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Title page

Title: Recovery of CYP3A Phenotype Following Kidney Transplantation

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Running title page

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CYP3A Phenotype After Kidney Transplantation

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ABBREVIATIONS: 4βOHC, 4β-hydroxycholesterol; CYP, cytochrome P450; eGFR, estimated glomerular filtration rate; ESRD, end stage renal disease; LLOQ, lower limit of quantification.

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Abstract

End-stage renal disease (ESRD) impairs drug metabolism via cytochrome P450 (CYP) 3A. However, it is unclear whether CYP3A activity recovers following kidney transplantation. Therefore, the aim of this study was to evaluate the change in CYP3A activity measured as 4β-hydroxycholesterol (4βOHC) concentration after kidney transplantation. In total, data from 58 renal transplant recipients with 550 prospective 4βOHC measurements were included in the study. One sample per patient was collected before transplantation, and 2-12 samples per patient were collected 1-82 days after transplantation. The measured pre-transplant 4βOHC concentrations ranged >7-fold with a median value of 22.8 ng/mL. Linear mixed model analysis identified a 0.16 ng/mL increase in 4βOHC concentration per day after transplantation (p<0.001), indicating a regain in CYP3A activity. Increasing estimated glomerular filtration rate after transplantation was associated with increasing 4βOHC concentration (p<0.001), supporting that CYP3A activity increases with recovering uremia. In conclusion, this study indicates that CYP3A activity is regained subsequent to kidney transplantation.

Introduction

End-stage renal disease (ESRD) impairs not only renal drug clearance, but also non-renal clearance via impairment of cytochrome P450 (CYP) enzymes and drug transport proteins (Ladda and Goralski, 2016). A number of CYP enzymes display reduced activity in ESRD patients (Ladda and Goralski, 2016). The mechanism behind this is not fully understood, but it might be related to direct inhibition of CYP metabolism or reduced protein expression caused by uremic toxins (Guevin et al., 2002; Barnes et al., 2014).

The CYP3A subfamily is involved in the metabolism of 30% of clinically used drugs, and CYP3A4 and CYP3A5 are the most relevant enzymes (Zanger and Schwab, 2013). Both enzymes display genetic polymorphisms, but the functional relevance is most evident for CYP3A5, where the *CYP3A5*3* variant allele – being the most frequent allele in most populations – encodes for a non-functional CYP3A5 enzyme (Kuehl et al., 2001). For CYP3A4, the *CYP3A4*22* variant allele is related to reduced enzyme activity (Wang et al., 2011), and has been associated with an approximately 30% reduced tacrolimus dose requirement (Elens et al., 2011). However, the general relevance of *CYP3A4*22* on CYP3A phenotype is unclear.

Due to the extensive and mainly non-genetic interindividual variability in CYP3A enzyme activity, phenotyping is likely to be more clinically useful than genotyping for dose individualization. Midazolam is considered the gold standard probe drug for measuring CYP3A4 phenotype, but endogenous phenotype markers have the advantage of not needing probe drug administration. 4β-Hydroxycholesterol (4βOHC) is metabolized from cholesterol by CYP3A4 and CYP3A5 (Bodin et al., 2001; Diczfalusy et al., 2008), and is a promising CYP3A phenotype marker. Plasma concentration of 4βOHC has proven sensitive to CYP3A induction and inhibition (Josephson et al., 2008; Lutjohann et al., 2009; Mangold et al., 2016), and correlates with oral and intravenous midazolam clearance (Tomalik-Scharte et al., 2009).

Several studies have shown reduced CYP3A metabolism in patients with impaired renal function (Dowling et al., 2003; Kirwan et al., 2009; Kirwan et al., 2012). Hemodialysis has been reported to rapidly improve hepatic CYP3A4 activity, as measured by the ^{14}C-erythromycin breath test (Nolin et al., 2006). However, there are conflicting study results regarding change in CYP3A phenotype after kidney transplantation, with reports of increased 4βOHC concentration (Suzuki et al., 2013) and decreased midazolam clearance (de Jonge et al., 2015). Furthermore, Suzuki *et al.* reported that post-transplant change in 4βOHC concentration was dependent on *CYP3A5* genotype, since only *CYP3A5*1* carriers – and not *CYP3A5*3/*3* homozygotes – experienced increased enzyme activity after transplantation (Suzuki et al., 2015). Thus, further elucidation of CYP3A-metabolizing status following kidney transplantation is needed.

