CLINICAL STUDY REPORT

STUDY TITLE PAGE

Study Title	The influence of *CYP3A5* and *ABCB1* genotype on the pharmacokinetics of twice daily tacrolimus and Advagraf
Sponsor	St. George's, University of London
Study Site	St. George's, St. Helier, Brighton, Guys and St Thomas' Hospitals
Chief Investigator	Prof. Iain MacPhee
EudraCT Number	2009-013461-25
Research Ethics Committee	London-West London Research Ethics Committee
REC Number	09/H0707/91
IMP	Tacrolimus (Advagraf)
Study start date (first patient enrolled)	13th October 2010
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Chief Investigator (Signature)	Prof. Iain MacPhee Iain MacPhee

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1. Title

Comparative pharmacokinetics assessments of tacrolimus Preparations: Evaluation of *CYP3A5* and *ABCB1* genotypes influence

2. Background

Scientific background and explanation of rationale

Tacrolimus is the primary immunosuppressive drug in use for solid organ transplantation. (Bouamar et al, 2013). Tacrolimus was initially available as a preparation requiring twice daily administration: Prograf®. A prolonged release preparation, Advagraf®, is now available with proven efficacy and safety following once daily dosing. Tacrolimus has a narrow therapeutic window and its high pharmacokinetic variability renders dose selection challenging. Therefore, therapeutic drug monitoring (TDM) is used routinely to direct tacrolimus dosing. To some extent, this variability is influenced by genetic factors. Enzymes in the cytochrome P4503A family (CYP3A) and the drug transporter P-glycoprotein (P-gp) play important roles in the absorption and metabolism of tacrolimus (MacPhee et al, 2002). The influence of the *CYP3A5*3* and *ABCB1 3435C>T* genotypes on the pharmacokinetics of immediate release tacrolimus; Prograf® is well-defined. However, it is unclear for prolonged release tacrolimus; Advagraf®. Recently identified polymorphisms *CYP3A4*22* and P450 Oxidoreductase (*POR*28*) were reported to have additional effects on tacrolimus pharmacokinetics and dose requirement (Jonge et al., 2011; Elens et al., 2013). Recently, 4β-hydroxycholesterol (4β-OHC) has been shown to be an endogenous marker of P450 3A activity in clinical practice (Diczfalusy et al., 2011). Prednisolone is a known inducer of both CYP3A and P-gp. The role of *CYP3A4*22* and *POR*28* in prednisolone metabolism is unknown. An inverse correlation between corticosteroid daily dose and tacrolimus exposure was demonstrated in renal transplant recipients (Anglicheau et al., 2003a). Achieving therapeutic trough concentration is of vital importance during the period immediately after transplantation. Therefore, the identification of parameters predictive of the optimal initial tacrolimus dosage would be of potential value in clinicla practice. Furthermore, high within-patient variability (WPV) in tacrolimus exposure is considered as a risk factor for allograft loss and late acute rejection (Wu, et al. 2011). The causes of this variability are not completely understood.

This study was carried out on stable renal transplant patients treated with twice daily tacrolimus (Prograf® or Adoport®) and were switched to the same total daily dose of Advagraf®. Twenty four hour pharmacokinetic profiles were performed before and two weeks after the change. In order to exclude the use of prednisolone as a confounding factor, only patients on not more than 5 mg prednisolone daily were included. The within-patient variability (WPV) was calculated based on the dose-normalized tacrolimus trough blood concentrations (C_0). Analysis of C_0 was also made during periods of stable tacrolimus doses. This study was designed to assess the influence of genetic polymorphisms *CYP3A5*3* and *ABCB1* 3435C >T on tacrolimus pharmacokinetics of immediate- and prolonged- release tacrolimus formulations and their correlation with tacrolimus dosing in 64 stable renal transplant recipients. Genotyping at *CYP3A4*22* and *POR*28* loci in this study was undertaken to ascertain any influence of these genes on the pharmacokinetics of twice and once daily tacrolimus formulations. The influence of switching stable renal transplant patients to once daily tacrolimus formulation (Advagraf®) on WPV was investigated. In a secondary exploratory study to investigate the potential utility of 4β-OHC

as a CYP3A biomarker in informing tacrolimus dosing, 4β-OHC concentrations in plasma samples was measured and the relationship between 4β-OHC, *CYP3A5*3* genotype and tacrolimus exposure was examined. As another secondary exploratory study, prednisolone plasma concentrations were measured to explore the relationship between the above mentioned genetic polymorphisms and prednisolone exposure and its effect on tacrolimus dose.

A significantly lower tacrolimus exposure was observed in CYP3A5 expressers compared with CYP3A5 non-expressers for both formulations. In contrast to *CYP3A5*3* genotype, *ABCB1 3435C>T* gene had a minor influence on tacrolimus exposure irrespective of tacrolimus formulation. When combined, tacrolimus pharmacokinetics and dose requirements were significantly correlated with the combined-genotype grouping. The *CYP3A4*22 CT* genotype was associated with significantly greater tacrolimus exposure ($AUC_{0\text{-}24}$, C_{max}) compared with the *CYP3A4*22 CC* genotype. *POR*28 CT/TT* genotype was associated with significantly lower tacrolimus exposure compared with the *POR*28 CC* genotype in CYP3A5 non-expressing subjects. Switching from immediate to prolonged release tacrolimus formulations in kidney transplant patients was associated with a significantly lower tacrolimus trough concentration (C_0), but had no influence on WPV. *CYP3A5* genotype had no impact on WPV. Plasma concentration of 4β-OHC was greater in CYP3A5 expressers. The 4β-OHC/C ratio was significantly correlated with tacrolimus exposure and dose requirement. Prednisolone exposure was not influenced by *CYP3A5*3, CYP3A4*22, ABCB1 3435C>T* or *POR*28* genotype.

Our results indicate that *CYP3A5*3*, *ABCB1 3435C>T* and *CYP3A4*22* polymorphisms are important determinants of tacrolimus disposition and may explain part of the clinically observed high between-individual variability in tacrolimus pharmacokinetics. *POR*28* is associated with tacrolimus dose requirement in CYP3A5 non-expressers. Thus, genotyping at these loci before renal transplantation may provide important information about the optimal initial dose of tacrolimus. Pharmacogenetic dosing strategies based on these genotypes are likely to be equally applicable to prescribing the once daily tacrolimus formulation, Advagraf®, as to twice daily formulations. Moreover, switching from immediate to prolonged release tacrolimus formulations had no influence on WPV. 4β-OHC/C ratio may be a useful biomarker for tacrolimus dosing in renal transplanted patients. Genotyping at *CYP3A5*3*, *CYP3A4*22*, *POR*28* and *ABCB1* 3435C>T loci is unlikely to allow individualization of prednisolone dose.

3. Methods

3.1 Objectives

The Objectives of the study are as follows:

1. Study the influence of *CYP3A5*3* and *ABCB1* SNPs on the pharmacokinetics of immediate release tacrolimus; Prograf® or Adoport® and prolonged release; Advagraf® within individual patients.
2. Investigating the influence of *CYP3A4*22* and *POR*28* SNPs on the pharmacokinetics of immediate release tacrolimus; Prograf® and Adoport® and prolonged release; Advagraf® within individual patients.
3. Investigate the relationship between genetically determined variation in CYP3A expression in comparison to the phenotypic marker 4β-OHC and tacrolimus pharmacokinetics and dose requirement.

4. Explore the relationship between the *CYP3A5, ABCB1, CYP3A4*22* and *POR*28* genotypes and prednisolone- prednisone exposure.

3.2 Trial design

The study is an open-label pharmacokinetic study with a crossover design.

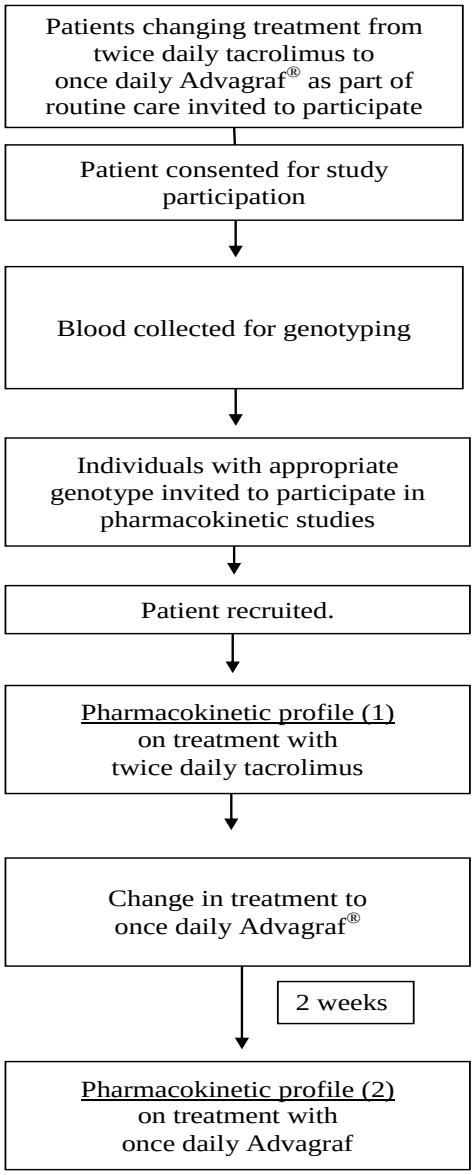


Figure 1: Study Flow Chart of Twice Daily Tacrolimus and Advagraf® Pharmacokinetic Profiles Using Crossover Design.

3.3 Participants

3.3.1 Inclusion Criteria

1) Renal transplant recipient at least 6 weeks after transplantation on treatment with twice daily Prograf (Tacrolimus) with planned change in treatment to once daily Advagraf (prolonged release tacrolimus) as part of standard care.
2) Aged at least 18 years
3) Treatment with 5 mg prednisolone daily
4) Signed and dated informed consent obtained before screening and before performance of any protocol-specific tests.
5)

3.3.2 Exclusion Criteria

Patients excluded from the study if they are

1) Unstable.
2) Under 18 years old.
3) Treated with more than 5 mg prednisolone daily.
4) Treated with potent cytochrome P4503A and P-glycoprotein inducers (such as Carbamazepine, Phenytoin, and Rifampicin) inhibitors (such as Diltiazem, Erythromycin, Fluconazole, and Verapamil) or any less commonly prescribed potent inducer or inhibitor.
5) Developing intolerance to either tacrolimus preparations.
6) Inability to obtain satisfactory venous access.
7) In the event of an adverse event where study continuation is considered to be inappropriate by the investigator.
8) Withdrawal of consent.

3.4 Study settings

This study is a multicentre, UK study. Subjects were recruited from the three centres that constitute the South West London, Surrey, Sussex Extended Renal Network:

- St. George's Healthcare NHS Trust
- Epsom and St. Helier University Hospitals NHS Trust
- Brighton and Sussex University Hospitals NHS Trust

3.5 Interventions

This study measured drug concentrations for patients' standard therapy. During twice daily tacrolimus treatment, tacrolimus dose is usually adjusted to achieve 12-hour post-dose whole blood concentrations of 8-12 µg/L up until three months after transplantation and thereafter 5-8 µg/L. When the treatment was changed from twice daily tacrolimus to Advagraf® the same total daily dose was administered and adjusted to maintain trough blood concentrations within the target range. In order to standardise prednisolone CYP3A inducing effect, only patients treated with no more than 5 mg prednisolone daily were recruited.

Patients involved in the study adopted a dosing schedule for one week prior to the study where the current dose of twice daily tacrolimus was administered twice daily at 08:00 and

20:00. An initial pharmacokinetic profile of the twice daily dose tacrolimus was measured. Treatment was then changed to the same daily dose of Advagraf®, which was administered once daily at 08:00 and then two weeks later, a further pharmacokinetic profile was measured after a single morning dose of Advagraf®.

A series of blood samples were collected at 0, 0.5, 1, 2, 4, 6, 8, 12, 12.5, 13, 14, 16 and 24 hours post-dose for twice daily tacrolimus and at 0, 1, 2, 3, 4, 6, 8, 10, 12 and 24 hours post-dose for Advagraf®. This required a 24 hour stay in the hospital for the first set of samples with the option to go home between the 12 hour and 24 hour samples on the second sampling day. Subjects were required to fast for 2 hours before taking the drug dose and for 1 hour afterwards. A venous cannula was placed into the hand or arm for blood sampling and was kept in place throughout the sampling period. Five mL of blood was collected at each time point into EDTA tubes. For patients on 5mg prednisolone, extra five mL blood samples were collected for prednisolone measurement at each time point into EDTA tubes. Patients' samples at 12.5 hour post-dose of twice daily tacrolimus administration were used for 4β-hydroxycholesterol analysis. Plasma was prepared by centrifugation for 10 minutes at 2500 g at room temperature. Blood and plasma samples were stored frozen at approximately -20ºC until the drug bioanalysis.

To study *CYP3A5*3* and *ABCB1* effect on tacrolimus preparations, subjects were divided into four groups according to their genotype, as shown in **Table 1**. The target size for each group was 30 patients. To study *CYP3A4*22* and *POR*28*, subjects were divided into two groups according to their genotype. Therefore *CYP3A4*22,* patients were divided into *CYP3A4*22 CC* and *CYP3A4*22 CT/TT* genotype groups and those for *POR*28* were divided into *POR*28 CC* and *POR*28 CT/TT* genotype groups.

Table 1: Study Genotype Groups of *CYP3A5* and *ABCB1* Alleles.

CYP3A5 genotype		ABCB1 genotype
***1/*1* or	CYP3A5 expressers	*CC*
***1/*1* or		*CT/TT*
***3/*3*	CYP3A5 non-expressers	*CC*
***3/*3*		*CT/TT*

3.6 Outcomes

Primary outcomes

- Determine the influence of *CYP3A5 and ABCB1* genotypes on Advagraf® pharmacokinetics
- Compare the influence of these genotypes on the comparison between twice daily tacrolimus and Advagraf® pharmacokinetics

Secondary outcomes

- Measurement of prednisolone concentration in samples collected for tacrolimus measurement to explore the relationship between the *CYP3A5* and *ABCB1* genotypes and prednisolone exposure.
- Measurement of plasma 4β-hydroxycholesterol as a biomarker of CYP3A4 and 3A5 activity to study relationship to *CYP3A5* genotype and tacrolimus pharmacokinetics.

- Determination of the influence of the *CYP3A4*22* and *P450 oxidoreductase*28* genotype on the pharmacokinetics of Prograf, Advagraf® and prednisolone.

I. Tacrolimus Analysis

This analysis procedure was conducted following the method previously validated at Analytical Services International Ltd, St George's - University of London titled "The Validation of an HPLC/MS Assay to Measure Tacrolimus and Everolimus in Human Blood". It allows the analysis of tacrolimus in whole blood at concentrations ranging from 1-50 ng/mL with a correlation coefficient of 0.998. The lower limit of quantitation was 0.25ng/mL.

II. 4β-hydroxycholesterol Analysis

This analysis procedure was conducted following the method previously validated at Clinical Chemistry Department; Erasmus MC University of Netherlands titled "Quantification of endogenous CYP3A marker 4β-hydroxycholesterol in human plasma by LC-ESI-MS/MS using picolinyl derivatisation." It allows the analysis of the plasma 4β-hydroxycholesterol at concentrations ranging from 4.28-137ng/mL with a correlation coefficient of 0.999. The lower limit of quantitation was 1.8 ng/mL.

III. Prednisolone and prednisone analysis in plasma

Plasma concentration of prednisolone and its metabolite in collected samples was analysed using validated liquid chromatography-mass spectrometry (LC- MS/ MS) method. Plasma samples were analysed using calibration range from 2.5-375 µg/ mL for prednisolone and 0.5-75.0 µg/L for prednisone. The correlation coefficient (r) between concentration and peak area ratio is ≥ 0.9976 for all curves.

IV. Genotyping Determination

Ethylene diamine tetra-acetic acid (EDTA) whole blood samples were collected. The whole blood samples were stored at −20°C until DNA isolation. DNA was extracted from peripheral blood samples using QIAamp DNA Blood Mini Kit (QIAGEN®, West Sussex, UK) and stored at −20°C until analysis. *CYP3A5*1/*3, ABCB1 3435C>T, CYP3A4*22 C >T* and *POR*28 C >T* polymorphisms were genotyped using real time polymerase chain reaction (PCR), a LightCycler® based technique. The samples were amplified using specific primer sequences.

V. Determination of ethnicity

Patients were classified by ethnicity based on the patient's transplant assessment records or their self-report as follows:

- White: any Caucasians, white British and any other white background
- Black: any ancestry from sub-Saharan Africa or any other black background including Caribbean
- Asian: ancestry from India and any other south Asian background, not including any East Asians (Koreans, Chinese and Japanese).

3.6.1 Changes to outcomes

Not applicable.

3.7 Sample size

Subjects were divided into four groups according to their genotype, as shown in Table **1**. The target size for each group was 30 patients. The sample size required was calculated using an anticipated CV of 20% and a power of 80%, within each of the four cohorts individually. From previous data, tacrolimus systemic exposure ($AUC_{0\text{-}24}$) for Prograf® was slightly higher than that for Advagraf® [ratio 1.10]. In order that the 90% confidence interval for the treatment ratio lies entirely within 0.8 – 1.25, the total number of subjects required in each group was 30. The sample size calculation was performed using nQuery Advisor 5.0 [table MTE3-1].

Table 2: Study Genotype Groups of *CYP3A5* and *ABCB1* Alleles.

CYP3A5 genotype		*ABCB1* genotype	Number of patients
**1/*1* or **1/*3*	CYP3A5 expressers	*CC*	30
**1/*1* or **1/*3*		*CT/TT*	30
**3/*3*	CYP3A5 non-expressers	*CC*	30
**3/*3*		*CT/TT*	30
Total			120

3.7.1 Interim analyses and stopping guidelines

Not applicable.

