Allelic frequency (%)	Hardy-Weinberg equilibrium* (p value)
-1032G>A	GG = 0.36	G = 0.66	0.17
	GA = 0.61	A = 0.34	
	AA = 0.03		
-361G>A	GG = 0.89	G = 0.95	0.96
	GA = 0.11	A = 0.05	
	AA = 0		
-189_-188InsA	-/- = 0.43	- = 0.69	0.37
	-/A = 0.54	A = 0.31	
	AA = 0.03		
516A>C	AA = 0.89	G = 0.95	0.96
	AC = 0.11	A = 0.05	
	CC = 0		
559G>A	GG = 0.89	G = 0.95	0.96
	GA = 0.11	A = 0.05	
	AA = 0		
-1105G>A	GG = 0.36	G = 0.66	0.17
	GA = 0.61	A = 0.34	
	AA = 0.03		

*Hardy-Weinberg equilibrium was tested by chi-squared test. The level of significance was $p \leq 0.05$

genotyped as AG for *SLCO1A2* -1105G>A and -1032G>A polymorphisms were homozygous for the wild-type *SLCO1A2* -189_-188InsA.

The kinetic disposition of ROC was evaluated in 30 patients undergoing elective surgery under general anesthesia. The administration of 0.3 to 0.9 mg/kg ROC resulted in median (25th–75th percentiles) AUC/D 417.6 (354.2–548.8) ($\mu\text{g}/\text{mL}/\text{min}$)/(mg/kg) and total body clearance of 154.3 (150.5–207.5) mL/min.

The relationship between *SLCO1A2* genetic polymorphisms and the kinetic disposition of ROC is shown in Table 3. Patients genotyped as -A or AA ($n = 17$) for

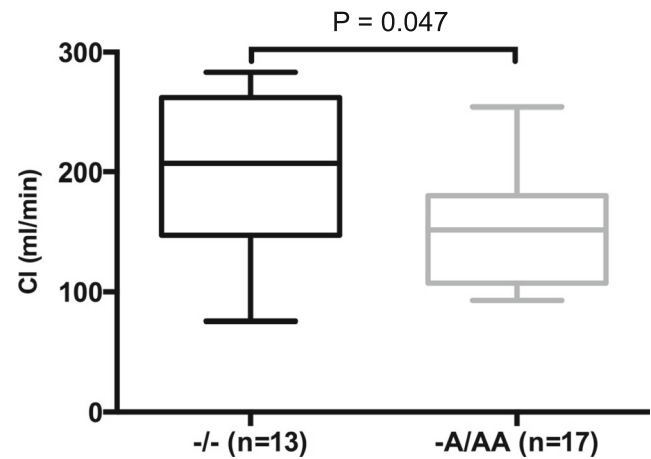


Fig. 1 Effect of *SLCO1A2* -189_-188InsA polymorphism on total clearance (CL) of rocuronium in patients undergoing elective surgery under general anesthesia. The line inside each box indicates the median; 25th and 75th percentiles are represented as the bottom and top lines of the box, respectively; error bars represent the maximum and minimum values. Groups were compared using the Mann-Whitney test for unpaired data

SLCO1A2 -189_-188InsA showed reduced total clearance of ROC compared to patients genotyped as -/- ($n = 13$) (151.6 vs 207.1 mL/min, $p \leq 0.05$) (Fig. 1). Patients genotyped as -A or AA for *SLCO1A2* -189_-188InsA did not differ from patients genotyped as -/- ($n = 13$) in terms of rocuronium dose, age, body weight, body mass index, and serum creatinine. The pharmacokinetics parameters of ROC were not significantly different between other *SLCO1A2* genotypes (Table 3).