The primary aim of this study was to evaluate the change in 4βOHC concentration after kidney transplantation in patients with ESRD. Furthermore, we wanted to assess the impact of *CYP3A4* and *CYP3A5* genotypes, demographic factors, pre-transplant dialysis status and post-transplant creatinine levels on the potential change in 4βOHC concentration following transplantation.

Materials and Methods

Patients. This study included patients from a previously described randomized controlled trial evaluating computerized tacrolimus dosing in renal transplant recipients (www.ClinicalTrials.gov, NCT02010320) (Storset et al., 2015). The patients underwent kidney transplantation at Oslo University Hospital Rikshospitalet between January and June 2014. All patients were >18 years and treated with a triple therapy immunosuppressive regime consisting of tacrolimus, mycophenolate mofetil and oral prednisolone (Storset et al., 2015). Induction therapy consisted of 20 mg basiliximab on day 0 and 4, and intravenous methylprednisolone on day 0. None of the included patients used CYP3A inducers or inhibitors during the study period, except for corticosteroids. Acute rejection episodes and concurrent methylprednisolone treatment was registered. The study was approved by the Regional Committee for Medical Research Ethics. All participants gave written informed consent.

Genotype analyses. Analyses of *CYP3A4*22* (rs35599367) and *CYP3A5*3* (rs776746) variant alleles were performed by real-time polymerase chain reaction and melt curve analysis with hybridization probes on the LightCycler® 480 instrument (Roche Applied Science, Penzberg, Germany).

4β-hydroxycholesterol analyses. Plasma concentrations of 4βOHC were determined by a previously described ultra-performance liquid chromatography tandem mass spectrometry method using atmospheric pressure chemical ionization (Gjestad et al., 2016), with an added filtration step (Størset et al., 2017). A quality control sample produced at the time of the oldest samples (January 2014) was included in each run to ensure stability during storage. Duplicate measurements were performed for each sample, and mean values of duplicates were used for statistical analyses. The lower limit of quantification (LLOQ) was 10 ng/mL, and 4βOHC measurements below LLOQ were excluded from statistical analysis. Intra- and interday

precision for the method was <8% at 10 ng/mL and <4% at 644 ng/mL (n = 6), while the corresponding intra- and interday accuracies were <15% and <2%, respectively (n = 6).

Creatinine analyses. Plasma creatinine was analyzed in fresh samples on the COBAS 8000 platform using the Roche CREP2 kit, a colorimetric based method (no. 05168589190, Roche, Basel Switzerland). The intra- and interpatient coefficients of variation were 5.3% and 14.2%, respectively.

Endpoints and statistical analyses. Kruskal-Wallis and Mann-Whitney *U* tests were used for comparison of pre-transplantation 4βOHC concentrations between subgroups. Correlations were evaluated using Spearman's signed rank test. Linear mixed modeling with random intercept and random slope was used to evaluate the effects of covariates on variation in 4βOHC concentration. An unstructured covariance type was used, and calculation of estimates was based on restricted maximum likelihoods. Evaluated fixed effects included *CYP3A4* and *CYP3A5* genotypes, days since transplantation, estimated glomerular filtration rate (eGFR) calculated by the Cockcroft-Gault-formula (Cockcroft and Gault, 1976), bodyweight, sex, age, whether the patient was subject to dialysis before transplantation (independent of previous transplantations), and whether the transplant originated from living or deceased donor. Two separate mixed model analyses were performed; one including samples from pre- and post-transplantation, where bodyweight was included as a covariate but not eGFR, and one including samples only from post-transplantation, where eGFR was included as a covariate but not bodyweight. Since bodyweight is incorporated in eGFR, we did not use both covariates in the same analyses. The final mixed models were chosen by Akaike's information criterion, and only statistically significant covariates were included. The impact of genotype on change in 4βOHC concentration over time was assessed by comparing the slope of change in 4βOHC concentration between genotype subgroups. Statistical significance was considered as $p<0.05$. Statistical analyses were performed using the IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, NY). SPSS and