3.8 Randomisation

No randomisation was required.

3.9 Blinding

This was an open label study

3.10 Statistical methods – may be provided by the trial statistician

Statistical analysis was performed using Minitab statistical software (Minitab 17). The log-transformed data were analysed using analysis of variance (ANOVA) with factors for genotype group and treatment. 90% confidence intervals around the ratio in means for twice-daily tacrolimus: Advagraf® within each genotyping group was compared to the bioequivalence margin of 0.8-1.25. 4β-hydroxycholesterol and cholesterol individual plasma concentrations and prednisolone-prednisone individual pharmacokinetic parameters were determined for

each genotype group. Statistical analysis was performed to assess the statistical significance of differences in 4β-OHC and 4β-OHC/C ratio in tacrolimus kinetics between different genotype groups and to assess the statistical significance of differences in prednisolone kinetics between different genotype groups.

4. Results

4.1 Participant Flow

Of 75 stable kidney transplant patients who were screened and considered eligible for participation, 11 withdrew before the study began. Therefore, 64 patients (43 men, 21 women; 39 White, 12 Black, 13 Asian; mean [SD] age, 55 [13] years; age range, 21-78 years; mean weight, 76.4 [15.2] kg; mean height, 170.4 [8.6] cm) was recruited, and all participants completed both study periods. The mean (SD) time post-transplant was 4.1 (4.6) years (median 1.8 years, range 0.3–22.8). 34 patients had received a graft from a deceased donor (59.3%). Of these 64 patients, 25% had diabetes mellitus, and 61% were receiving maintenance steroids. 19 patients (29.7%) were receiving mycophenolate and 14 were receiving Azathioprine (21.9%) at baseline and throughout the study. Forty-eight patients (75%) were receiving with Prograf® and 16 patients (25%) were receiving Adoport®. The demographic characteristics and immunosuppression therapy are shown in **Table 3.**

Table 3: Population Characteristics and Immunosuppression Therapy

Characteristics	Results
Age (years), mean (SD)	55 (13)
Male gender, n (%)	43 (67.2%)
Ethnic group, n (%)	
White	39 (60.9%)
Black	12 (18.8%)
Asian	13 (20.3%)
Body weight (kg), mean (SD)	76.4 (15.2)
Height (cm), mean (SD)	170.4 (8.6)
Diabetes mellitus, n (%)	16 (25%)
Time since transplantation (years)	
Mean (SD)/ Median (range)	4.1 (4.6) / 1.8 (0.3-22.8)
Donor type, n (%)	
Living / Deceased	26 (40.6%) / 38 (59.3%)
Immunosuppression at baseline:	
Tacrolimus, n (%)	
Prograf®/ Adoport®	48 (75%) / 16 (25%)
Corticosteroids, n (%)	39 (61%)
Azathioprine, n (%)	14 (21.9%)
Mycophenolate, n (%)	19 (29.7%)

4.2 Summary of results with tables

4.2.1 *CYP3A5* effect on tacrolimus pharmacokinetic profiles

The *CYP3A5* SNP was a significant predictor of tacrolimus dose. Individuals possessing at least one *CYP3A5*1* allele (CYP3A5-expressers) required higher tacrolimus doses compared with *CYP3A5*3/*3* carriers (CYP3A5 non-expressers). Furthermore, we observed that the presence of the *CYP3A5*1* allele was strongly associated with lower dose-normalised tacrolimus blood concentrations (**Table 4**).

Table 4. Tacrolimus PK parameters according to their *CYP3A5*3* genotypes

PK-parameter	CYP3A5 Expressers n= (30)	CYP3A5 Non-expressers n= (34)	p-value
Dose **(mg/Kg/day)**	0.11± 0.05	0.05± 0.03	P<0.001
C_{max} **(µg/L/mg/Kg)**	19.1 ± 10.6	33.5 ± 13.8	P<0.001
$AUC_{0\text{-}24}$ **(µg*h/L/mg/Kg)**	223.4 ± 140.3	458.0 ± 214.2	P<0.001
C_0 **(µg/L/mg/Kg)**	6.9 ± 5.1	15.1 ± 8.7	P<0.001

Tacrolimus pharmacokinetic parameters following administration of twice-daily Prograf® or Adoport® and once-daily Advagraf® in CYP3A5 expresser and non-expresser patients are summarized in **Table 5**. The blood concentration-time profiles of tacrolimus in 30 stable kidney transplant recipients are presented in

Figure 2. After the switch from twice to once daily tacrolimus, a slight decrease in the mean dose-normalized C_{max} and $AUC_{0\text{-}24}$ was observed, regardless of the CYP3A5 genotype. The mean dose-normalized C_{max} and $AUC_{0\text{-}24}$ were comparable between tacrolimus formulations in CYP3A5 expresser and non-expresser groups. In the CYP3A5 expresser group the mean dose-normalized C_0 was comparable for both formulations; however, in the CYP3A5 non-expresser patients, there was a significant reduction in the mean dose-normalized C_0 after the switch to once daily tacrolimus (**Table 5**).

The ratio of AUC_{0-24} , C_{max}, dose-normalized AUC_{0-24} and C_{max} means (90% CI) for Tac-OD versus Tac-TD are within the 90% CI of 80, 125. Hence bioequivalence was achieved with tacrolimus formulations in both CYP3A5 groups **(Table 6).**

Table 5. Influence of *CYP3A5*3* polymorphism and Form on Tacrolimus Pharmacokinetic Parameters.

PK-parameter	CYP3A5 Expressers n= (30)			CYP3A5 Non-expressers n= (34)		
	TD-Tac	Advagraf®	p-value	TD-Tac	Advagraf®	p-value
Dose (mg/Kg/day)	0.11 ± 0.05	-		0.05 ± 0.03	-	
AUC_{0-24} (µg*h/L/mg/Kg)	223.7 ± 152.1	223.0 ± 130.1	P= 0.72	478.7 ± 241.5	437.4 ±184.4	p= 0.14
C_{max} (µg/L/mg/Kg)	20.1 ± 11.8	18.1 ± 9.2	P= 0.33	35.8 ±15.9	31.2 ±11	P= 0.06
C_0 (µg/L/mg/Kg)	6.7 ±4.5	7.2 ± 5.7	P= 0.46	16.6 ± 10.2	13.6 ±6.7	P < 0.05

Table 6: Ratios of geometric means and 90% CI for AUC_{0-24}, C_{max}, dose-normalized AUC_{0-24} and dose-normalized C_{max} for Tacrolimus formulations in CYP3A5 genotype groups

Parameter	CYP3A5 Expressers		CYP3A5 Non-expressers	
	Ratio of geometric means (%)	90% CI	Ratio of geometric means (%)	90% CI
AUC_{0-24}	101%	(93% –110%)	92%	(86% –99%)
C_{max}	93%	(82% –104%)	88%	(80% –97%)
Dose-normalized AUC_{0-24}	102%	(93% –111%)	94%	(88% –101%)
Dose-normalized C_{max}	93%	(82% –105%)	90%	(82% –98%)

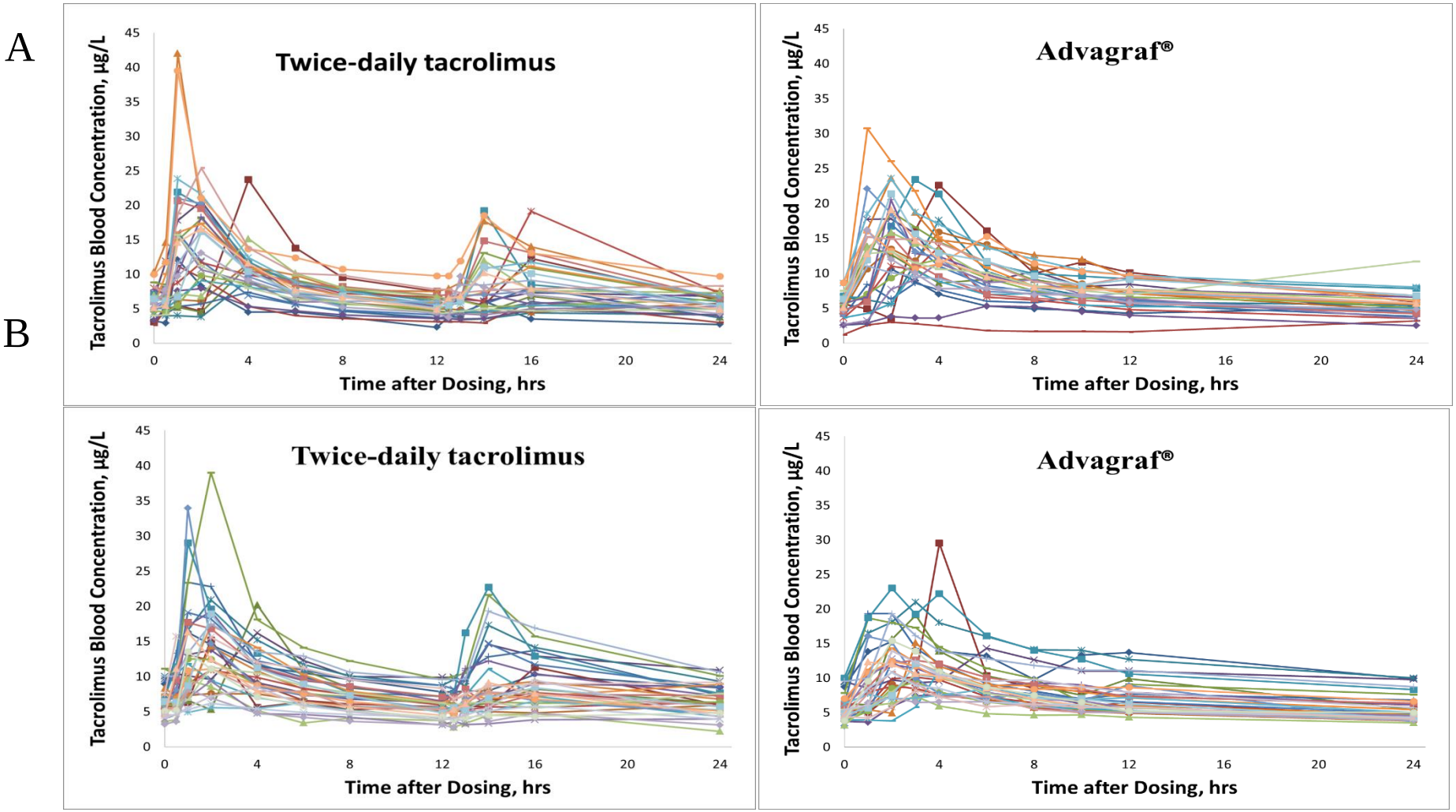


Figure 2: Summary of blood tacrolimus concentration time profiles in 30 stable adult kidney transplant recipients on twice-daily tacrolimus (before the switch) and Advagraf (after the switch) for CYP3A5 expressers (A) and CYP3A5 non-expressers (B).

4.2.2 *ABCB1* effect on tacrolimus pharmacokinetic profiles

The tacrolimus pharmacokinetic parameters according to *ABCB1* polymorphisms are shown in **Table 7**. A significant difference was observed at dose-normalized tacrolimus pharmacokinetic parameters (C_{max}, AUC_{0-24} and C_0) between the *ABCB1 CC* and the *CT/TT* genotypes. Moreover, we found that recipients with C/C genotype required a higher tacrolimus dose compared to those with *CT/TT* genotypes (0.10 ± 0.06 vs 0.07 ± 0.04, P <0.001, **Table 7**).

Table 7. Tacrolimus PK parameters according to their *ABCB1* genotypes

PK-parameter	*ABCB1 CC* n= (15)	*ABCB1 CT/TT* (49)	p-value
Dose (mg/Kg/day)	0.10 ± 0.06	0.07 ± 0.04	P<0.001
C_{max} (µg/L/mg/Kg)	21.3 ± 14.6	28.4 ± 13.8	P<0.001
AUC_{0-24} (µg*h/L/mg/Kg)	254.2 ± 189.6	376.8 ± 217.9	P<0.001
C_0 (µg/L/mg/Kg)	7.6 ± 6.0	12.4 ± 8.6	P<0.001

In both *ABCB1* groups, there was a slight decrease in tacrolimus dose-normalized C_{max}, AUC_{0-24} and C_0 after the switch to once-daily tacrolimus. Regardless of the *ABCB1* genotype group, the mean dose-normalized C_{max}, as well as mean dose-normalized AUC_{0-24} and C_0 were comparable for both tacrolimus formulations (P >0.05; **Table 8**). The blood concentration-time profiles of tacrolimus in 30 stable kidney transplant recipients are presented in

Figure 3.

In both *ABCB1* groups, the ratio of Tac-OD/Tac-TD AUC_{0-24} and dose-normalized AUC_{0-24} are within the 90% CI of 80, 125 and bioequivalence was achieved with both tacrolimus formulations. The ratio of means (90% CI) of C_{max} for Tac-OD versus Tac-TD in *ABCB1 CT/TT* carriers is within the 90% CI of 80, 125 and both formulations are bioequivalent. While the ratio of Tac-OD / Tac-TD C_{max} and dose-normalized C_{max} ratio in *ABCB1 CC* group was outside the 90% CI of 80, 125. C_{max}

and dose-normalized C_{max} ratio was not bioequivalent between both formulations (**Table 9).**

Table 8: Influence of *ABCB1* polymorphism and Form on Tacrolimus Pharmacokinetic Parameters.

PK-parameter	*ABCB1 CC* n= (15)			*ABCB1 CT/TT* n= (49)		
	TD-Tac	Advagraf®	P-value	TD-Tac	Advagraf®	P-value
Dose (mg/Kg/day)	0.10 ± 0.06			0.07 ± 0.04		
C_0 (µg/L/mg/Kg)	8.6 ± 7.4	6.6 ± 4.3	P= 0.21	13.3 ± 9.9	11.5 ± 7.0	P= 0.07
C_{max} (µg/L/mg/Kg)	23.8 ± 17.6	18.7 ± 10.8	P= 0.17	29.8 ± 15.6	27.0 ± 11.8	P= 0.15
AUC_{0-24} (µg*h/L/mg/Kg)	275.3 ± 224.7	233.0 ± 151.9	P= 0.37	384.8 ± 241.0	368.7 ± 194.2	P= 0.82

Table 9: Ratios of geometric means and 90% CI for AUC_{0-24}, C_{max}, dose-normalized AUC_{0-24} and dose-normalized C_{max} for Tacrolimus formulations in *ABCB1* genotype groups.

Parameter	*ABCB1 CC*		*ABCB1 CT/TT*	
	Ratio of geometric means (%)	90% CI	Ratio of geometric means (%)	90% CI
AUC_{0-24}	93%	(81% –108%)	97%	(92% –103%)
C_{max}	85%	(68% –105%)	92%	(85% –100%)
Dose-normalized AUC_{0-24}	93%	(80% –107%)	99%	(94% –105%)
Dose-normalized C_{max}	84%	(68% –104%)	94%	(87% –101%)

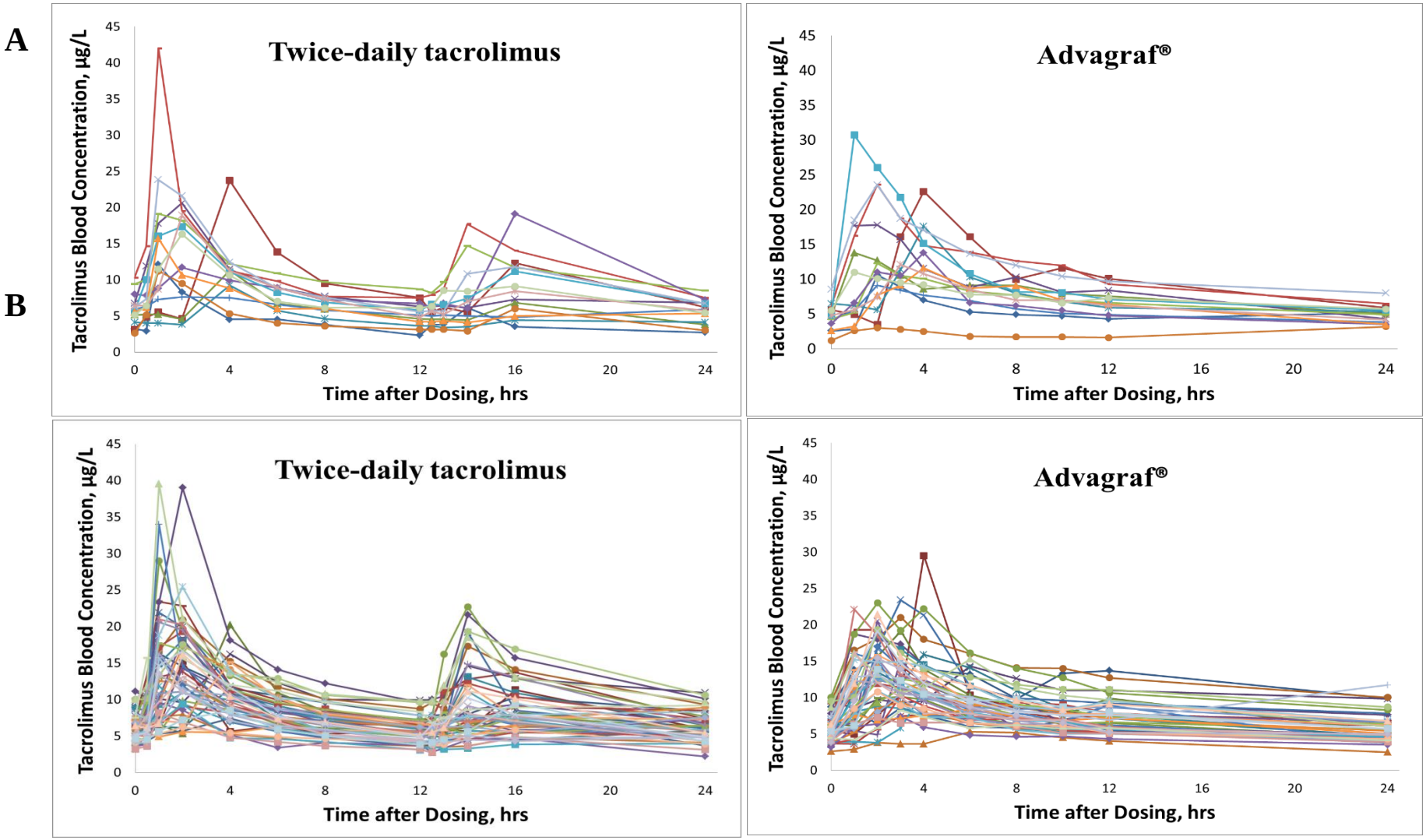


Figure 3: Summary of blood tacrolimus concentration time profiles in 30 stable adult kidney transplant recipients on twice-daily tacrolimus (before the switch) and Advagraf (after the switch) for *ABCB1 CC* (A) and *ABCB1 CT/TT* (B)groups.