Moderate correlation was observed between age and CL of ROC (correlation coefficient = -0.520 , $p \leq 0.05$) and between *SLCO1A2* -189_-188InsA genotype and CL ROC (correlation coefficient = -0.535 , $p \leq 0.05$). A weak correlation was

Table 3 Effects of *SLCO1A2* polymorphisms on rocuronium total clearance (mL/min)

Polymorphism	Genotypes	Clearance (mL/min)		p value
		Median	25th–75th percentiles	
-1032/-1105G>A	GG ($n = 8$)	183.0	152.3–219.9	0.19
	GA/AA ($n = 22$)	154.0	108.4–199.9	
-361G>A	GG ($n = 27$)	158.8	143.0–207.4	0.35
	GA/AA ($n = 3$)	107.5	91.6–166.3	
-189_-188InsA	-/- ($n = 13$)	207.1	150.5–256.7	0.047*
	-A/AA ($n = 17$)	151.6	107.168.2	
516A>C	AA ($n = 26$)	157.1	139.3–219.5	0.33
	AC/CC ($n = 4$)	147.1	120.4–167.2	
559G>A	GG ($n = 27$)	153.2	123.2–207.4	0.20
	GA/AA ($n = 3$)	202.4	180.6–234.6	

*Statistically significant ($p \leq 0.05$)

observed between sex and CL of ROC (correlation coefficient = -0.365 , $p \leq 0.05$). Multiple regression analysis revealed that 55% of the variation in total clearance of ROC was explained by three variables: age, *SLCO1A2* -189_-188InsA genotype, and sex, accounting for 28.3, 16.3, and 10.4% of the variation of ROC clearance, respectively (Table 4).

Discussion

This study suggests that OATP1A2 is involved in hepatic uptake of ROC required for its elimination, which is primarily mediated by biliary excretion. The active transport of ROC into cholangiocytes is considered the determining factor for the duration of neuromuscular blocking effect, and in vitro and clinical studies have reported the important contribution of OATP1A2 for the hepatic uptake of the ROC [5–7, 19].

Genetic variation of drug transporters has important consequences for the kinetic disposition and the response of several drugs. In *SLCO1A2*, the non-silent single nucleotide polymorphisms (SNPs) in the coding region T38C (I13T), A516C (E172D), G559A (A187T), A382T (N128Y), A404T (N135I), and C2003G (T668S) define the allelic variants OATP1A2*2, *3, *4, *5, *6, and *7, respectively [2]. However, clinical studies investigating the influence of *SLCO1A2* polymorphisms on the kinetic disposition and drug response are still scarce [20].

The uptake of OATP1A2 substrates estrone sulfate, deltorphin II, [D-penicillamine(2,5)]-encephalin (DPDPE), and imatinib in HeLa cells was inhibited in OATP1A2*3 (516A>C) variant [2, 21]. The minor allele frequency (MAF) for *SLCO1A2* 516A>C (OATP1A2*3) in exon 5 was 5% in our work (Table 2) and was the same found in Europeans (5%) and in Brazilians (5%) [2, 22]. In African, Chinese, and Hispanic populations, the MAF for *SLCO1A2* 516A>C was 2.1, 0, and 2.1%, respectively [2]. Patients with gastrointestinal stromal tumors carrying 516A>C (OATP1A2*3) polymorphism showed no change in imatinib kinetic disposition [21]. Likewise, in the present investigation, the presence of 516A>C (OATP1A2*3) polymorphism did

not change the kinetic disposition of ROC in patients undergoing elective surgeries under general anesthesia.

The uptake of estrone sulfate was reduced in OATP1A2*4 (559G>A) variant compared to the reference OATP1A2*1 [2]. In the present study, three patients were genotyped as heterozygous for *SLCO1A2* 559G>A (OATP1A2*4; MAF = 0.05). However, *SLCO1A2* 559G>A polymorphism was not found previously in European, African, Chinese, or Japanese populations [2, 23]. The pharmacokinetic disposition of ROC was not altered in the presence of 559G>A (OATP1A2*4).

The functional characterization of OATP1A2*6 (404A>T) using transfected HeLa cells showed reduced uptake of estrone sulfate, deltorphin II, and [D-penicillamine(2,5)]-encephalin (DPDPE) compared to that of the reference [2]. Reduced OATP1A2 activity in carriers of *6 (404A>T) variant is due to the reduced OATP1A2 expression [2, 24]. In the investigation of Yamakawa et al. (2011) and in this study, none of the patients had heterozygous or homozygous variant of 404A>T (*6) [23].