GraphPad Prism for Windows, version 6.01 (GraphPad Software, La Jolla, CA), were used for graphical presentations.

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Results

Patient characteristics. One patient was carrier of both *CYP3A4**22 and *CYP3A5**1 alleles, and was excluded from the study. Clinical and demographic characteristics of the 58 remaining patients are listed in Table 1. Thirty-nine patients were male (67%) and median age was 56 years (range 24 to 76). This was the first kidney transplantation for 54 of the patients (93%). There were 40 patients (69%) who had been subject to dialysis treatment prior to transplantation, of which one patient was receiving a re-transplant. Regarding genotype distributions in the population, 5 patients (9%) had the *CYP3A4*1/*22* genotype, while 12 patients (21%) had the *CYP3A5*1/*3* genotype. The remaining 41 patients (71%) were *CYP3A4*1/*1* and *CYP3A5*3/*3* homozygotes. Three of the patients carrying the *CYP3A5*1* allele were of non-Caucasian origin.

Pre-transplantation 4βOHC concentration. One 4βOHC measurement was available from the period immediately prior to transplantation (1-10 days) for all but 3 patients; two patients lacked measurements due to shortage of plasma, and one measurement was excluded due to level below LLOQ. Pre-transplant 4βOHC concentrations of the remaining 55 patients ranged >7-fold (10.0 to 73.1 ng/mL) with a median value of 22.8 ng/mL. The median pre-transplantation 4βOHC concentration in *CYP3A4*22* carriers was 16.1 ng/mL (range 12.8-27.7 ng/mL, n=5), in combined *CYP3A4*1/*1* and *CYP3A5*3/3* homozygotes it was 22.3 ng/mL (10.3-71.2 ng/mL, n=39), and in *CYP3A5*1* carriers it was 33.5 ng/mL (10.0-73.1 ng/mL, n=11) (Kruskal-Wallis p=0.04). The Mann-Whitney U test revealed a significant difference in 4βOHC concentration between *CYP3A5*1* carriers and patients with combined *CYP3A4*1/*1* and *CYP3A5*3/*3* genotypes (p=0.03), but not between *CYP3A4*22* carriers and patients with combined CYP3A4*1/*1 and CYP3A5*3/*3 genotypes (p=0.3) (Figure 1A). As shown in Figure 1B, female patients had a median 4βOHC concentration of 31.3 ng/mL (11.1 to 71.2 ng/mL) compared to 22.3 ng/mL in male patients (10.0 to 73.1 ng/mL)

(Mann-Whitney p=0.049). Pre-transplantation 4βOHC concentration was not different between dialysis and non-dialysis patients (Mann-Whitney p=0.5). Pre-transplant bodyweight correlated with 4βOHC concentration (Spearman's r = -0.58, p<0.001).

Change in 4βOHC concentration after transplantation. A median of nine 4βOHC measurements per patient (range 2 to 12) were available from the post-transplantation period (1-82 days). Nine post-transplant 4βOHC measurements were below LLOQ, and were not included in the statistical analysis. In total 550 pre- and post-transplantation 4βOHC measurements from 58 patients were included in the study (Figure 2). There were 39 missing bodyweight measurements and 27 missing creatinine concentration measurements during follow-up after transplantation.