4.2.3 *CYP3A5* and *ABCB1* combined effect on tacrolimus pharmacokinetic profiles

Four genotype combinations were identified in our study population: CYP3A5 expresser recipients with *ABCB1 CC* genotype (n=12); CYP3A5 expressers recipients with *ABCB1 CT/TT* genotype (n=18); CYP3A5 non-expresser recipients with *ABCB1 CC* genotype (n=4) and CYP3A5 non-expresser recipients with *ABCB1 CT/TT* genotype (n=30).

After excluding the four subjects with *CYP3A5 *3*3* and *ABCB1 CC* genotype from the analysis, significant differences in tacrolimus pharmacokinetic parameters were observed between groups. The influence of tacrolimus parameters increased progressively from CYP3A5 expresser */ABCB1CC* group to CYP3A5 non-expresser */ABCB1 CT/TT* group. When CYP3A5 non-expresser/*ABCB1 CT/TT* group compared with CYP3A5 expressers; *ABCB1CC* and the *CT/TT* genotype subgroup, significant differences in TAC dose requirements, dose-normalized AUC_{0-24}, dose-normalized C_{max} and dose-normalized C_0 were evident (**Table 10**).

Additionally, when we compared changes in these different genotype groups with the form, no difference was found between twice-daily tacrolimus and Advagraf® in mean weight-adjusted dose, dose-normalized C_0, dose-normalized C_{max} and dose-normalized AUC_{0-24} between the different genotype groups (**Table 11**). The blood concentration-time profiles of tacrolimus are presented in **Figure 5**.

The ratio of Tac-OD / Tac-TD AUC_{0-24} and dose-normalized AUC_{0-24} in all our study groups is within the 90% CI of 80, 125 and both formulations are bioequivalent. Whereas the ratio of C_{max} and dose-normalized C_{max} means (90% CI) for OD-Tac versus TD-Tac in CYP3A5 expressers *ABCB1 CC* group was outside the 90% CI of 80, 125 and both formulations were not bioequivalent (**Table 12**).

Table 10. Tacrolimus PK parameters according to their *CYP3A5*3* and *ABCB1*genotypes

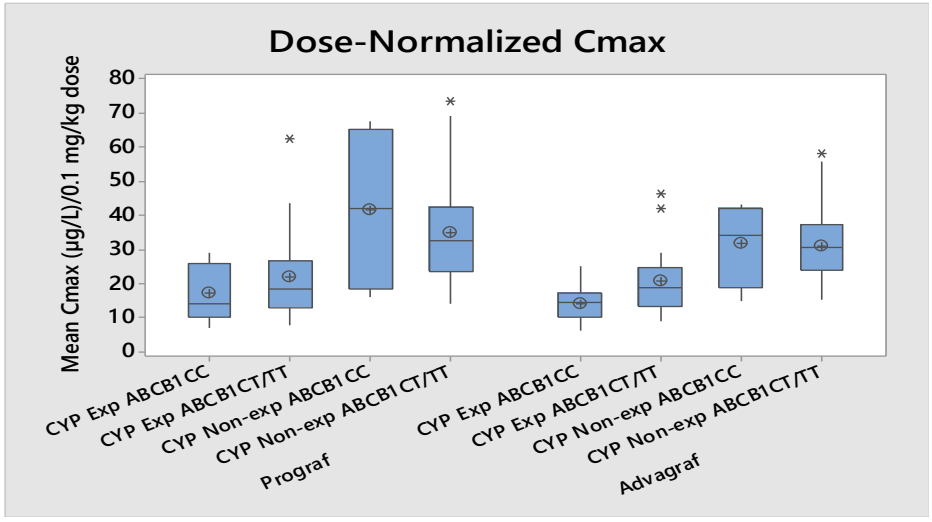
PK-parameter	CYP3A5 Expressers		CYP3A5 Non-expressers		p-value [a]	p-value [b]
	ABCB1 CC *n= 12*	*ABCB1 CT/TT* *n= 18*	*ABCB1 CC* *n= 4*	*ABCB1 CT/TT* *n= 30*		
Dose (mg/Kg/day)	0.12 ± 0.05	0.10 ± 0.05	0.05 ± 0.05	0.05 ± 0.02	P<0.001	P<0.05
C_{max} (µg/L/mg/Kg)	15.6 ± 6.8	21.4 ± 12.0	36.7 ± 19.0	33.1 ± 13.1	P<0.001	P<0.05
AUC_{0-24} (µg*h/L/mg/Kg)	172.3 ± 74.4	257.4 ± 163.0	477.6 ± 230.3	455.4 ± 213.9	P<0.001	P<0.05
C_0 (µg/L/mg/Kg)	5.0 ± 2.2	8.2 ± 6.0	14.7 ± 7.5	15.1 ± 8.9	P<0.001	P<0.01

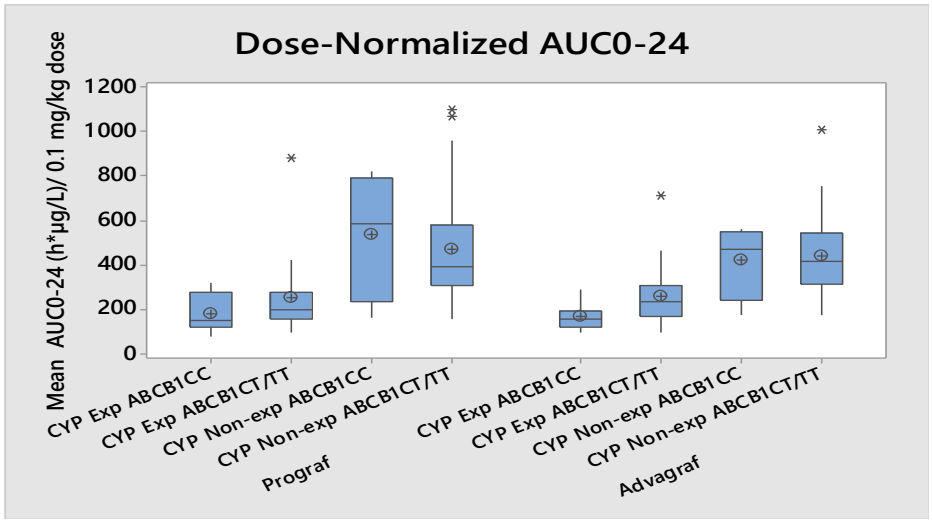
[a] p-Values refer to comparisons of CYP3A5 Expressers subgroups to CYP3A5 non-expresser/*ABCB1 CT/TT* group.

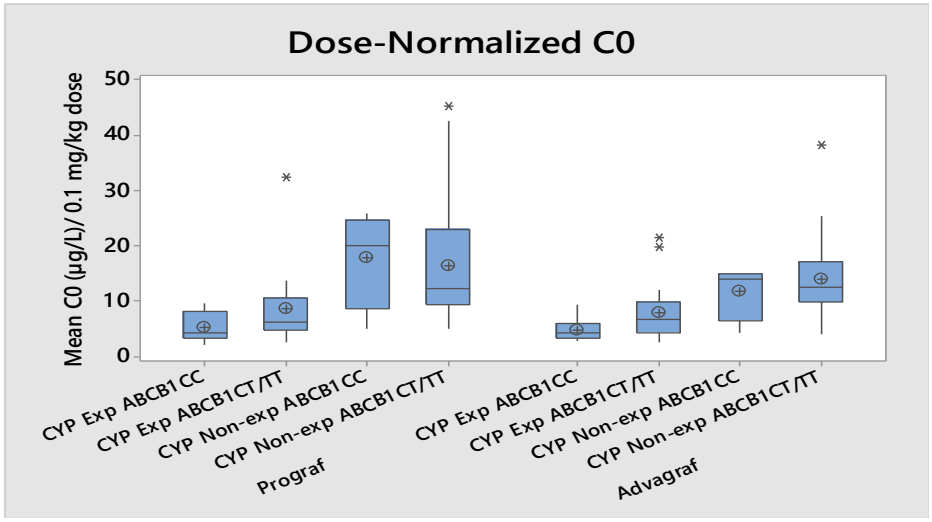
[b] p-Values refer to comparisons between CYP3A5 Expressers subgroups.

Table 11. Tacrolimus Pharmacokinetic Parameters for different combination of *CYP3A5*3* and *ABCB1*genotypes in both once- and twice daily-tacrolimus.

PK-parameter	CYP3A5 Expressers					
	ABCB1 CC			*ABCB1 CT/TT*		
	TD-Tac	Advagraf®	P-value	TD-Tac	Advagraf®	P-value
Dose (mg/Kg/day)	0.12 ± 0.05			0.10 ± 0.05		
C_0 (µg/L/mg/Kg)	5.3 ± 2.6	4.7 ± 1.8	0. 7	8.5 ± 6.8	8.0 ± 5.4	0. 6
C_{max} (µg/L/mg/Kg)	17.2 ±8.0	14.1 ± 5.2	0.3	22.0 ± 13.7	20.8 ±10.4	0.8
AUC_{0-24} (µg*h/L/mg/Kg)	178.0 ± 86.0	166.0 ± 64.0	0.8	253.8±179.6	261.0 ± 9.7	0.4
PK-parameter	CYP3A5 Non-expressers					
	ABCB1 CC			*ABCB1 CT/TT*		
	TD-Tac	Advagraf®		TD-Tac	Advagraf®	P-value
Dose (mg/Kg/day)	0.05 ± 0.05			0.05 ± 0.02		
C_0 (µg/L/mg/Kg)	17.7 ± 9.0	11.7 ± 5.1		16.4 ± 10.5	13.9 ± 6.9	0.06
C_{max} (µg/L/mg/Kg)	41.8 ± 25.0	31.6 ± 12.2		35.0 ± 14.8	31.1±11.0	0.1
AUC_{0-24} (µg*h/L/mg/Kg)	536 ± 291	418.8 ± 172.8		471.0 ± 238.9	439.9 ± 188.5	0.3


Dose-Normalized Cmax
Mean Cmax (µg/L)/0.1 mg/kg dose
0
10
20
30
40
50
60
70
80
CYP Exp ABCB1CC
CYP Exp ABCB1CT/TT
CYP Non-exp ABCB1CC
CYP Non-exp ABCB1CT/TT
Prograf
CYP Exp ABCB1CC
CYP Exp ABCB1CT/TT
CYP Non-exp ABCB1CC
CYP Non-exp ABCB1CT/TT
Advagraf


Dose-Normalized AUC0-24
Mean AUC0-24 (h*µg/L)/ 0.1 mg/kg dose
0
200
400
600
800
1000
1200
CYP Exp ABCB1CC
CYP Exp ABCB1CT/TT
CYP Non-exp ABCB1CC
CYP Non-exp ABCB1CT/TT
Prograf
CYP Exp ABCB1CC
CYP Exp ABCB1CT/TT
CYP Non-exp ABCB1CC
CYP Non-exp ABCB1CT/TT
Advagraf


Dose-Normalized C0
Mean C0 (µg/L)/ 0.1 mg/kg dose
0
10
20
30
40
50
CYP Exp ABCB1CC
CYP Exp ABCB1CT/TT
CYP Non-exp ABCB1CC
CYP Non-exp ABCB1CT/TT
Prograf
CYP Exp ABCB1CC
CYP Exp ABCB1CT/TT
CYP Non-exp ABCB1CC
CYP Non-exp ABCB1CT/TT
Advagraf

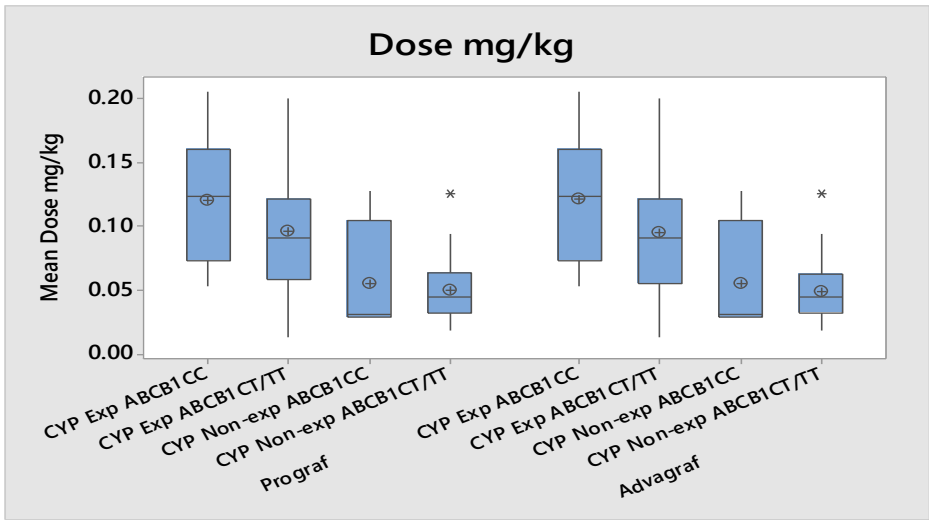
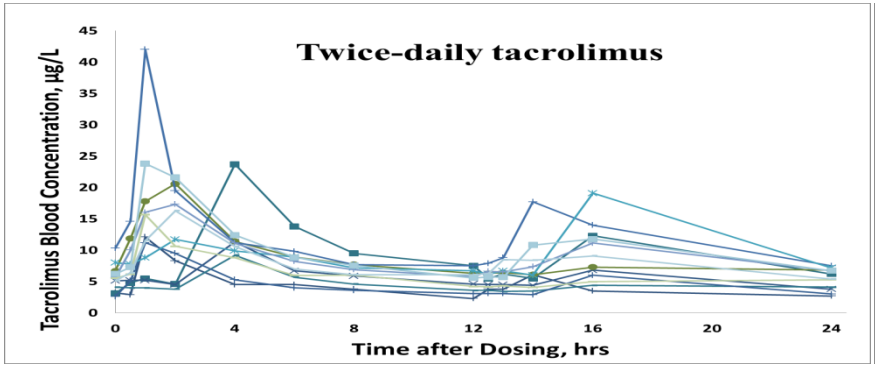

Dose mg/kg
Mean Dose mg/kg
0.00
0.05
0.10
0.15
0.20
CYP Exp ABCB1CC
CYP Exp ABCB1CT/TT
CYP Non-exp ABCB1CC
CYP Non-exp ABCB1CT/TT
Prograf
CYP Exp ABCB1CC
CYP Exp ABCB1CT/TT
CYP Non-exp ABCB1CC
CYP Non-exp ABCB1CT/TT
Advagraf

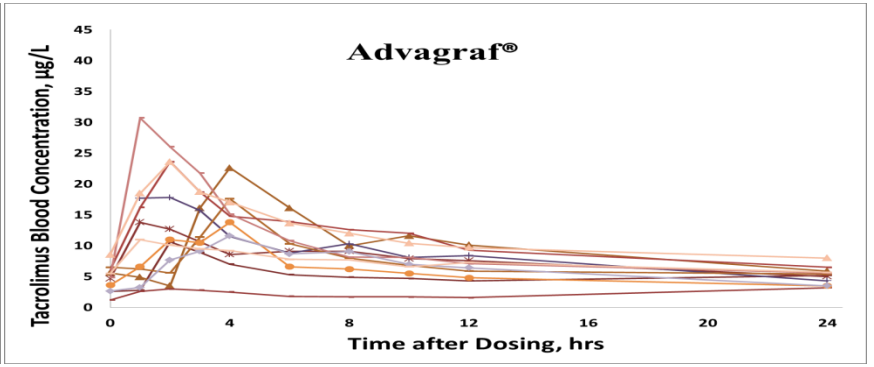
Figure 4: Effects of CYP3A5 and *ABCB1* genotypes on Tacrolimus Pharmacokinetic Parameters of twice-daily tacrolimus and Advagraf®.

Table 12: Ratios of geometric means and 90% CI for AUC_{0-24}, C_{max}, dose-normalized AUC_{0-24} and dose-normalized C_{max} for Tacrolimus formulations in CYP3A5 and *ABCB1* genotype groups.