Inhibition of uptake of OATP1A2 substrates was also reported for the variant *7 (2003C>G). The inhibition was pH-dependent with 77% reduction of uptake at pH 7.4 and 46% reduction at pH 5 [21]. Lee et al. (2005) suggested that reduced OATP1A2 activity in carriers of *7 (2003C>G) variant is related to reduced OATP1A2 expression [2, 24]. The *SLCO1A2* 2003C>G polymorphism was not investigated in this study due to its very low frequency in Hispanic Americans (1%) and Mexican Americans (1%), and its absence in European Americans, Asian Americans, and Chinese Americans [2, 25].

Considering the SNPs in the promoter region, the linkage disequilibrium between *SLCO1A2* -1105G>A and -1032G>A observed in this study has been previously reported in the investigation of 34 patients with chronic myeloid leukemia (CML). The MAF for *SLCO1A2* -1105G>A and -1032G>A polymorphisms was approximately 0.3 in the investigation of 100 healthy volunteers and 34 patients with chronic myeloid leukemia [23]. Similarly, the MAF of 0.34 for *SLCO1A2* -1105G>A and -1032G>A polymorphisms was found in this work (Table 2). In the evaluation of imatinib clinical pharmacokinetics, no differences in drug clearance were observed in the presence of -1105G>A/- or 1032G>A ($p \geq 0.05$).

Table 4 Multiple regression analysis to estimate the contribution of age, weight, and *SLCO1A2* -189_-188InsA genotype to the clearance of rocuronium in patients submitted to elective surgeries ($n = 30$)

Covariate	Contribution of covariates	p value
<i>SLCO1A2</i> -189_-188InsA genotype (%)	16.3	0.002*
Age (years) (%)	28.3	0.003*
Sex (male = 0; female = 1) (%)	10.4	0.036*
Weight (kg)	–	0.61
R^2	0.585	0.002

*Statistically significant ($p \leq 0.05$)

Similarly, no differences were found on ROC clearance in terms of *SLCO1A2* -1105G>A and -1032G>A genotypes (Fig. 1).

Within Japanese patients, the MAF for *SLCO1A2* -189_-188insA varied from 0.10 to 0.12 [23], while the MAF of 0.31 was found in this work (Table 2). In the evaluation of imatinib clinical pharmacokinetics, no differences in drug clearance were observed in the presence of -189_-188insA polymorphisms ($p \geq 0.05$). However, our data showed that patients carrying -A/AA genotypes ($n = 17$) for *SLCO1A2* -189_-188insA presented significant reduction in clearance of rocuronium ($p \leq 0.05$).

The presence of *SLCO1A2* -361G>A polymorphism increased significantly the clearance of imatinib in Japanese patients with chronic myeloid leukemia ($p \leq 0.05$) [23]. On the other hand, in the investigation of Brazilian patients undergoing elective surgery under general anesthesia, *SLCO1A2* -361G>A had no impact on ROC kinetic disposition (Fig. 1, Table 3).

Multiple regression analysis showed that age, *SLCO1A2* -189_-188insA genotype, and sex accounted for 28.3, 16.3, and 10.4% of the variation of ROC clearance, respectively. Collinearity was observed between age and eGFR, which are highly correlated. Age was chosen to the final regression model because it has shown to be a better predictor than eGFR (Table 4).

Conclusions

Patients carrying -A/AA genotypes for *SLCO1A2* -189_-188insA presented significant reduction on ROC clearance. When the present findings are compared to previous literature, the different influences of *SLCO1A2* -361G>A and *SLCO1A2* -189_-188insA polymorphisms on the pharmacokinetics of imatinib and rocuronium emphasize the importance of investigating the influence of pharmacogenetics of OATP1A2 in the clinical pharmacology of its substrates.

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Authors' contributions NVM, GRL, and VLL designed this study. ACCC, EBC, and JTA performed the pharmacogenetics analysis.

ACCC, BVC, and NVM performed the pharmacokinetics and statistical calculations. ACCC and NVM wrote the manuscript. All authors approved the final version of the manuscript.

Compliance with ethical standards The study was performed in accordance with the revised Declaration of Helsinki and the Good Clinical Practice of the International Conference on Harmonization (ICH-GCP). The Ethics Committee of the School of Pharmaceutical Sciences of Ribeirão Preto and the School of Medicine of Ribeirão Preto,

University of São Paulo (USP) approved the clinical protocol on September 13th, 2012 (CAAE 02583412.2.0000.5403). All patients provided written informed consent.

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