As shown in Figure 3, correlation between plasma creatinine and 4βOHC concentrations from week one post-transplantation was borderline significant (Spearman's r = -0.36, p=0.053, n=30). Linear mixed modeling analysis including both pre- and post-transplantation samples identified 'days since transplantation', bodyweight and *CYP3A5* genotype as significant covariates with impact on 4βOHC concentration (Table 2). The final model predicted a 0.16 ng/mL increase in 4βOHC per day after transplantation (p<0.001). This corresponds to a 13.1 ng/mL increase in 4βOHC concentration from before transplantation to day 82 after transplantation, or a 46% increase for a 70kg person. A bodyweight increase of 1 kg predicted a 0.4 ng/mL reduction in 4βOHC concentration (p<0.001), which corresponds to a pre-transplantation 4βOHC concentration of 33 ng/mL for a 60kg patient, and 16 ng/mL for a 100kg patient who did not express *CYP3A5*. Carrying the *CYP3A5*1* allele indicated a 7.8 ng/mL higher 4βOHC level than being homozygous for *CYP3A5*3/*3* (p=0.02). Being carrier of the *CYP3A4*22* allele did not have a significant impact on 4βOHC levels (p=0.5), nor did sex, age, pre-transplant dialysis status or whether the transplanted kidney originated from a living or deceased donor (p>0.2). The slope of change in 4βOHC concentration according to

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days after transplantation was not significantly different between the genotype subgroups (p>0.5). When excluding all samples from patients after a rejection episode and concurrent methylprednisolone treatment, the results of the mixed model analysis were essentially the same (no differences in significance levels of the tested variables, results not shown). A linear mixed model analysis based solely on post-transplantation samples further identified an association between 4βOHC concentration and eGFR (Table 3). A 10 mL/min increase in eGFR was associated with a 1.0 ng/mL increase in 4βOHC levels (p<0.001).

Discussion

Our study of 58 kidney transplant recipients showed a gradual increase in CYP3A activity from immediately before to 82 days after kidney transplantation, suggesting that CYP3A impairment due to ESRD is recovered subsequent to transplantation. Furthermore, post-transplantation increase in 4βOHC concentration was associated with increasing eGFR, supporting that uremia or uremic toxins impairs CYP3A activity. However, the degree of increase in CYP3A activity was not statistically associated with *CYP3A4* or *CYP3A5* genotypes.

The gradual increase in 4βOHC concentration with increasing time after transplantation coincides with findings in Japanese kidney transplant recipients, where a 30-50% increase in 4βOHC concentration was found on day 90 and 180 after transplantation compared with pre-transplant 4βOHC concentration (Suzuki et al., 2013; Suzuki et al., 2015). Furthermore, *in vitro* studies of rat hepatocytes incubated with serum from chronic renal failure patients reported a reduction of CYP3A activity and enzyme expression prior to, but not after, transplantation (Michaud et al., 2005). Clinical implications could be that dose reductions of CYP3A metabolized drugs in ESRD patients may prevent overdosing and concentration-dependent side effects, and correspondingly that post-transplantation dose increase of CYP3A drugs may prevent therapeutic failure. However, it should be mentioned that one study actually reported decreased clearance of the CYP3A substrate midazolam 1 to 12 months after transplantation compared to 7 days after transplantation (de Jonge et al., 2015). Although midazolam is considered a gold standard CYP3A probe drug, it is also highly bound to plasma proteins (Kirwan et al., 2010). Albumin levels are reported to be reduced in patients with chronic kidney disease and after recent surgery (Meijers et al., 2008; Hubner et al., 2016), and increase with time after transplantation (Størset et al., 2014). Thus, it is possible that the reported decreasing midazolam plasma clearance mainly reflects increasing protein

binding rather than decreasing CYP3A activity. Nevertheless, further studies are needed to elucidate the change in CYP3A activity after kidney transplantation, and the potential clinical relevance of such a change.