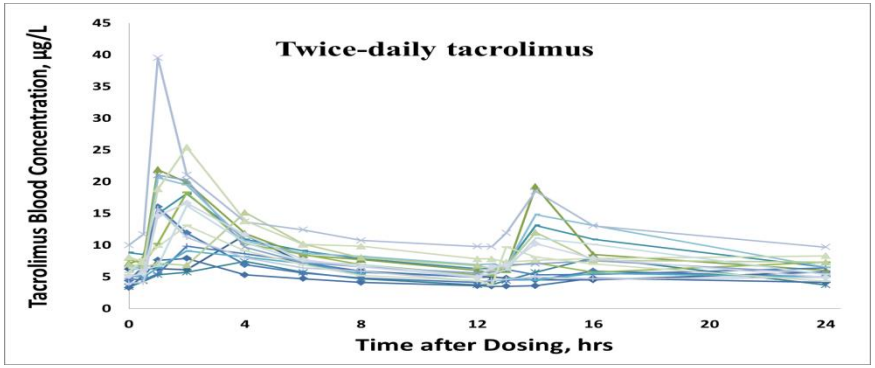
Parameter	CYP3A5 Expressers				CYP3A5 Non-expressers	
	ABCB1 CC		*ABCB1 CT/TT*		*ABCB1 CT/TT*	
	Ratio of geometric	90% CI	Ratio of geometric	90% CI	Ratio of geometric	90% CI
AUC_{0-24}	98%	(82% –117%)	104%	(94% –113%)	87%	(94% –101%)
C_{max}	86%	(66% –112%)	97%	(85% –110%)	89%	(80% –99%)
Dose-normalized AUC_{0-24}	97%	(82% –115%)	105%	(94% –117%)	96%	(89% –103%)
Dose-normalized C_{max}	86%	(66% –111%)	98%	(87% –112%)	91%	(82% –100%)

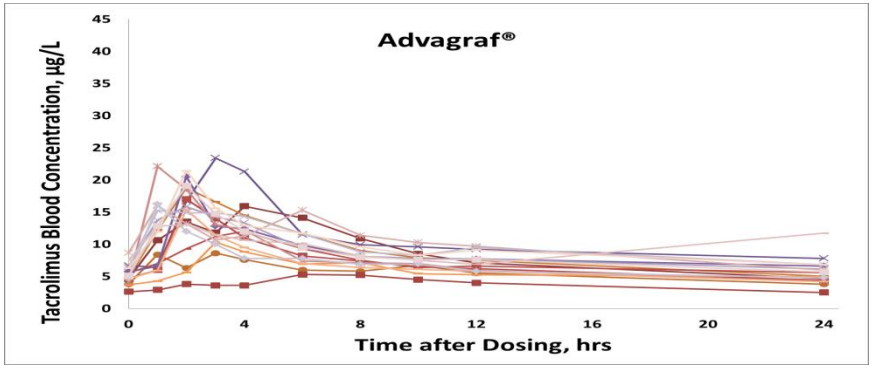
A


Twice-daily tacrolimus
Tacrolimus Blood Concentration, µg/L
Time after Dosing, hrs

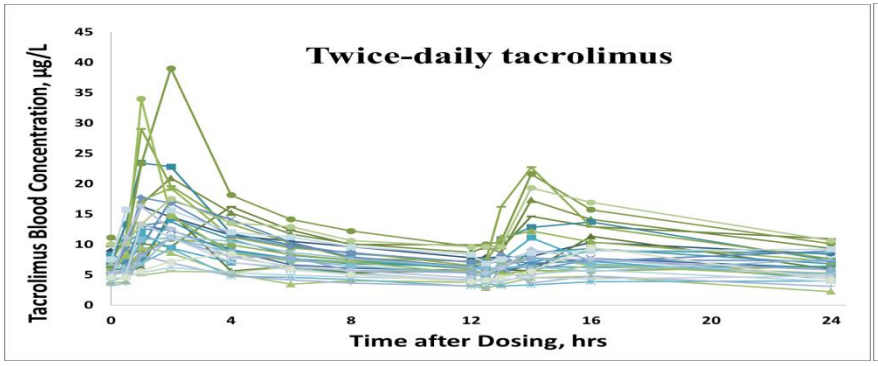

Advagraf®
Tacrolimus Blood Concentration, µg/L
Time after Dosing, hrs

B


Twice-daily tacrolimus
Tacrolimus Blood Concentration, µg/L
Time after Dosing, hrs


Advagraf®
Tacrolimus Blood Concentration, µg/L
Time after Dosing, hrs

C


Twice-daily tacrolimus
Tacrolimus Blood Concentration, µg/L
Time after Dosing, hrs

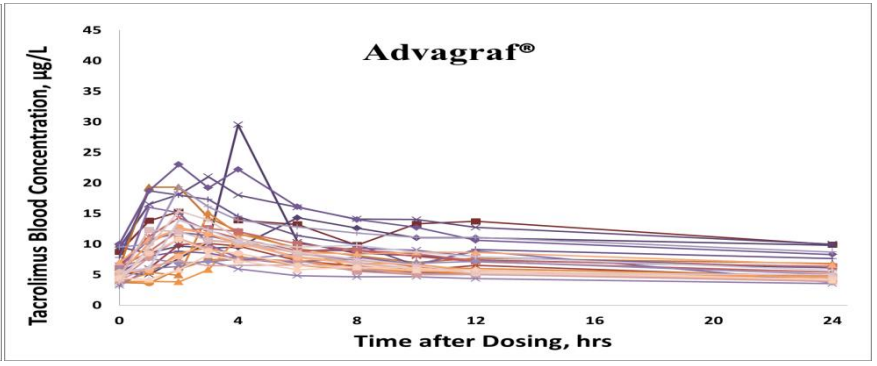

Advagraf®
Tacrolimus Blood Concentration, µg/L
Time after Dosing, hrs

Figure 5: Summary of blood tacrolimus concentration time profiles for *CYP3A5-ABCB1* genotypes . (A) CYP3A5 expressers / *ABCB1CC* group, (B) CYP3A5 expressers / *ABCB1CT/TT* group and (C) CYP3A5 non-expressers/ *ABCB1CT/TT* group .

4.2.4 *CYP3A4*22* & tacrolimus disposition

As shown in **Table 13**, a significant decrease in dose-normalized C_0 was observed according to the patients *CYP3A4*22* allelic status. The mean daily dose requirement of tacrolimus per body weight was 38.2% lower for T-variant allele carriers compared to CC allele carriers.

Table 13: Tacrolimus PK parameters according to their *CYP3A4*22* genotypes

PK-parameter	*CYP3A4*22 CC* n= (59)	*CYP3A4*22 CT* n= (5)	P-value
Dose (mg/Kg/day)	0.08 ± 0.05	0.05 ± 0.04	P<0.01
C_{max} (µg/L/mg/Kg)	25.8 ± 13.9	37.2 ± 15.4	P<0.05
AUC_{0-24} (µg*h/L/mg/Kg)	329.9 ± 198.6	562.0 ± 313.3	P<0.01
C_0 (µg/L/mg/Kg)	10.6 ± 7.4	19.5 ± 13.0	P<0.01

Upon comparing the influence of *CYP3A4*22* polymorphism on both tacrolimus formulations, we found no association between the dose-normalized AUC_{0-24}, C_{max}, C_0 and *CYP3A4*22* genotypes for either Tac formulation ($p > 0.05$), see **Table 14** and **Figure 6**.

In *CYP3A4*22 CC* group, the ratio of OD-Tac/TD-Tac AUC_{0-24} and dose-normalized AUC_{0-24} was within the 90% CI of 80, 125 and both formulations are bioequivalent. The ratio of the OD-Tac/TD-Tac for dose-normalized C_{max} falls outside 80% to 125% bioequivalence limits. These data are summarized in **Table 15**.

Table 14 :Influence of *CYP3A4*22* polymorphism and form (twice-daily tacrolimus; TD-Tac, and Advagraf®) on Tacrolimus Pharmacokinetic Parameters.

PK-parameter	*CYP3A4*22 CC* n= (59)			*CYP3A4*22 CT* n= (5)		
	TD-Tac	Advagraf®	P-value	TD-Tac	Advagraf®	P-value
Dose (mg/Kg/day)	0.08 ± 0.05			0.048± 0.045		
AUC_{0-24} (µg*h/L/mg/Kg)	340 ± 223	320 ± 173	P= 0.85	582 ± 351	542 ± 311	P=0.92
C_{max} (µg/L/mg/Kg)	27.5 ± 15.8	24.2 ± 11.6	P= 0.37	39.2 ± 17.6	35.3 ± 14.6	P= 0.79
C_0 (µg/L/mg/Kg)	11.4 ± 8.8	9.7 ± 5.7	P= 0.45	20.9 ± 14.5	18.2 ± 12.8	P =0.81

Table 15: Bioequivalence statistics for AUC_{0-24} and C_{max} for twice-daily tacrolimus (TD-Tac) and Once-daily tacrolimus OD-Tac in *CYP3A4*22 CC* Carriers.

Parameter	*CYP3A4*22 CC*	
	Ratio of geometric means (%)	90% CI
AUC_{0-24}	98%	(82% –117%)
C_{max}	90%	(79% –103%)
Dose-normalized AUC_{0-24}	98%	(82% –117%)
Dose-normalized C_{max}	91%	(78% –108%)

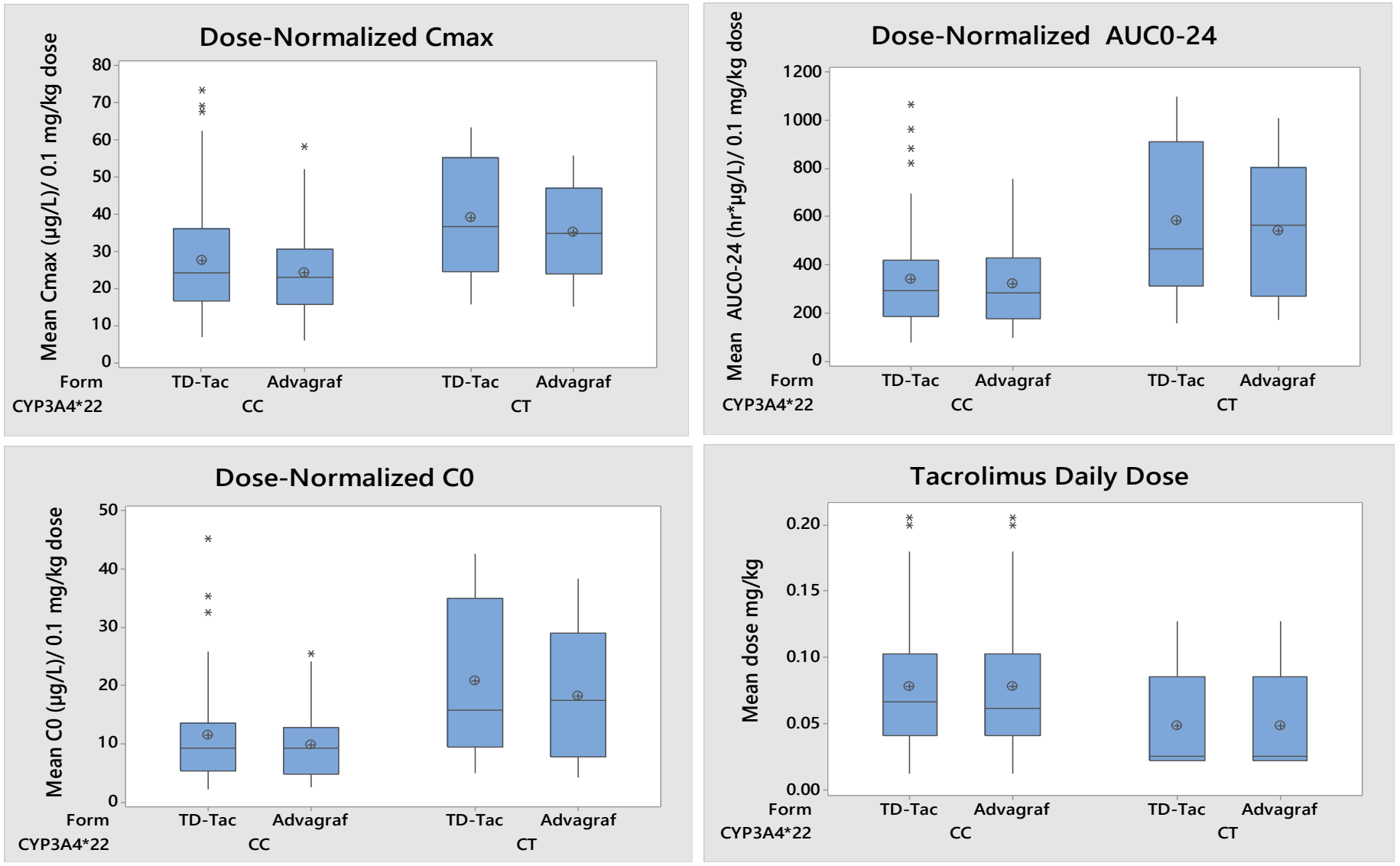


Figure 6: Effects of *CYP3A4*22* on Tacrolimus Pharmacokinetic Parameters of Twice-daily Tacrolimus (TD-Tac) and Advagraf®.

4.2.5 *CYP3A5*3* and *CYP3A4*22* combined genotypes & tacrolimus disposition

Three genotype groups were identified in our study population: extensive CYP3A metabolizers (CYP3A5 expressers/*CYP3A4*22CC*, n=30), intermediate CYP3A metabolizers (CYP3A5 non-expressers/*CYP3A4*22CC*, n=29) and poor CYP3A metabolizers (CYP3A5 non-expressers/*CYP3A4*22CT*, n=5).

Extensive CYP3A metabolizers showed a significantly higher daily dose of tacrolimus compared to the other two groups; the intermediate CYP3A metabolizers group (P< 0.001) and poor CYP3A metabolizers group (P< 0.001). However, the difference between the intermediate metabolizers and poor metabolizers wasn't statistically significant (p= 0.2). On the other hand, poor CYP3A metabolizers showed 95% higher dose-normalized tacrolimus C_{max} compared to extensive metabolizers (p < 0.0001) and 13% higher dose-normalized tacrolimus C_{max} compared to intermediate metabolizers (p= 0.4). The same trend was observed at tacrolimus dose-normalized AUC_{0-24} and C_0. The intermediate CYP3A metabolizers had a slight, but not statistically significant, decrease in dose-normalized AUC_{0-24} (p= 0.3) and C0 (p= 0.4) compared to poor CYP3A metabolizers, see **Table 16** & **Figure 7**.

No significant differences were found in tacrolimus dose and pharmacokinetic parameters between tacrolimus formulations in each group of the combined CYP3A genotypes (**Figure 8; Table 17**). The ratio of dose-normalized means (90% CI) of AUC_{0-24} for Tac-OD versus Tac-TD is within the 90% CI of 80, 125. Hence bioequivalence was achieved with tacrolimus formulations. Whereas The ratio of (90% CI) of C_{max} for Tac-OD versus Tac-TD was outside the 90% CI of 80, 125 and both formulations were not bioequivalent (**Table 18**).

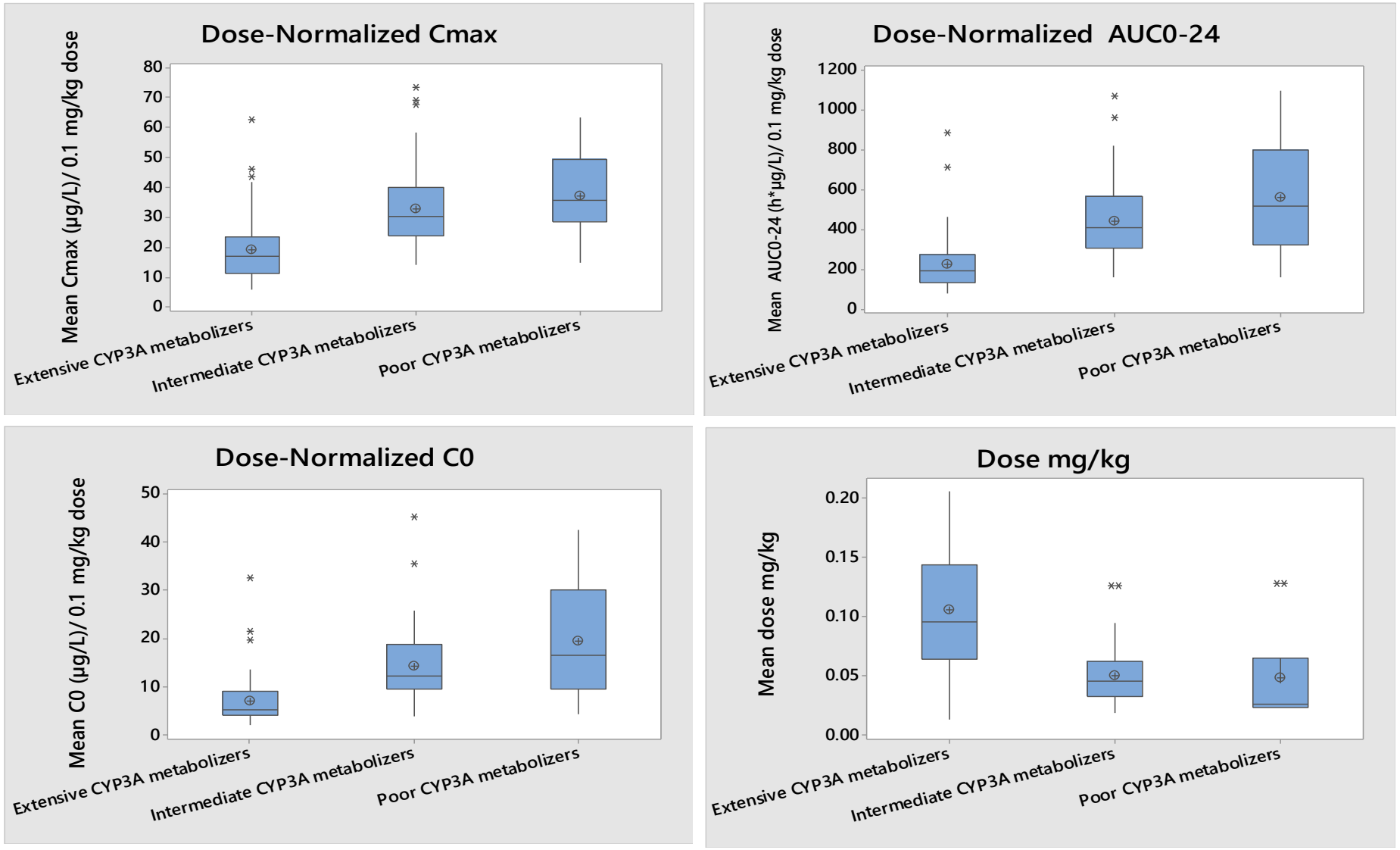


Figure 7: Combined effects of CYP3A5 polymorphism and *CYP3A4*22* genotypes on tacrolimus exposure and dose.

