# TITLE PAGE

Manuscript Title: Evaluation of the relationship between concentrations of tacrolimus metabolites, 13-O-demethyl tacrolimus and 15-O-demethyl tacrolimus, and clinical and biochemical parameters in kidney transplant recipients

My manuscript is submitted for the following meeting: Polish Transplantation Society 2017 Congress

**Authors:**

Zegarska Jolanta1, Hryniewiecka Ewa2,1, Zochowska Dorota1, Samborowska Emilia3, Jazwiec Radoslaw3, Maciej Kosieradzki4, Nazarewski Slawomir 5, Dadlez Michal3, Paczek Leszek1

# Affiliations:

1 Department of Immunology, Transplant Medicine and Internal Diseases, Medical University of Warsaw, Transplantation Institute, 59 Nowogrodzka St., 02-006 Warsaw, Poland, tel. 0048225021461; fax 0048225022127

2 Department of Clinical Nursing, Medical University of Warsaw, 27 Ciolka St., 01-445 Warsaw, Poland, tel. 0048228360972; fax 0048228360972

3 Institute of Biochemistry and Biophysics Polish Academy of Sciences, 5a Pawińskiego St., 02-106 Warsaw, Poland, tel. 0048225923476; fax 0048226584766

4 Department of General and Transplantation Surgery, Medical University of Warsaw, Transplantation Institute, 59 Nowogrodzka St., 02-006 Warsaw, Poland, tel. 0048225021126; fax 0048225022155

5 Department of General, Vascular and Transplant Surgery, Medical University of Warsaw, 1A Banacha St., 02-097 Warsaw, Poland, tel. 0048225992467; fax 0048225991468

**Email addresses of all authors:**

Jolanta Zegarska1, [zegarska@yahoo.com](mailto:zegarska@yahoo.com), Ewa Hryniewiecka2,1, [elhryniewiecka@gmail.com](mailto:elhryniewiecka@gmail.com), Dorota Zochowska1, [d\_zochowska@wp.pl](mailto:d_zochowska@wp.pl), Emilia Samborowska3, [emi.sambor@gmail.com](mailto:emi.sambor@gmail.com), Radoslaw Jazwiec3, [rjazwiec@ibb.waw.pl](mailto:rjazwiec@ibb.waw.pl), Kosieradzki Maciej4, [mkosieradzki@wp.pl](mailto:mkosieradzki@wp.pl), Nazarewski Slawomir 5, [slawomir.nazarewski@wum.edu.pl](mailto:slawomir.nazarewski@wum.edu.pl), Michal Dadlez3, [michald@poczta.ibb.waw.pl](mailto:michald@poczta.ibb.waw.pl), Leszek Paczek1, leszek.paczek@wum.edu.pl

**Corresponding author:**

Leszek Paczek, Department of Immunology, Transplant Medicine and Internal Diseases, Medical University of Warsaw, Transplantation Institute, 59 Nowogrodzka St., 02-006 Warsaw, Poland, leszek.paczek@wum.edu.pl, tel.: +48 22 5021641, Fax: +48 22 5022127

**Grant information:**

This work was supported by the Polish National Center of Research and Development grant NR13014410 and the Polish National Science Centre grant 2013/09/B/NZ2/00275.

**Key words:**

tacrolimus, metabolites, kidney transplantation, anaemia

**Abbreviations:**

AlAT, alanine aminotransferase

CYP3A, cytochrome P450 3A

eGFR, estimated glomerular filtration rate

ESI, electrospray ionization mode

LC/MS/MS, liquid chromatography combined with tandem mass spectrometry

M-I, 13-O-demethyl tacrolimus

M-II, 31-O-demethyl tacrolimus

M-III, 15-O-demethyl tacrolimus

M-IV, 12-hydroxyl tacrolimus

MDRD, Modification of Diet in Renal Disease

MLC, mixed lymphocyte culture assay

MRM, multiple-reaction monitoring

PLT, blood platelet count

RBC, red blood cell count

Tac, tacrolimus

WBC, white blood cell count

**Tables:**

**Figures:**

**Introduction**

Tacrolimus (Tac) is an essential component of immunosuppressive therapy after solid organ transplantation. Tac exhibits a narrow therapeutic index and variable pharmacokinetic characteristics. Therefore, Tac regimen requires therapeutic drug monitoring to prevent either graft rejection or toxic effects. In clinical practice, monitoring of Tac predose trough blood concentrations is routinely used [1].Unfortunately, it sems to be not always sufficient for guiding optimal long-term dosing of this drug. Toxic effects of Tac include nephrotoxicity, neurotoxicity, diabetogenic effect, and hypertension. Tac undergoes extensive biotransformation that is mediated by hepatic and intestinal cytochrome P450 3A (CYP3A), especially CYP3A5, into at least eight metabolites [2]. Four of them are considered to be first- generation metabolites formed directly from the parent drug, 13-O-demethyl (M-I), 31-O-demethyl (M-II), 15-O-demethyl (M-III), and 12-hydroxyl (M-IV), of varying immunosuppressive activity [3,4(4\*)]. Second generation metabolites, M-V to M-VIII are formed from the first generation metabolites. It has been found the mean concentrations of M-I, M-II, and M-III to be 10%, 4%, and 6%, respectively, of the Tac concentration [5(4)]. Most Tac metabolites, with exeption of M-II, are pharmacologically inactive. However, their toxicological activity is not known. The clinical importance of Tac metabolites remains unclear.