The median value of pre-transplantation 4βOHC concentration in the present population was 22.8 ng/mL, which is approximately 10% lower than the reported median concentration in a Norwegian patient population of psychiatric patients (Hole et al., 2017), and approximately 15-25% lower than mean concentrations in healthy Scandinavian volunteers (Bodin et al., 2001; Diczfalusy et al., 2008). This supports that CYP3A activity is impaired in ESRD patients. The mechanism through which ESRD impairs CYP3A activity is not fully elucidated, but is considered to involve uremic toxins (Ladda and Goralski, 2016). Possible mechanisms include direct enzymatic inhibition and/or reduced CYP gene expression by uremic toxins (Barnes et al., 2014; Ladda and Goralski, 2016). Suzuki *et al.* reported a negative correlation of plasma indoxyl sulfate with 4βOHC concentration in stable kidney transplant recipients (Suzuki et al., 2014), indicating that a post-transplant decline of uremic toxin was linked to regain of CYP3A activity. This coincides with our findings that a post-transplantation increase in eGFR was associated with an increase in 4βOHC concentration.

We observed a significant impact of *CYP3A5*, but not *CYP3A4* genotype on pre-transplant 4βOHC concentration. There were only 5 *CYP3A4*22* carriers, which may explain why the 4βOHC concentration was not significantly lower in this group compared to the other genotype groups. It is also possible that non-genetic factors such as inflammation and uremic toxins mask the effects of *CYP3A4* genotype on CYP3A phenotype in this population (Morgan, 1997; Suzuki et al., 2014). Suzuki *et al.* reported identical pre-transplantation 4βOHC levels in *CYP3A5*1* carriers and non-carriers, with a mean concentration of 38 ng/mL (Suzuki et al., 2015). They further reported a post-transplantation increase in 4βOHC concentration only among *CYP3A5*1* carriers, and not among *CYP3A5*3/*3* homozygotes

(Suzuki et al., 2015). We found no difference in the post-transplantation increase in 4βOHC concentration when comparing *CYP3A4*22* carriers, *CYP3A4*1/*1* and *CYP3A5*3/*3* homozygotes, and *CYP3A5*1* carriers, indicating a recovery of CYP3A activity independent of genotype. The inconsistent findings regarding impact of *CYP3A4/5* genotype on metabolizing phenotype both before and after kidney transplantation might be related to ethnic differences, but this is a speculation which needs to be investigated in further studies.

In this study we used 4βOHC concentrations as a measure of *in vivo* CYP3A activity without adjusting for total cholesterol levels. Total cholesterol has only minor impact on the variation in 4βOHC concentration (Diczfalusy et al., 2008), except for in statin treated patients (Bjorkhem-Bergman et al., 2016). None of the patients included in our study received statins. Thus, it is not expected that using 4βOHC:cholesterol ratio would have led to different conclusions. The elimination half-life of 4βOHC is considered to be between 3 and 17 days (Bodin et al., 2002; Diczfalusy et al., 2009; Ngaimisi et al., 2014), which is a limitation when studying changes in CYP3A activity over short time periods. However, the fact that use of a CYP3A inducer for a week doubles 4βOHC concentration indicates that this biomarker rapidly captures increased enzyme activity (Marschall et al., 2005; Wide et al., 2008). We are therefore confident that 4βOHC can reflect recovery in CYP3A activity within the time span of our study.

Studying dynamic changes early after kidney transplantation is complex, and there are several factors that were not accounted for in our analysis, such as delayed graft function, albumin levels, and corticosteroid doses. All patients received methylprednisolone on the day of transplantation, and prednisolone in the immunosuppressive regimen. CYP3A induction by corticosteroids is reported in the literature, most potently by dexamethasone, but also by prednisolone and methylprednisolone (Matoulkova et al., 2014). Clinical observations of increasing tacrolimus concentrations concurring with tapering of corticosteroid dosing has

been interpreted as indirect evidence that corticosteroid induction of CYP3A and P-glycoprotein is of clinical relevance. However, we recently showed that CYP3A activity measured as 4βOHC did not account for the increasing tacrolimus concentration with time after kidney transplantation (Størset et al., 2017). The conclusions regarding corticosteroid CYP3A induction are conflicting, and the clinical evidence limited (Matoulkova et al., 2014). Therefore, we do not suspect that the post-transplant increase in CYP3A activity is due to corticosteroid CYP induction, but both oral and intravenous corticosteroids given after transplantation may still have been an interfering factor on 4βOHC levels that our study did not completely account for. In future studies, it is important to account for interfering factors, such as corticosteroid use, on post-transplant regain of CYP3A phenotype.