Table 16: Combined effects of CYP3A5 polymorphism and *CYP3A4*22* genotypes on tacrolimus exposure and dose.

PK-parameter	Extensive metabolizers n= (30)	Intermediate metabolizers n= (29)	Poor metabolizers n= (5)	p-value [a]	p-value [b]
Dose (mg/Kg/day)	0.11 ±0.05	0.05±0.02	0.05 ±0.04	P<0.001	P=0.2
AUC_{0-24} (µg*h/L/mg/Kg)	19.1 ±10.6	32.8 ±13.5	37.2 ±15.4	P<0.001	P=0.4
C_{max} (µg/L/mg/Kg)	223.4 ±140.3	440.1 ±190.3	562 ±313.3	P<0.001	P=0.3
C_0 (µg/L/mg/Kg)	6.9 ±5.1	14.3 ±7.6	19.5 ±13.0	P<0.001	P=0.4

[a] p-Values refer to comparisons to extensive metabolizers.

[b] p-Values refer to comparisons to Poor metabolizers

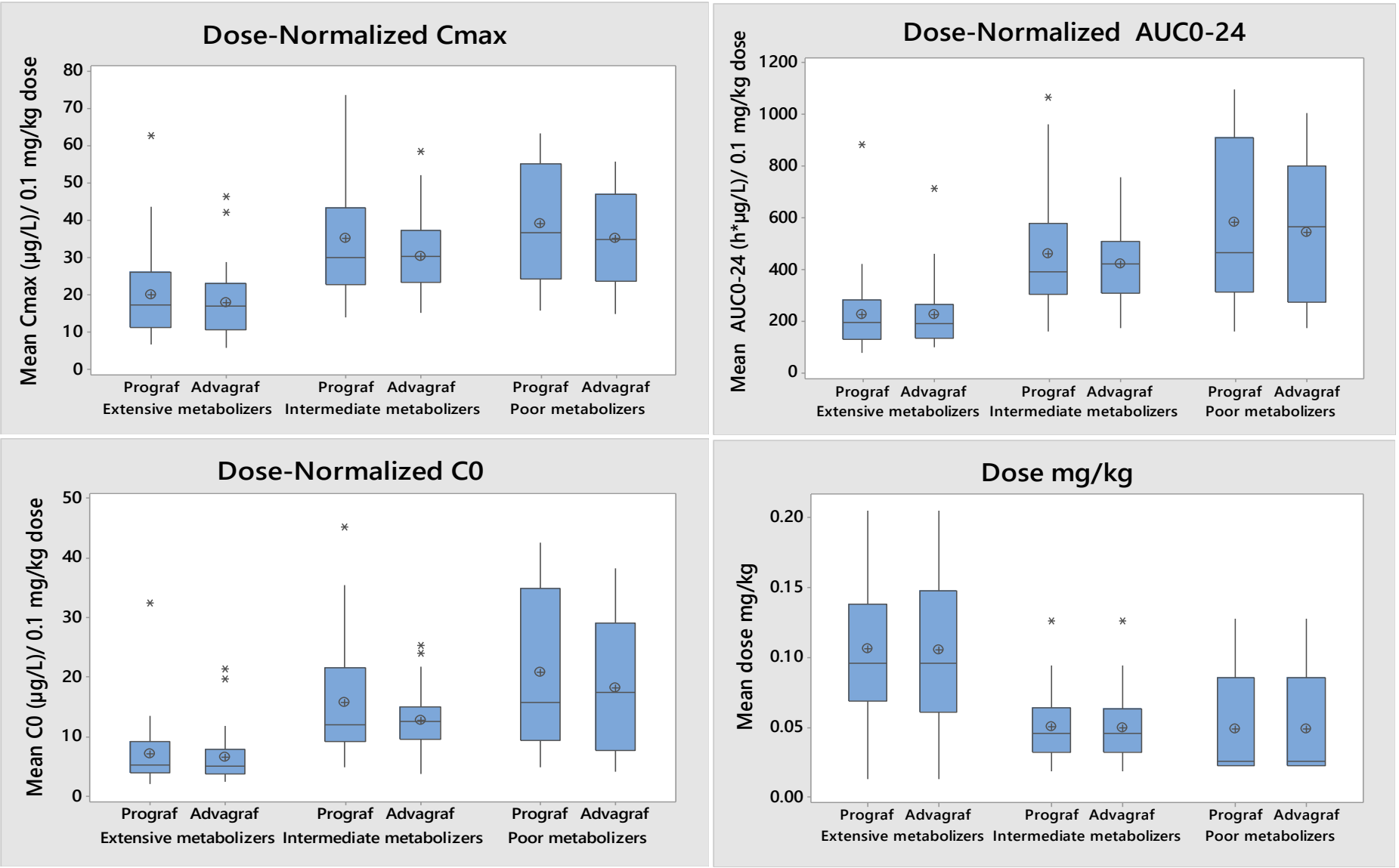


Figure 8: Combined effects of CYP3A5 polymorphism and *CYP3A4*22* genotypes on tacrolimus exposure and dose in both tacrolimus formulations.

Table 17: Tacrolimus pharmacokinetic parameters for twice-daily tacrolimus (TD-Tac) and Advagraf®: all patients and CYP3A combined genotype groups

PK-parameter	Extensive metabolizers n= (30)			Intermediate metabolizers n= (29)			Poor metabolizers n= (5)		
	TD-Tac	Advagraf®	p-value	TD-Tac	Advagraf®	p-value	TD-Tac	Advagraf®	p-value
Dose (mg/Kg/day)	0.11 ±0.05			0.05 ±0.02			0.05 ±0.05		
AUC_{0-24} (µg*h/L/mg/Kg)	223 ±130	224 ±152	p=0.9	419 ±155	461 ±221	p= 0.6	542 ±311	582 ±351	p= 0.9
C_{max} (µg/L/mg/Kg)	18.1 ±9.2	20.1 ±11.8	p= 0.6	30.5 ±10.4	35.2 ±15.9	P= 0.3	35.3 ±14.6	39.2 ±17.6	p= 0.8
C_0 (µg/L/mg/Kg)	6.7±4.5	7.2 ±5.7	P= 0.7	12.8 ±5.1	15.8 ±9.4	P= 0.3	18.2 ±12.8	20.9 ±14.5	p= 0.8

Table 18: Bioequivalence statistics for AUC_{0-24} and C_{max} for twice-daily tacrolimus (TD-Tac) and Once-daily tacrolimus OD-Tac in CYP3A combined genotype groups.

Parameter	Extensive metabolizers		Intermediate metabolizers	
	Ratio of geometric means (%)	90% CI	Ratio of geometric means (%)	90% CI
AUC_{0-24}	101%	(88% –116%)	92%	(81% –104%)
C_{max}	93%	(77% –111%)	88%	(74% –104%)
Dose-normalized AUC_{0-24}	102%	(82% –127%)	94%	(78% –113%)
Dose-normalized C_{max}	93%	(75% –116%)	90%	(76% –106%)

4.2.6 Effect of *POR*28* polymorphism on tacrolimus pharmacokinetics

The effect of *POR*28* polymorphism on pharmacokinetic parameters of tacrolimus are shown in **Table 19**. When considering only the *POR*28* allelic status, no significant difference in the dose-normalized AUC_{0-24}, C_{max}, C0 values and the daily of tacrolimus was observed between the *POR*28* genotype groups ($p > 0.05$). When considering both *POR*28* allelic status and tacrolimus formulations, this difference remained not significant. Despite that similar trend, Advagraf® have a slight decrease in tacrolimus pharmacokinetic parameters compared to twice-daily tacrolimus in both *POR*28* genotype groups ($p > 0.05$;

Table 20

In addition, the AUC_{0-24} OD-Tac / AUC_{0-24} TD-Tac ratio was bioequivalent in *POR*28 CC* group (97% with 90% CI 85% -112%) and in *POR*28 CT/TT* group (96% with 90% CI 84% -109%). The C_{max} OD-Tac / C_{max} TD-Tac ratio was not bioequivalent (**Table 21**).

Table 19: Pharmacokinetic tacrolimus in renal transplant recipients with different *POR*28* genotypes. Data are shown as mean (SD).

PK-parameter	*POR*28 CC* n= (30)	*POR*28 CT/TT* n= (34)	p-value
Dose (mg/Kg/day)	0.08 ± 0.05	0.07 ± 0.05	p=0.72
C_{max} (µg/L/mg/Kg)	26.6 ± 14.5	26.9 ± 14.2	p=0.96
AUC_{0-24} (µg*h/L/mg/Kg)	353 ± 239	344 ± 198	p= 0.68
C_0 (µg/L/mg/Kg)	11.8 ± 9.6	10.8 ± 7.0	p= 0.89

Table 20: Influence of *POR*28* polymorphism and Form on Tacrolimus Pharmacokinetic Parameters.

PK-parameter	*POR*28 CC* *n= (30)*			*POR*28 CT/TT* *n= (34)*		
	TD-Tac	Advagraf®	p-value	TD-Tac	Advagraf®	p-value
Dose (mg/Kg/day)	0.08 ± 0.05			0.07 ± 0.05		
AUC_{0-24} (µg*h/L/mg/Kg)	361 ± 260	344 ± 221	p=0.94	357 ± 226	331 ± 168	p= 0.81
C_{max} (µg/L/mg/Kg)	28.7 ± 16.4	24.5 ± 12.3	p=0.44	28.2 ± 16.2	25.5 ± 12.0	p= 0.59
C_0 (µg/L/mg/Kg)	12.5 ± 10.9	11.0 ± 8.1	p=0.76	11.9 ± 8.4	9.8 ± 5.3	p= 0.40

Table 21: Bioequivalence statistics for AUC_{0-24} and C_{max} for TD-Tac and OD-Tac in *POR*28* genotypes.

Parameter	*POR*28 CC*		*POR*28 CT/TT*	
	Ratio of geometric means (%)	90% CI	Ratio of geometric means (%)	90% CI
AUC_{0-24}	97%	(85% –112%)	96%	(84% –109%)
C_{max}	88%	(75% –103%)	93%	(77% –111%)
Dose-normalized AUC_{0-24}	99%	(74% –132%)	97%	(78% –121%)
Dose-normalized C_{max}	89%	(69% –115%)	94%	(77% –115%)

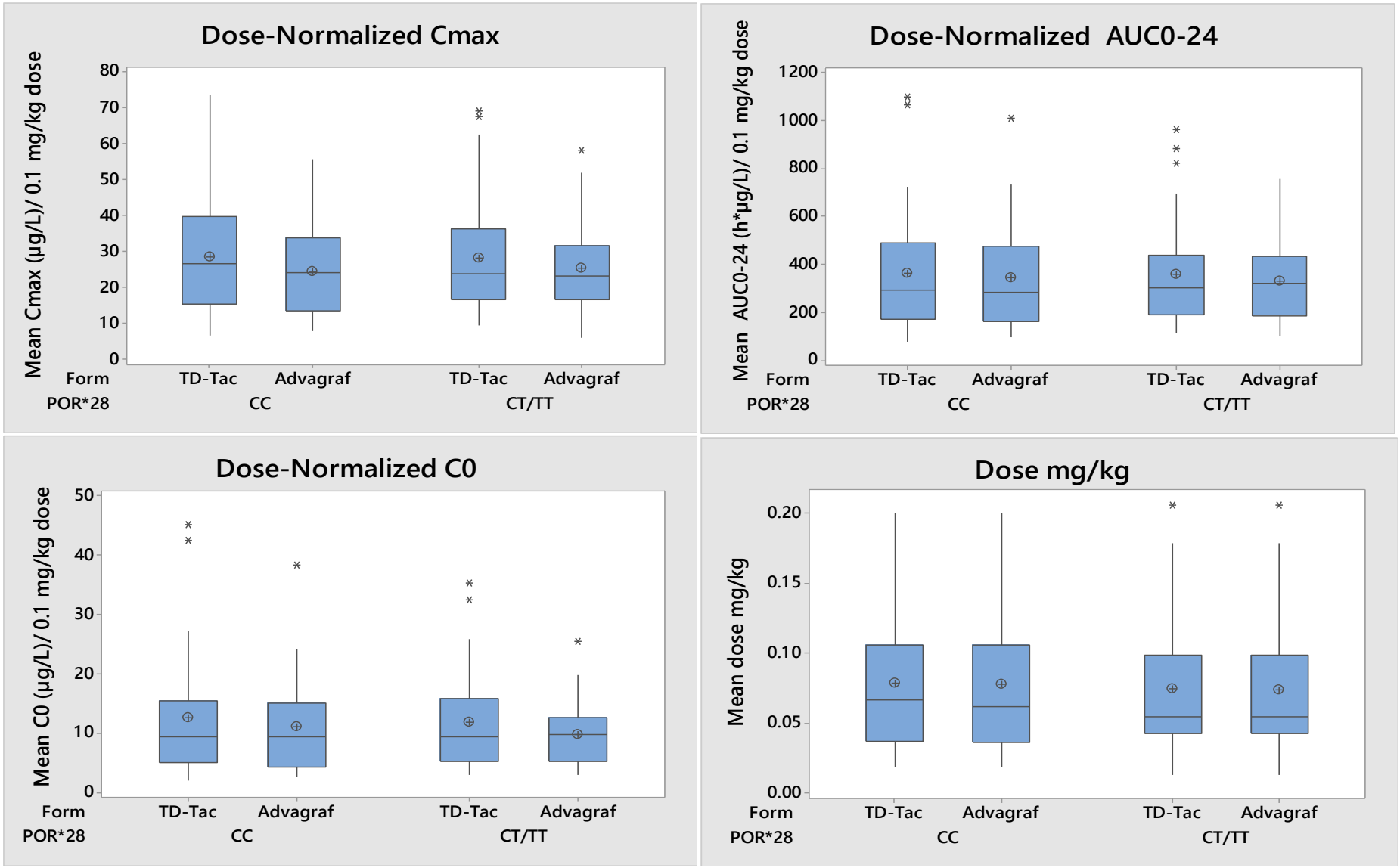


Figure 9: The Mean pharmacokinetic parameters of tacrolimus with different *POR*28* genotypes in once- and twice-daily tacrolimus.

4.2.7 Effect of *POR*28* polymorphism on tacrolimus pharmacokinetics in CYP3A5 expressers and non-expressers.

According to the level of CYP3A5 expression, subjects were divided into CYP3A5 expressers (*CYP3A5*1* allele carriers) and CYP3A5 non-expressers (*CYP3A5*3/*3* carriers). The effect of the *POR*28* polymorphism on the pharmacokinetics of tacrolimus were studied in each group.

CYP3A5 non-expressers carrying at least one *POR*28 T* allele had a significant increase in tacrolimus daily dose compared to CYP3A5 non-expressers carrying the *POR*28CC* genotype. In contrast, CYP3A5 expressers with *POR*28 T* variant allele had a tendency for a slightly lower tacrolimus daily dose when compared to *POR*28 CC* homozygous patients. In CYP3A5 non-expressers, the dose-normalized tacrolimus C_0 was significantly higher in *POR*28 CC* carriers than *POR*28 T* variant allele carriers. Alternatively, within the CYP3A5 expressers, carriers of the *POR*28 T* variant allele tended to have a 6% lower dose-normalized tacrolimus C_0 compared with *POR*28 CC* carriers (P=0.4, Table 22).

Additionally, when considering both the combined effects of *CYP3A5*3* and *POR*28* allelic status and tacrolimus formulations, no significant difference in tacrolimus dose-normalized pharmacokinetic parameters and dose was found between the *POR*28* genotypes within both the CYP3A5 expressers and non-expressers groups (P >0.05; Table 23 &

Figure 10). The confidence intervals of the AUC_{0-24} and C_{max} ratios are summarized in Table 24. All ratios were falling outside 80% to 125% bioequivalence limits except in CYP3A5 expressers carrying *POR*28 CC* genotype and in CYP3A5 non-expressers carrying *POR*28 CT/TT* genotype the AUC_{0-24} ratios were falling inside 80% to 125% bioequivalence limits.

Table 22: Tacrolimus PK parameters according to their *CYP3A5*3* and *ABCB1*genotypes.

PK-parameter	CYP3A5 Expressers		p-value	CYP3A5 Non-expressers		p-value
	*POR*28 CC*	*POR*28 CT/TT*		*POR*28 CC*	*POR*28 CT/TT*	
	n= 17	*n= 13*		*n= 13*	*n= 21*	
Dose (mg/Kg/day)	0.11± 0.05	0.10 ± 0.05	p= 0.56	0.04 ± 0.02	0.06 ± 0.03	p=0.006
C_{max} (µg/L/mg/Kg)	19.1 ± 10.2	19.1 ± 11.2	p= 0.72	36.4 ± 13.6	31.7 ± 13.8	p= 0.16
$AUC_{0\text{-}24}$ (µg*h/L/mg/Kg)	212 ± 105.7	238 ± 177	p= 0.41	537 ± 241	409 ± 183	p= 0.02
C_0 (µg/L/mg/Kg)	6.5 ± 4.0	7.5 ± 6.3	p= 0.40	18.7 ± 10.3	12.9 ± 6.7	p= 0.007

Table 23: Tacrolimus Pharmacokinetic Parameters for different combination of *CYP3A5*3* and *POR*28* genotypes in both once- and twice daily-tacrolimus.

PK-parameter	CYP3A5 Expressers					
	*POR*28 CC* (n=17)			*POR*28 CT/TT* (n=13)		
	TD-Tac	Advagraf®	P-value	TD-Tac	Advagraf®	P-value
Dose (mg/Kg/day)	0.11±0.05			0.10±0.05		
C_0 (µg/L/mg/Kg)	6.4±3.4	6.6±4.7	0.96	8.2±7.8	6.8±4.5	0. 66
C_{max} (µg/L/mg/Kg)	19.9±10.5	18.3±10.0	0. 71	20.2±13.8	17.9±8.3	0.70
$AUC_{0\text{-}24}$ (µg*h/L/mg/Kg)	207±105	216±110	0. 77	245±201	232±158	0.92
PK-parameter	CYP3A5 Non-expressers					
	*POR*28 CC* (n=13)			*POR*28 CT/TT* (n=21)		
	TD-Tac	Advagraf®	P-value	TD-Tac	Advagraf®	P-value
Dose (mg/Kg/day)	0.04±0.02			0.06±0.03		
C_0 (µg/L/mg/Kg)	20.5±12.2	16.8±8.1	0.51	14.1±8.1	11.6±4.9	0.38
C_{max} (µg/L/mg/Kg)	40.1±15.8	32.7±10.3	0.21	33.1±15.8	30.2±11.6	0.64
$AUC_{0\text{-}24}$ (µg*h/L/mg/Kg)	563±266	511 ±220	0.57	427±216	392±146	0.78

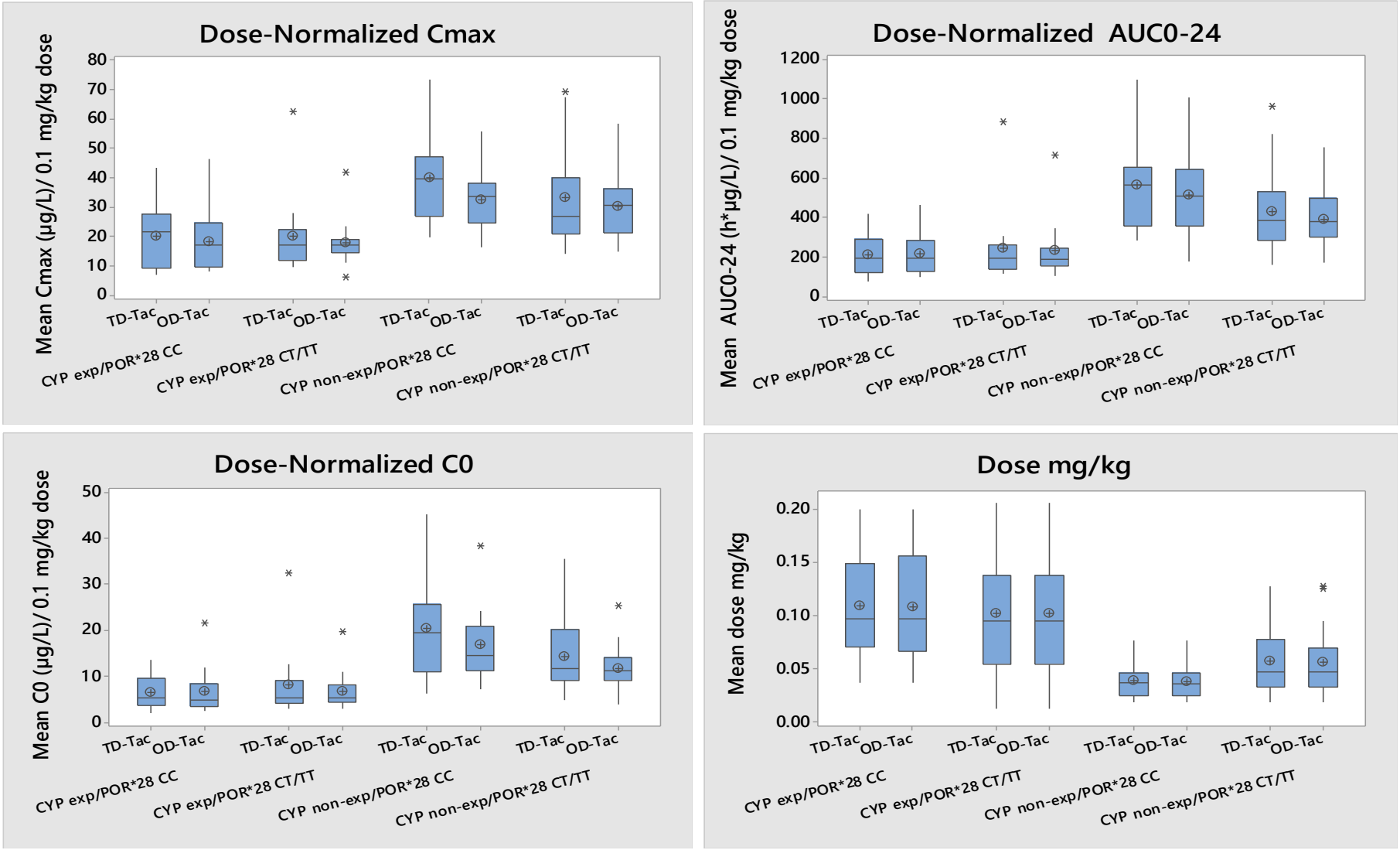


Figure 10: The Mean pharmacokinetic parameters of tacrolimus with different *CYP3A5*3* and *POR*28* combined genotypes in once- and twice-daily tacrolimus.

Table 24: Ratios of geometric means and 90% CI for AUC_{0-24}, C_{max}, dose-normalized AUC_{0-24} and dose-normalized C_{max} for Tacrolimus formulations in CYP3A5 and *POR*28* genotype groups

Parameter	CYP3A5 Expressers			
	*POR*28 CC*		*POR*28 CT/TT*	
	Ratio of geometric means (%)	90% CI	Ratio of geometric means (%)	90% CI
AUC_{0-24}	104%	(88% –123%)	98%	(71% –136%)
C_{max}	92%	(74% –115%)	93%	(59% –146%)
Dose-normalized AUC_{0-24}	105%	(74% –149%)	98%	(64% –150%)
Dose-normalized C_{max}	93%	(64% –136%)	93%	(63% –137%)
Parameter	CYP3A5 Non-expressers			
	*POR*28 CC*		*POR*28 CT/TT*	
	Ratio of geometric means (%)	90% CI	Ratio of geometric means (%)	90% CI
AUC_{0-24}	98%	(71% –136%)	94%	(80% –112%)
C_{max}	82%	(64% –106%)	92%	(73% –117%)
Dose-normalized AUC_{0-24}	91%	(64% –128%)	96%	(73% –127%)
Dose-normalized C_{max}	83%	(63% –111%)	94%	(72% –122%)

4.2.8 Factors associated with dose requirements of tacrolimus

Factors associated with dose requirements of tacrolimus were studied using univariate regression analysis. The P value was highly significant with ethnicity, sex, *CYP3A5*3* genotype and the combined *CYP3A5* and *ABCB1* genotype (p<0.001) and hematocrite and *ABCB1* genotype (p=0.001). The P values for ag and time since transplant was less than 0.05. Although diabetic patients had higher tacrolimus dose-normalized C_{max} and required lower tacrolimus dose than non-diabetic patients, the difference was not statistically significant. The p-value was 0.098 and 0.056, respectively. The *CYP3A5*3* genotype explains 33.9% of tacrolimus dose requirements variability. Similarly, the combined *CYP3A5* and *ABCB1* genotype accounts for 36.9% of the between-individual variability in tacrolimus dose requirements. *CYP3A4*22* served as a borderline significant factor (p= 0.06) and *POR*28* have no association with tacrolimus dose requirement.

Multiple regression analysis by stepwise selection identified the combined *CYP3A5*3* and ABCB1 genotype, age, ethnicity, heamatocrite and diabetic status as independent variables associated with tacrolimus dose (**Table 25**). These factors explain 59.2% of the variability in tacrolimus dose requirements.

Table 25: Factors associated with tacrolimus dose (mg/kg) requirements (Multiple regression analysis)

Stepwise regression equation	R^2	R^2(adj)	Independent variables with statistical significance
0.2112 - 0.000579 Age - 0.1906 Hematocrite - 0.01915 Asian + 0.0204 Black - 0.02678 Diabetic + 0.0111 Steroids + 0.0021 *CYP3A5*1/*1/*1/*3 /ABCB1CT/TT* - 0.0483 *CYP3A5*3/*3 /ABCB1CC* - 0.0557 *CYP3A5*3/*3*	59.16 %	56.04%	Age (p=0.018) Ethnicity (p=0.003) Hematocrit (p=0.002) Diabetic status (p=0.001) *CYP3A5/ABCB1* Genotype (p<0.001

4.2.9 *CYP3A5* Genotype Effect on 4β-OHC and 4β-OHC/C ratio

As shown in **Table 26**, a significant difference was observed between *CYP3A5*1/*1* and **1/*3* in comparison with the **3/*3* carriers (p<0.01). However, there was no significant difference between *CYP3A5*1/*1* and **1/*3* genotypes (p>0.05). Based on these results patients were divided into two groups CYP3A5 expressers (*CYP3A5*1/*1* and **1/*3*) and CYP3A5 non-expressers (*CYP3A5*3/*3*). We found significant differences in 4β-OHC and 4β-OHC/C ratio in the CYP3A5 expresser group (**1/*3* and **1/*1*) compared to the CYP3A5expresser group (**3/*3*). By studying the ethnicity influence on the 4ß-OHC plasma concentration, it was found that the 4ß-OHC/C ratio was higher in Black subjects than White and Asian ethnic groups. The analysis was repeated excluding black patients. Again, CYP3A5 expressers *(*1/*1*, n=3 and **1/*3*) demonstrated a significant increase in 4β-OHC/C ratio compared with the CYP3A5 non-expressers (**3/*3*).

Table 26. Plasma concentrations of 4β-hydroxycholesterol in stable kidney transplant recipients with different *CYP3A5*3* genotypes.

	4β-OHC (ng/mL)	p-value	4β-OHC/C Ratio	p-value
*CYP3A5*3*				
All patients				
**1/*1*	32.1±12.6		7.6±2.3	
**1/*3*	28.2±16.5	P=0.25	7.1±4.1	P=0.33
**3/*3*	20.9±8.7	P=0.01	5.0±2.0	P=0.007
**1/*1 + *1/*3*	29.9±14.8		7.3±3.4	
**3/*3*	20.9±8.7	P=0.001	5.0±2.0	p<0.001
Non-black patients				
**1/*1 + *1/*3*	25.3±7.0		6.4 ± 2.1	
**3/*3*	20.9±8.7	P=0.02	5.0 ± 2.0	p<0.01
Black patients				
**1/*1*	34.0±13.6		8.0±2.6	
**1/*3*	50.6±47.8	P= 0.71	12.1±11.3	P=0.70
Ethenicity				
Black	36.9 ± 6.2		8.8 ± 4.6	
White	22.7 ± 1.4	P<0.01	5.4± 1.9	P<0.01
Asian	21.5 ± 2.6	P<0.05	5.8 ± 2.7	P<0.05

The results were compared with the top group in each sub-table.

4.2.10 The relation of 4β-OHC with tacrolimus exposure and dose requirement

As shown in **Table 27**, highly significant differences in tacrolimus exposure and dose requirements were observed between CYP3A5 expresser and CYP3A5 non-expresser patients (

Figure 11).

A significant positive correlation was observed between 4β-OHC/C ratio and tacrolimus dose ($r = 0.45$, $p < 0.001$). On the other hand, A significant negative correlation was observed between 4β-OHC/C ratio and tacrolimus pharmacokinetic parameters; dose-normalized C_{max} ($r = -0.35$, $p < 0.01$), dose-normalized $AUC_{0\text{-}24}$ ($r = -0.41$, $p < 0.01$) and dose-normalized C_0 ($r = -0.41$, $p < 0.01$,**Figure 12**).

Factors associated with dose requirements of tacrolimus were studied using univariate regression analysis. The P value was highly significant with ethnicity, *CYP3A5*3* genotype and log-transformed 4β-OHC/C ratio (p<0.001) and *ABCB1* genotype (p<0.01). The P values for sex and haematocrit was less than 0.05 and it was 0.071 for age and 0.09 for *CYP3A4*22* genotype. Multiple regression analysis by stepwise selection; alpha to enter or remove was 0.15; identified 4β-OHC/C ratio, *CYP3A5*3* genotype, age, ethnicity, heamatocrit and *CYP3A4*22* genotype, as independent variables associated with tacrolimus dose (**Table 28**) and this explains 62.48% of the

variability in tacrolimus dose. Furthermore, when we repeat the multiple regression analysis by using the *CYP3A5*3/ABCB1*combined genotype instead of *CYP3A5*3* and *ABCB1* separately, the percentage of tacrolimus dose variability explained by these variables increased to be 63.37% (**Table 28**).

Table 27. *CYP3A5*3* genotype effect on tacrolimus PK parameters and dose.

PK-parameter	CYP3A5 Expressers	CYP3A5 Non-expressers	p-value
Dose (mg/Kg/day)	0.11±0.05	0.05±0.02	P<0.001
C_{max} (µg/L/mg/Kg)	20.4±12.2	34.5±14.3	P<0.001
AUC_{0-24} (µg*h/L/mg/Kg)	227±157	462±215	P<0.001
C_0 (µg/L/mg/Kg)	7.4±5.8	16.0±9.3	P<0.001

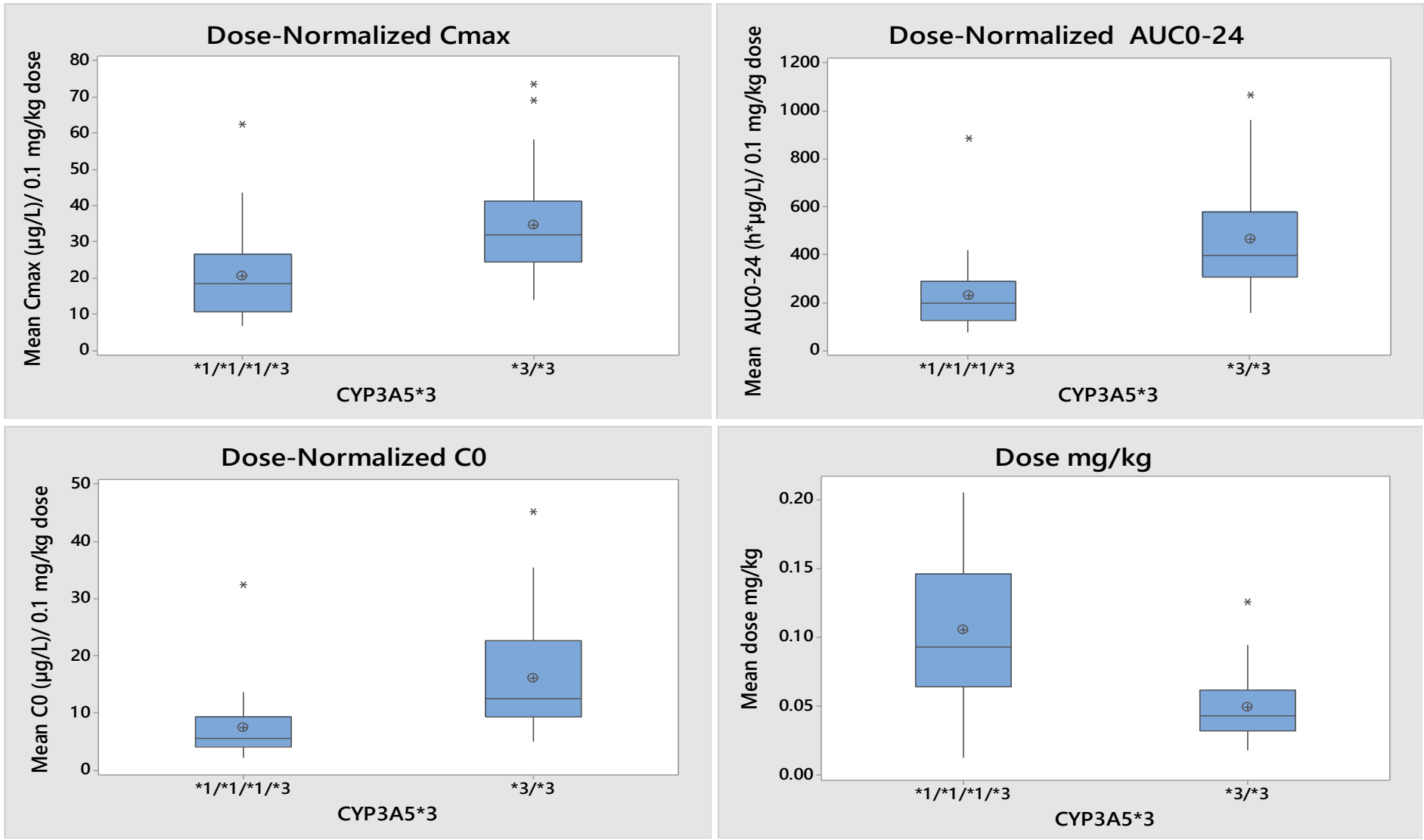


Figure 11: *CYP3A5*3* Effects on Tacrolimus Pharmacokinetic Parameters and dose requirement.