CYP enzymes, including CYP3A isoforms, are known to be expressed in kidneys (Aleksa et al., 2005). The increased metabolic activity following transplantation might therefore in theory reflect higher renal CYP3A activity in the donor kidney than in the impaired kidney. CYP3A5 expression has been shown to be lower in renal transplant patients with calcine urine-induced nephrotoxicty compared to controls, which could support that metabolic activity is declined during renal failure (Joy et al., 2007). However, Aleksa *et al.* have reported that total expression of CYP enzymes in the kidneys is 80-90% lower than in the liver (Aleksa et al., 2005). Overall, we therefore consider it likely that the raise in 4βOHC level following transplantation mainly reflects a gradual regain of CYP3A metabolism in liver/intestine, but it is possible that higher metabolic activity in the donor vs. the failing kidney may have contributed to the observed increase in 4βOHC concentration. Regarding causality behind the post-transplant increase in 4βOHC concentrations, it is necessary with further investigation to clarify the possible mechanisms behind the suppression of CYP3A phenotype in ESRD patients. Another limitation in our study is the low number of *CYP3A4*22* carriers. Since the *CYP3A4*22* allele codes for a reduced function enzyme, and patients rarely are homozygous, a larger number of patients would probably be needed to find significant impact on CYP3A phenotype. Furthermore, there were no *CYP3A5*1/*1*

homozygotes, so we could not investigate whether there was a stepwise increase in 4βOHC concentration according to number of *CYP3A5*1* alleles. The main strength of our study is the high number of samples from the early post-transplant period, which allowed us to investigate intra- and interindividual change in 4βOHC concentration after transplantation. The study would have been further strengthened by investigating the clinical effect of change in 4βOHC concentration on clearance of CYP3A metabolized drugs. We have previously reported that 4βOHC concentration did not improve tacrolimus dose predictions (Størset et al., 2017). However, it would be interesting to see whether 4βOHC concentration in ESRD patients and early after transplantation is associated with clearance of other CYP3A drugs with less complex pharmacokinetic profiles than tacrolimus.

In conclusion, we found that CYP3A activity increased after kidney transplantation, indicating a regain of enzyme activity impaired by ESRD. Clinically, this implicates that dosing of CYP3A-metabolized drugs may need to be reduced in patients with ESRD, and increased following kidney transplantation.

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Authorship contributions

Participated in research design: Hole, Størset, Olastuen, Midtvedt, Åsberg, Molden.

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Wrote or contributed to the writing of the manuscript: Hole, Størset, Olastuen, Haslemo, Kro, Midtvedt, Åsberg, Molden.

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Footnotes

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Figure Legends

Fig. 1. Pre-transplantation 4β-hydroxycholesterol concentrations in A) *CYP3A4*22* carriers (*n*=5), *CYP3A4*1/*1* and *CYP3A5*3/*3* homozygotes (*n*=39), and *CYP3A5*1* carriers (*n*=11), and B) males (*n*=36) and females (*n*=19). Medians are expressed as solid lines, and *p*-values are derived from Mann-Whitney U-Tests.

Fig. 2. Measured 4β-hydroxycholesterol concentrations in all patients according to days after transplantation and stratified by *CYP3A4* and *CYP3A5* genotypes.

Fig. 3. Correlation between 4β-hydroxycholesterol concentration and creatinine concentration one week after transplantation (*n*=30). *P*- and r-values are estimated from Spearman's test. Linear trend line is added for visual purposes.

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TABLE 1

Clinical and demographic patient characteristics

	Patients with *CYP3A4*1/*22*	Patients with *CYP3A4*1/*1* and *CYP3A5*3/*3*	Patients with *CYP3A5*1/*3*
Number of patients	5	41	12
Sex (male / female)	4 / 1	27 / 14	8 / 4
Age, years	63 (47-66)	54 (24-76)	61 (30-74)
Bodyweight before transplantation, kg	89 (76-104)	76 (58-116)	79 (63-118)
Ethnicity (Caucasian / other)	5 / 0	40 / 1	9 / 3
Donor (living / deceased)	1 / 4	12 / 29	3 / 9
Previous transplantations (0 / 1 / 2)	5 / 0 / 0	37 / 3 / 1	12 / 0 / 0
Pre-transplant dialysis (HD / PD / none)	2 / 1 / 2	19 / 9 / 13	7 / 2 / 3

CYP, cytochrome P450; HD, haemodialysis; PD, peritoneal dialysis. Data are expressed as numbers or median (range).

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TABLE 2

Linear mixed model estimates for predictors of 4β-hydroxycholesterol concentration (ng/mL) including pre- and post-transplantation samples

		95% confidence interval		
Variables	Estimate	Lower bound	Upper bound	*p*-value
Intercept	59.58	46.78	72.38	<0.001
Time since transplantation, days	+0.16	+0.11	+0.22	<0.001
Bodyweight, kg	-0.44	-0.59	-0.29	<0.001
*CYP3A5*1* carrier[a]	+7.78	+1.23	+14.33	0.02

[a]Vs. non-carrier

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TABLE 3

Linear mixed model estimates for predictors of 4β-hydroxycholesterol concentration (ng/mL) including solely post-transplantation samples

		95% confidence interval		
Variables	Estimate	Lower bound	Upper bound	*p*-value
Intercept	15.64	11.25	20.03	<0.001
Time since transplantation, days	+0.18	+0.12	+0.24	<0.001
Estimated glomerular filtration rate, mL/min	+0.10	+0.05	+0.16	<0.001
*CYP3A5*1* carrier[a]	+10.43	+3.65	+17.20	0.003

[a]Vs. non-carrier

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Figures

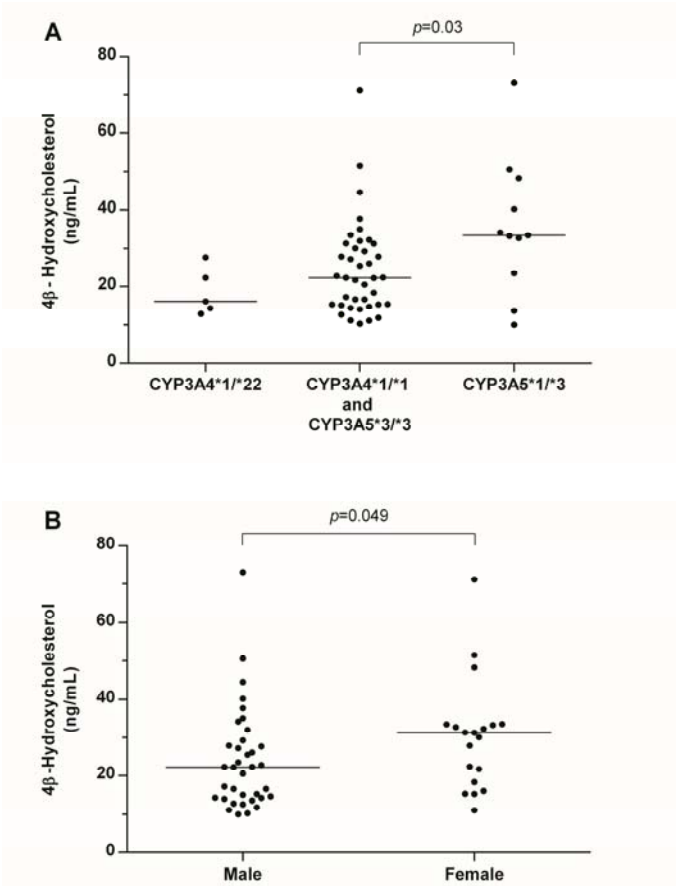

Fig. 1.