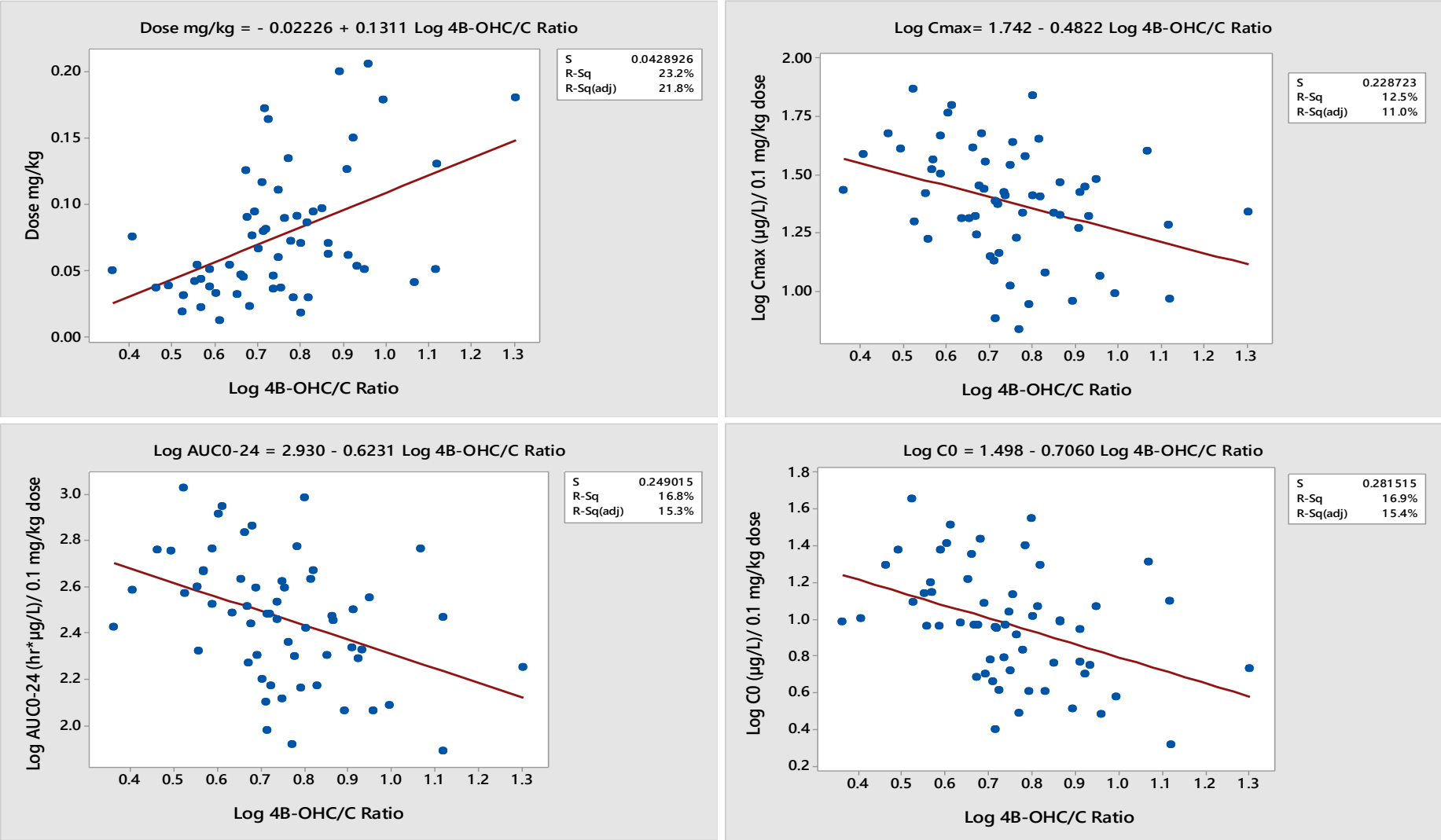


Figure 12: The fitted line plot for 4β-OHC/C ratio effects on tacrolimus pharmacokinetic parameters and dose requirement.

Table 28: Stepwise regression equation of tacrolimus dose (mg/kg) requirement after renal transplantation.

Stepwise regression equation	R^2	R^2(adj)	Independent variables with statistical significance
0.1209 + 0.0838 Log 4β-OHC/C Ratio - 0.000880 Age - 0.248 Haematocrit - 0.0391 Asian -0.0104 White + 0.0374 *CYP3A5*1/*1/*1/*3* + 0.0369 *CYP3A4*22 CC*	62.48%	57.33%	Age (p=0.021) Ethnicity (p=0.013) Haematocrit (p=0.018) *CYP3A5*3* genotype (p=0.001) Log 4β-OHC/C Ratio (p=0.006)
0.1112 + 0.0835 Log 4β-OHC/C Ratio - 0.000951 Age - 0.235 Haematocrit - 0.0322 Asian - 0.0032 White + 0.0399 CYP3A4*22 CC + 0.045 CYP3A5*1/*1/*1/*3/ABCB1CC + 0.0342 CYP3A5*1/*1/*1/*3 /ABCB1CT/TT - 0.0207 CYP3A5*3/*3 /ABCB1CC	63.37%	56.65%	Age (p=0.016) Ethnicity (p=0.039) Haematocrit (p=0.028) Log 4β-OHC/C Ratio (p=0.007) *CYP3A5/ABCB1* Genotype (p=0.011)

4.2.11 *CYP3A5*3 and ABCB1* on prednisolone and prednisone pharmacokinetics.

In the overall study population, no significant association was observed between the different CYP3A5 genotypes with prednisolone pharmacokinetic parameters; AUC_{0-24}, C_{max} and trough concentration (C_0). Disimilar to the prednisolone results, there were significant differences in the mean AUC_{0-24} and C_{max} of prednisone between these genotype groups. The mean prednisone C_{max} and AUC_{0-24} in recipients having the *CYP3A5*3/*3* genotype were significantly lower than in those patients having the *CYP3A5*1/*1 + *1/*3* genotype. However, there was no significant change in prednisone C_0 between CYP3A5 expressers and non-expressers. After adjustment to the body weight, we continued to have the same statistic results **Table 29**.

Table 29: Pharmacokinetic parameters of prednisolone-prednisone in CYP3A5 genotype groups

Study group	CYP3A5 Expressers n= (18)	CYP3A5 Non-expressers n= (20)	p-value
Prednisolone			
C_{max} (µg/L)	171.0 ± 43.0	158.3 ± 36.6	P= 0.38
AUC_{0-24} (µg*h/L)	1170.6 ± 278.7	1072.8 ± 243.1	P= 0.29
C_0 (µg/L)	4.3 ± 4.3	3.9 ± 4.0	P= 0.47
C_{max}/BW (µg/L/kg)	2.47 ± 0.78	2.19 ± 0.86	P= 0.31
AUC_{0-24}/ BW (µg*h/L/kg)	16.9± 5.5	14.1 ± 4.2	P= 0.16
Prednisone			
C_{max} (µg/L)	17.0 ± 3.3	13.4 ± 3.2	P= 0.001
AUC_{0-24} (µg*h/L)	139.3 ± 31.7	113.7 ± 26.5	P= 0.027
C_0 (µg/L)	0.58 ± 0.61	0.43 ± 0.58	P= 0.1
C_{max}/BW (µg/L/kg)	0.24 ± 0.06	0.18 ± 0.07	P= 0.004
AUC_{0-24}/ BW (µg*h/L/kg)	2.0 ± 0.6	1.5 ± 0.5	P= 0.026

The values are shown as the mean±S.D. Cmax, Maximum plasma concentration; AUC0–24, area under the plasma concentration–time curve from 0 to 24h; BW, body weight.

The prednisolone-prednisone pharmacokinetic parameters according to *ABCB1* polymorphisms are shown in **Table 30**. No significant change was found in the AUC_{0-24} and C_{max} of prednisolone between the different genotypes of *ABCB1* gene. However, a significant difference was observed at prednisone pharmacokinetic parameters (C_{max} and AUC_{0-24}) between the *ABCB1 CC* and the *CT/TT* genotypes. The mean prednisone C_{max} for *ABCB1* CC genotype was significantly higher than for *ABCB1 CT/TT* genotype. Moreover, *ABCB1* CC carriers had a significantly higher AUC_{0-24} compared to *ABCB1 CT/TT* carriers.

While there was no difference in prednisolone C_0 between *ABCB1*genotype groups (**Table 30**).

Table 30: Pharmacokinetic parameters of prednisolone- prednisone in *ABCB1* genotype groups.

Study group	*ABCB1 CC* Carriers n= (9)	*ABCB1 CT/TT* Carriers n= (29)	p-value
Prednisolone			
C_{max} (µg/L)	165.2 ± 52.1	164.0 ± 36.2	P= 0.79
AUC_{0-24} (µg*h/L)	1187.0 ± 333.0	1095.9 ± 239.9	P= 0.16
C_0 (µg/L)	3.3 ± 1.5	4.3 ± 4.6	P= 0.66
Prednisone			
C_{max} (µg/L)	18.0 ± 3.4	14.2 ± 3.4	P= 0.002
AUC_{0-24} (µg*h/L)	153.9 ± 31.6	116.9 ± 26.3	P= 0.004
C_0 (µg/L)	0.49 ± 0.22	0.50 ± 0.67	P= 0.135

Prednisolone and prednisone pharmacokinetic parameters in the two different CYP3A5 genotype groups in relation to ABCB1 polymorphisms are shown in **Table 31**. Hence there was only one patient in CYP3A5 Non-expressers / *ABCB1 CC* group, it was excluded from the analysis. Regarding prednisolone, no significant difference was found in prednisolone C_{max}, AUC_{0-24} and C_0 between the three genotype groups. However, the mean C_{max} and AUC_{0-24} of prednisone in CYP3A5 non-expresser patients having the *ABCB1 CT/TT* genotype were lower than in CYP3A5 expresser patients having either *ABCB1 CC* or *ABCB1 CT/TT* genotypes.

Table 31: Pharmacokinetic parameters of prednisolone- prednisone in *ABCB1* genotype groups.

Study group	*CYP3A5* Expressers/ *ABCB1 CC* Carrers n= (8)	*CYP3A5* Expressers / *ABCB1 CT/TT* Carriers n= (10)	*CYP3A5* Non-expressers / *ABCB1 CT/TT* Carriers n= (19)	p-value
Prednisolone				
C_{max} (µg/L)	168.8 ± 54.5	172.8 ± 34.2	159.4 ± 37.3	P > 0.05
AUC_{0-24} (µg*h/L)	1189 ± 373	1157 ± 222	1065 ± 250.5	P > 0.05
C_0 (µg/L)	3.3 ± 1.6	5.2 ± 5.7	3.9 ± 4.1	P > 0.05
Prednisone				
C_{max} (µg/L)	18.2 ± 3.6	16.1 ± 3.0	13.2 ± 3.2	P< 0.001
AUC_{0-24} (µg*h/L)	155 ± 35	128 ± 26	111 ± 25	P= 0.004
C_0 (µg/L)	0.5 ± 0.2	0.6 ± 0.8	0.4 ± 0.6	P > 0.05

4.2.12 *POR*28* on prednisolone and prednisone pharmacokinetics.

No statistically significant differences in prednisolone pharmacokinetics were observed between patients carrying *POR*28 CC* genotype and those had *CT/TT* genotype (**Table 32**). The mean Prednisolone C_{max} for *POR*28 CC* carriers and for *CT/TT* patients were 169.0 ± 47.0 and 161.2 ± 35.0 ng/mL, respectively. The mean Prednisolone AUC_{0-24} for *POR*28 CC* and for *CT/TT* carriers were 1063 ± 304 h*ng/mL, 1084.3 ± 279 h*ng/mL and 1158 ± 219 h*ng/mL, respectively. Similarly, we found no difference in prednisone pharmacokinetic profile between *POR*28* different genotypes (P> 0.05).

Table 32: Pharmacokinetic parameters of prednisolone- prednisone in *POR*28* genotype groups.

Study group	*POR*28 CC* Carriers n= (15)	*POR*28 CT/TT* Carriers n= (23)	p-value
Prednisolone			
C_{max} (µg/L)	169.0 ± 47.0	161.2 ± 35.0	P= 0.52
AUC_{0-24} (µg*h/L)	1063 ± 304	1158 ± 219	P= 0.95
C_0 (µg/L)	3.4 ± 4.0	4.5 ± 4.2	P= 0.32
Prednisone			
C_{max} (µg/L)	18.0 ± 3.4	14.2 ± 3.4	P= 0.61
AUC_{0-24} (µg*h/L)	153.9 ± 31.6	116.9 ± 26.3	P= 0.66
C_0 (µg/L)	0.49 ± 0.22	0.50 ± 0.67	P= 0.46

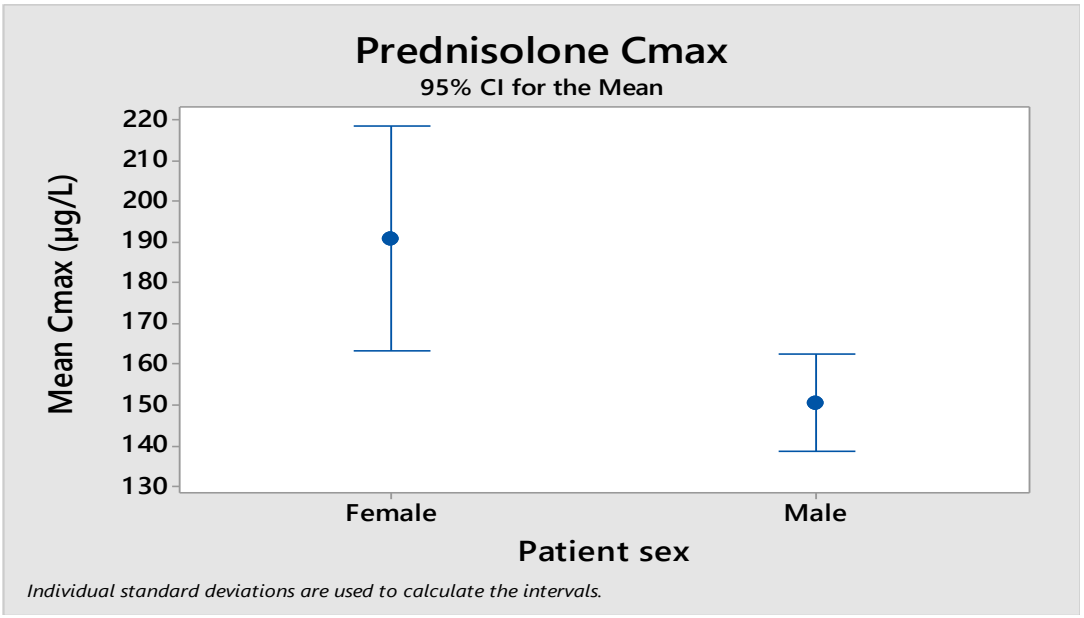
4.2.13 Non-genetic Factors associated with prednisolone and prednisone plasma concentrations.

No correlation was seen among different Ethnic backgrounds for prednisolone or prednisone blood concentrations. However, patient sex have been shown to influence prednisolone and prednisone C_{max} , but have no effect on the AUC_{0-24} and the trough concentration (

Table 33). In the current study, thirty-eight patients were analyzed; Twenty-five of them were males, and they all had a significantly lower C_{max} compared to females for both prednisolone and prednisone ($p<0.01$ and $p<0.05$, respectively; **Figure 13**). After adjustment to the body weight, we continued to have the same statistic results. In addition, there was no correlation between patient age and prednisolone and prednisone blood concentrations. Multiple regression analysis by stepwise selection; alpha to enter or remove was 0.15; identified patient weight and gender, but not tacrolimus dose, as independent variables associated with prednisolone C_{max} and identified tacrolimus dose as independent variables associated with prednisolone AUC_{0-24}. However, multiple regression analysis for prednisone identified patient gender as independent variables associated with prednisone C_{max} and patient age and weight for prednisone AUC_{0-24}.

Table 33: Prednisolone and prednisone pharmacokinetic parameters in renal transplant recipients in accordance with ethnicity and sex

Study group	Prednisolone		
	C_{max} (µg/L)	AUC_{0-24} (µg*h/L)	C_0 (µg/L)
Ethnicity			
Black	163.4 ± 60.8	1117 ± 407	2.9 ± 1.6
White	162.8 ± 35.6	1125 ± 249	4.2 ± 4.5
Asian	171.3 ± 32.8	1085 ± 148	4.9 ± 4.8
p-value	P= 0.93	P= 0.67	P= 0.80
Sex			
Male	150.5 ± 28.6	1066 ± 243	3.54 ± 4.12
Female	190.8 ± 45.6	1260 ± 266	5.12 ± 4.09
p-value	P= 0.003	P=0.59	P= 0.38
Study group	**Prednisone**		
	C_{max} (µg/L)	AUC_{0-24} (µg*h/L)	C_0 (µg/L)
Ethnicity			
Black	17.7 ± 3.7	151 ± 38	0.42 ± 0.22
White	14.1 ± 3.1	118 ± 25	0.52 ± 0.69
Asian	16.4 ± 4.5	125 ± 37	0.53 ± 0.50
p-value	P= 0.03	P= 0.11	P= 0.93
Sex			
Male	14.1 ± 3.3	125.1 ± 29.2	0.40 ± 0.57
Female	17.2 ± 3.6	125.2 ± 38.7	0.69 ± 0.60
p-value	P= 0.017	P= 0.93	P= 0.24



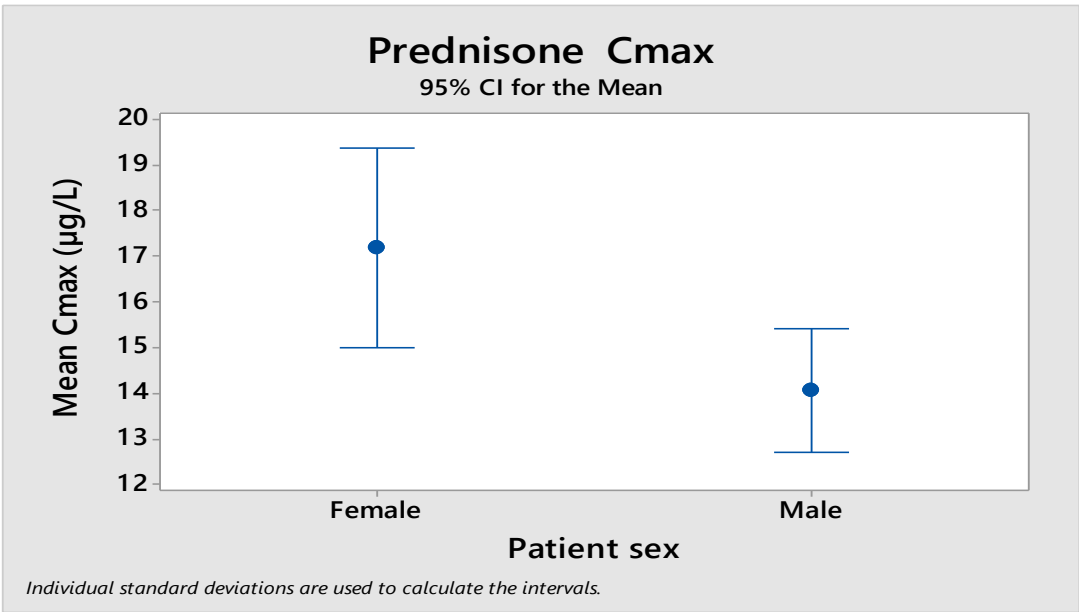


Figure 13: The interval plot of the mean prednisolone and prednisone C_{max} in renal transplant recipients in accordance with patient sex.

5. Discussion

5.1 Limitations

This study has some limitations. This is a relatively small sample size with a failure to recruit to the planned target participant number. A further limitation is that concentration time points may not be accurately reflective with a relatively small shift in the collection time.

5.2 Generalisability

The findings of the studies presented have demonstrated that the between-patient variability in tacrolimus daily dose requirement was related to *CYP3A5*3*, *CYP3A4*22* and *ABCB1 3435* gene polymorphisms and that *CYP3A5 *3* genotype is a key factor in the prediction of tacrolimus blood concentrations and dose requirement. Furthermore, the influence of *CYP3A5*3*, *CYP3A4*22* and *ABCB1 3435* genotypes on tacrolimus exposure was the same for once- and twice daily tacrolimus. *CYP3A5*3* polymorphism cannot be replaced by 4B-OHC to predict tacrolimus dose requirement, even though they are strongly linked. Moreover, our data showed that switching from immediate to extended-release tacrolimus has an impact on between-patient variability of tacrolimus exposure in this cohort of patients. On the other hand, conversion from immediate to extended-release tacrolimus did not make any significant difference in WPV of dose-normalized C_0 and *CYP3A5*3* genotype had no impact on within-patient variability of tacrolimus clearance in once- and twice-daily tacrolimus formulations. In addition to the genetic factors associated with tacrolimus dose, some non-genetic factors, including age, ethnicity, haematocrit, diabetic status and steroid treatment seems to have an influence in tacrolimus dose. This can be applied for both tacrolimus formulations and could be generalised to a broad range of renal transplant recipients.

5.3 Interpretation

In this study, Advagraf® showed a comparable pharmacokinetic profile to twice-daily tacrolimus confirming that once-daily tacrolimus, Advagraf® is bioequivalent to twice-daily tacrolimus preparations according to the FDA guidelines; 80-125% (FDA., 2003). It also met the bioequivalence acceptance criteria of the European Medicines Agency for tacrolimus; (90-111%) for AUC and (80-125%) for C_{max} (EMA, 2015). We confirmed the repeatedly reported strong correlation between AUC_{0-24} and C_0 for immediate and modified release tacrolimus indicating that measurement of C_0 is appropriate for therapeutic monitoring of Advagraf®. Our results are in accordance with the previous findings in Phase II studies on Advagraf® showing that a given dose of Advagraf® delivered 90% of the area under the concentration-time curve (AUC) obtained with Prograf® (EMEA, 2007) . However, the mean tacrolimus ratio for C_{max} following the administration of Advagraf was up to 20% less when compared with Prograf®. They noted that the AUC_{0-24} may need to be monitored to ensure maintenance of similar systemic exposure and they found a good correlation between AUC_{0-24} and C_0 for Advagraf® and Prograf® at steady state, as found in the current study. They also observed less between- and within-

subject variability in exposure when compared Advagraf® to Prograf®(EMEA, 2007).

Our data showed that switching from immediate to extended-release tacrolimus has an impact on between-patient variability of tacrolimus exposure in this cohort of patients. Advagraf® has less between-individual differences in tacrolimus exposure when compared to immediate release tacrolimus.

In addition, we demonstrated that the between-patient variability in tacrolimus daily dose requirement was related to *CYP3A5*3*, *CYP3A4*22* and *ABCB1 3435* gene polymorphisms in stable kidney transplant recipients, as reported previously, suggesting that the pharmacogenetic assessment of *CYP3A5*3*, *CYP3A4*22* and *ABCB1 3435* genotypes may offer an effective tool for individualizing drug therapy by optimizing tacrolimus dosage for both twice daily tacrolimus and Advagraf®.
It is becoming apparent that all individuals express CYP3A4 and CYP3A4 poor metabolizers are rare. However, CYP3A5 expression varies between different individuals. The *CYP3A5*3* allele reduces CYP3A5 production and results in the loss of hepatic CYP3A5 activity (Hustert et al., 2001, Kuehl et al., 2001). Thus, it has been repeatedly reported that patients with the *CYP3A5*3/*3* genotype (CYP3A5 non-expressers) require lower doses to reach similar dose-normalized tacrolimus trough concentrations than patients carrying at least one *CYP3A5*1* allele (CYP3A5 expressers) (Macphee et al., 2005, Vannaprasaht et al., 2013, Ferraris et al., 2011). In our study, we clearly could confirm this effect. Several studies have examined the effect of P-gp on tacrolimus exposure, and conflicting results have been obtained. Some studies reported no correlation between *ABCB1 3435* genotypes and tacrolimus dose and pharmacokinetics (Haufroid et al., 2004, Jun et al., 2009, Vannaprasaht et al., 2013). However, other studies displayed a significant influence of *ABCB1 3435* genotypes on tacrolimus pharmacokinetics and dose requirements (Zheng et al., 2003, Lopez-Montenegro Soria et al., 2010, Yu et al., 2011), which is in line with our findings. In addition, upon evaluation of *CYP3A5*3* and *ABCB1 3435* genotypes in combination, significant differences in tacrolimus pharmacokinetics were evident between *ABCB1 3435* polymorphisms in CYP3A5 expressers suggesting that *ABCB1 3435* genotype is an important factor in tacrolimus pharmacokinetics particularly in the case of CYP3A5 expressers. These findings support previous findings by Loh and colleagues who reported the same outcome between *ABCB1 3435* and *CYP3A5*3* variants (Loh et al., 2008) and contrast with other studies demonstrating no significant differences in tacrolimus bioavailability between the *ABCB1 3435* polymorphisms in both CYP3A5 expressers and non-expressers (Rong et al., 2010, Tada et al., 2005). Additionally, our data showed the contribution between *CYP3A4*22* polymorphisms and tacrolimus pharmacokinetics confirming the findings of the recently published studies (Elens et al., 2011a, Tavira et al., 2013, Kurzawski et al., 2014).

Interestingly, we found that the influence of *CYP3A5*, *CYP3A4* and *ABCB1 3435* genotypes on tacrolimus exposure was the same for once- and twice daily tacrolimus. No significant difference was observed between these polymorphisms and tacrolimus pharmacokinetics and dose requirements in both tacrolimus preparations.

It has been reported that CYP3A expression reduces progressively along the length of the gut. However, the level of cellular expression of Pg-p increases continuously along the gut length (Thorn et al., 2005). We hypothesised that the influence of these genotypes would apply differently between tacrolimus formulations. Our assumption was that CYP3A5 polymorphisms may have less effect on the oral bioavailability of extended release tacrolimus formulation, Advagraf® which is mostly absorbed lower down the gut than the immediate release preparations of tacrolimus such as Prograf® and Adoport® that are absorbed in the upper part of the gastrointestinal tract (GIT), mainly around the stomach and proximal small intestine (MacPhee, 2012). Our data showed that the impact of *CYP3A5, CYP3A4* and *ABCB1 3435* polymorphisms and their combinations had no clear difference between twice-daily tacrolimus and Advagraf®. This is in accordance with the recently published studies finding that tacrolimus exposure was significantly higher in CYP3A5 non-expressers than in CYP3A5expressers and the degree of difference was similar between Prograf® and Advagraf® (Benkali et al., 2010, Glowacki et al., 2011b, Niioka et al., 2012, Wehland et al., 2011). This may indicate a dominant effect of the liver CYP3A5 on the first-pass metabolism of tacrolimus and a minor influence of intestinal enzymes in tacrolimus metabolism. However, earlier studies in liver transplant recipients have revealed the influence of the intestinal CYP3A5 on tacrolimus absorption. A study by Uesugi et al indicates that intestinal CYP3A5, as well as hepatic CYP3A5, plays an essential role in the first-pass metabolism of orally administered tacrolimus in liver transplantation (Uesugi et al., 2006). Another study in liver transplantation recipients found that tacrolimus pharmacokinetics is mainly influenced by the intestinal CYP3A5 and P-gp expression during the first week; after that, it is mostly affected by the hepatic metabolism (Goto et al., 2004). This can be explained by the minor effect of the intestinal CYP3A5 on tacrolimus metabolism that only appears in the absence of the liver CYP3A5 enzymes. It is also possible that the gradient of CYP3A5 and P-gp expression along the length of the gut was over-estimated in previously published reports (Thorn et al., 2005).

Moreover, this study showed that switching from immediate to extended-release tacrolimus formulations did not make any significant difference in WPV of dose-normalized C_0 in both tacrolimus preparations. Similar observations were made in other conversion studies (van Hooff et al., 2012, Wehland et al., 2011, Shuker et al., 2014). However, other studies showed that conversion from Prograf® to Advagraf® was associated with a significantly lower WPV of Tac C_0 (Wu et al., 2011, Alloway et al., 2005). Our findings showed that neither patients treated with twice daily tacrolimus nor patients treated with once- daily tacrolimus show any significant association between WPV of dose-normalized Tac C_0 and *CYP3A5* genotype. This in line with previous published studies (Pashaee et al., 2011, Ro et al., 2012, Wu et al., 2014). The balance of published evidence suggests that conversion from twice daily tacrolimus to Advagraf® is unlikely to impact significantly on WPV in routine renal transplantation.

Additionally, in our data, we found that in spite of being a known substrate of CYP3A and P-glycoprotein (Anglicheau et al., 2003a), prednisolone pharmacokinetics were not associated with *CYP3A5*3* and *ABCB1 3435* polymorphisms. Prednisone behaved differently and *CYP3A5*3* and *ABCB1*

3435 genotypes were strongly associated with prednisone pharmacokinetics. CYP3A5 expressers had higher concentrations of prednisone, presumably reflecting preferential metabolism of prednisolone to prednisone. It is worth noting that patient sex had a significant effect on prednisolone C_{max}. Ethnic factors and tacrolimus dose were shown to have no influence on the C_{max} and $AUC_{0\text{-}24}$ of prednisolone and prednisone. This indicates that neither the genetic factors nor ethnicity can predict prednisolone plasma concentration. Given the wide variation between individuals in prednisolone blood concentration achieved by a dose of 5 mg prednisolone daily, it may actually be appropriate to consider using TDM, in particular for patients with efficacy failure or toxicity.

Moreover, our study showed that 4β-OHC concentration increased significantly in *CYP3A5*1* allele carriers compared to recipients having *CYP3A5*3*3* genotype (Diczfalusy et al., 2008, Suzuki et al., 2014). The 4β-OHC/C ratio was significantly correlated with tacrolimus exposure and dose requirement. 4β-OHC/C ratio may be a useful biomarker for tacrolimus dosing in renal transplanted patients. While the effect of *CYP3A5*3* genotype and CYP3A activity measured by plasma 4β-OHC/C ratio on tacrolimus exposure were closely linked, they were both found to be independent predictors and would be additive in developing an algorithm for predicting optimal initial tacrolimus dose.
Of note, tacrolimus dose requirement may be modified and its pharmacokinetics can be affected by several parameters including genetic and non-genetic factors. In order to secure the optimal tacrolimus administration, both genetic and non-genetic factors must be taken into account. Most studies search for the genetic polymorphisms that affect the response of individuals to tacrolimus. However, non-genetic factors may have an influence in tacrolimus pharmacokinetics and dose requirements. Hence, we tried to model tacrolimus kinetics based on both genetic and non-genetic factors.

In the present study, we demonstrated that *CYP3A5*3* genotype is a key factor in the prediction of tacrolimus blood concentrations and dose requirement. Many studies highlighted the influence of CYP3A5 in tacrolimus pharmacokinetics and dose requirement and reported that CYP3A5 could be useful to predict the optimal tacrolimus dose (Niioka et al., 2015, Thervet et al., 2008, Birdwell et al., 2015). In a randomized controlled study, kidney transplant recipients receiving tacrolimus doses according to the CYP3A5 genotype reached the target C_0 significantly earlier than recipients used a standard regimen. Although more patients were within the desired tacrolimus target range early after transplantation, a considerable proportion of patients still did not have tacrolimus C_0 levels within the target range points (Thervet et al., 2010) indicating that *CYP3A5* genotype alone is unlikely to be sufficient for successful individualisation of initial tacrolimus dose. Another study found no association between pharmacogenetic adaptation of tacrolimus daily dose and earlier achievement of the tacrolimus target exposure range. No improvement in the clinical outcome was observed (Shuker et al., 2015). In addition to *CYP3A5*3* genotype, our results also confirm a minor role of the *ABCB1 3435* variant allele. This supports previous studies showing a weak association between *ABCB1 3435* polymorphism and tacrolimus dose requirements (Li et al., 2006a, MacPhee et al., 2002, Anglicheau et al., 2003b). However, other studies failed to identify such an association (Tsuchiya et al., 2004, Haufroid et

al., 2004, Quteineh et al., 2008, Shi et al., 2013). The reason for the discrepancies between these studies is unclear, but may be due to the food, the genetic effect of other genes, or studies lacking sufficient statistical power.
Moreover, we studied some non-genetic factors, including age, sex, haematocrit, ethnicity, diabetic status, steroid therapy, donor type, time since transplantation and tacrolimus formulation. We found that the donor type, time since transplantation and tacrolimus formulations had no significant effect. Although sex had a significant effect on tacrolimus dose in univariate analysis, this effect was diminished in multivariate regression analysis. Stratta et al reported that sex differences affect tacrolimus dose requirements (Stratta et al., 2012).

Furthermore, we demonstrated that the haematocrit value was strongly correlated with tacrolimus dose, consistent with previous reports showing the influence of haematocrit values in tacrolimus blood concentration (de Jonge et al., 2012, Stratta et al., 2012). Tacrolimus is extensively bound to FK-binding proteins in red blood cells. Hence the haematocrit plays an important role in tacrolimus pharmacokinetics and may need to be considered in tacrolimus dosage regimens especially with significant changes in their levels. In multivariate analysis, diabetic status was significantly associated with tacrolimus dose requirements, confirming previous findings by Chitnis et al who found that diabetic patients have significantly higher dose adjusted tacrolimus blood concentrations compared to non-diabetic patients (Chitnis et al., 2013). Our findings confirm the effect of age on tacrolimus dose requirement. Younger patients required higher tacrolimus dose than older patients. This is in accordance with previous findings indicating the strong correlation of age with tacrolimus dose in both adults (Kim et al., 2012) and paediatric patients (Gijsen et al., 2011). We also demonstrated that ethnicity had a significant effect on tacrolimus dose, black patients required higher tacrolimus dose than white and Asian subjects, which is consistent with previous findings (Macphee et al., 2005). However, the *CYP3A5*3* polymorphism cannot be replaced by ethnicity to predict the tacrolimus dose requirement, even though they are strongly linked.
In this study, different tacrolimus formulation has no influence on tacrolimus dose either in univariate or multivariate analysis. This means that factors were reported to influence tacrolimus dose including age, sex, ethnicity, haematocrit, diabetic status, corticosteroid treatment and *CYP3A5*3/ ABCB1 3435* polymorphisms are the same for both tacrolimus formulations. From our study prediction of tacrolimus dose can be achieved from the following equation:

Dose (mg/kg) = 0.2199 - 0.000622 * Age - 0.1636 * Haematocrit - 0.0387 (if Asian) - 0.0217 (if White) - 0.02665 (if diabetic) + 0.01043 (if treated with corticosteroids) + 0.00974 (if female) + 0.0017 (if *CYP3A5*1/*1/*1/*3 /ABCB1CT/TT* genotype) - 0.0457 (if *CYP3A5*3/*3 /ABCB1CC* genotype) - 0.0534 (if *CYP3A5*3/*3 /ABCB1CT/TT* genotype).

Our findings suggest that taking all these aforementioned factors into consideration may account for 59.9% of the between-individual variability in tacrolimus dose requirements. However, the impact of other factors, including different diet habits, comorbidity and concomitant treatment schemes could not be estimated. These findings may have potential clinical application for

initiation and adjustment of tacrolimus therapy. Given the modest impact, if any, of using *CYP3A5* genotype to predict the optimal initial dose of tacrolimus, it may now be appropriate to test algorithms including genetic and non-genetic factors as described here. As a first step, it would be useful to test the predictive value of this equation in an independent group of transplant recipients. Demonstration of clinical utility of an algorithm would require a clinical trial statistically powered to demonstrate improvement in hard clinical endpoints.

6. Other Information

6.1 Archiving

The trial essential documents along with the trial database were archived in accordance with the sponsor SOP. The agreed archiving period for this trial was 10 years.

6.2 Funding

This study was sponsored and monitored by the Joint Research and Enterprise Office, St. George's University of London and it was supported by an unrestricted research grant from Astellas Pharma Ltd.

6.3 Statement of Compliance

The study was carried out in conformation with the spirit and the letter of the declaration of Helsinki, and in accord with the ICH Good Clinical Practice Guidelines

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