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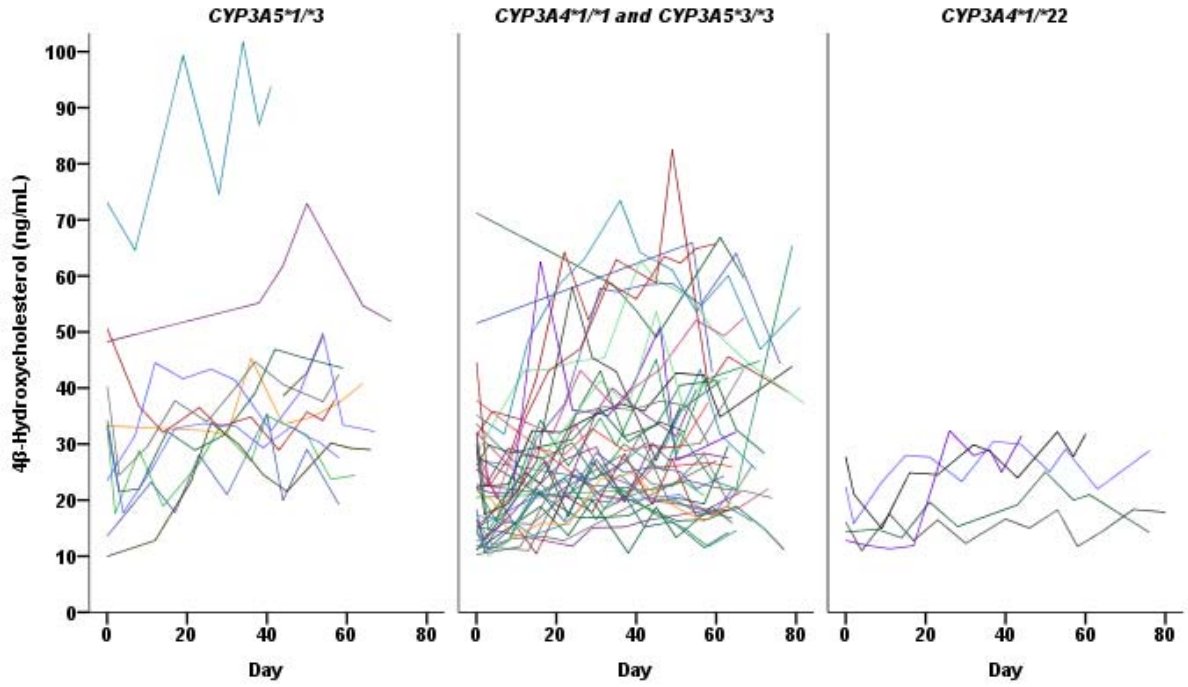

Fig. 2.

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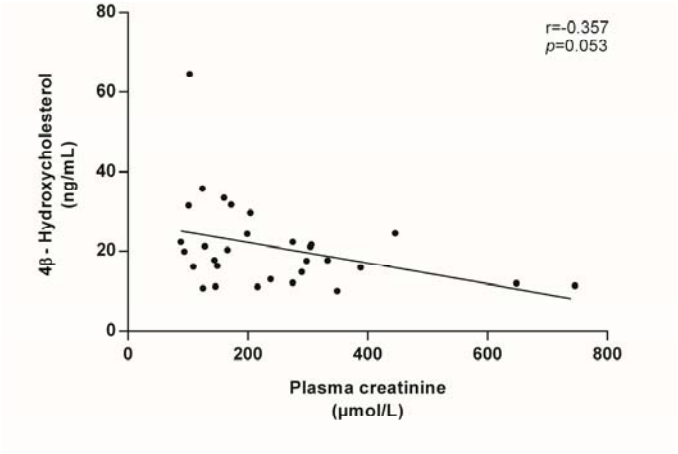

Fig. 3.