The objective of this study was to evaluate the concentrations of the three major tacrolimus metabolites, M-I, M-II and M-III, using liquid chromatography combined with tandem mass spectrometry (LC/MS/MS) in kidney transplant recipients. We tried to link tacrolimus metabolites’ levels with clinical and biochemical parameters.

**Material and Methods**

Blood samples were obtained from patients who gave their written informed consent to participate in the study. 81 stable patients who underwent kidney transplantation were included in the study. The mean age was 51 years, ranging from 22 to 73. The study group included 36 male and 27 female. The mean postransplant time was 36 months, ranging from 1 to 209. Trough blood samples of 2 ml were collected during routine outpatients visits into EDTA-containing tubes and stored at -80oC until analysis. The study protocol was approved by local Ethics Committee and is in accordance with the revised Declaration of Helsinki.

Blood sampling was accompanied by the collection of relevant biochemical and clinical data. Serum glucose, creatinine, bilirubin, total cholesterol, triglycerides levels, alanine aminotransferase **(**ALAT) and aspartate aminotransferase (AspAT) activity, white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin and blood platelet (PLT) count were measured in parallel. Renal function was assessed with the use of estimated glomerular filtration rate (eGFR) by Modification of Diet in Renal Disease (MDRD) [6(5)]. Data on the arterial blood pressure measured on the date of blood sampling were collected as well as on the previous visit and on the next visit. The mean systolic and diastolic arterial pressure measured durind the three visits was calculated. Additionally, body mass index (BMI) was calulated.

Samples were prepared by protocol described before [7(6)]. The analyzes were performed using Waters Acquity Ultra Performance Liquid Chromatograph coupled with Waters TQ-S triple-quadrupole mass spectrometer. Tac and its metabolites were measured by LC/MS/MS method as described earlier [8(16)]. Separation was performed using a Waters BEH C18 column (1.7µm, 2.1 mm x 50 mm). LC/MS/MS analysis was performed in a positive electrospray ionization mode (ESI) and the mass spectrometer was operated in a multiple-reaction monitoring (MRM) mode. The calibration curves range was 1.1-30.8 ng/ml for tacrolimus, and 0.01-1.5 ng/ml for M-I. Concentration of M-III tacrolimus was quantified using M-I tacrolimus calibration curve. Tacrolimus metabolite concentrations obtained were shown as the ratio of the metabolite to the parent drug.

The Kolmogorov-Smirnov test was applied to assess variables’ normality. The continuous data were described as a mean ± standard deviation for normal distribution and as a median and range for data with non-normal distribution. Correlations between the parameters were calculated with the Pearson and Spearman correlation coefficient for normally and non-normally distributed variables, respectively. The Student’s t test and U Mann-Whitney test were used to determine the differences between normally and not-normally distributed variables, respectively. A p-value of <0.05 was considered as significant. The statistical analyses were performed using Statistica software (version 12.0, StatSoft Inc., Tulsa, US).

**Results**

Patients’ demographics, concentrations of Tac and its metabolites are shown in Table 1. None of the patients had M-II in the blood.There was a positive correlation between M-I/Tac and ALAT (r=0,29, p=0,046) (Fig. 1).Also we have found a negative correlation between M-I/Tac and hemoglobin(r=-0,33, p=0,008) (Fig, 2). A similar relationship was observed betweenTac dose/kg body weight and hemoglobin (r=-0,27, p=0,032). There was no relationship between M-I/Tac or M-III/Tac and: age, BMI, arterial pressure, time posttransplant, serum glucose, bilirubin, cholesterol, triglycerides, WBC, RBC, PLT or eGFR. A significant positive correlation between Tac concentrations and PLT count is evident (r=0,30, p=0,017). A similar relationship was found with respect to the Tac dose/kg body weight (r=0,43, p=0,0006).We observed significantly higher Tac concentrations in the subgroup with hypercholesterolaemia in comparison with the subgroup without hypercholesterolaemia(6,45±2,32 vs 5,16±2,12, p=0,043) (Tab. 2). Patients with hypertriglycerydaemia compared to those without hypertriglycerydaemia had significantly higher Tac concentrations (6,60±2,30 vs 5,34±2,20, p=0,033) (Tab. 2).

**Discussion**

We have evaluated the concentrations of Tac and its main metabolites using LC/MS/MS in kidney transplant recipients. In the literature there are some reports concerning quantification of tacrolimus metabolites using the same method [4, 7, 8]. However, data on clinical importance of these metabolites is limited.

We observed a positive correlation between M-I/Tac and ALAT. It could be explained by the fact that liver dysfunction leads to accumulation of Tac and its metabolites. Over 95% of Tac is eliminated as metabolites by the bile and less than 5% of the drug is excreted unchanged in the urine. Gonschior et al. have proved that impaired liver function resulted in increased concentrations of Tac and its metabolites, indicating accumulation of metabolites. Serum activities of ALAT, gamma-glutamyltransferase and alkaline phosphatase were predictors for increased concentrations of Tac and its metabolites. [11]. Surprisingly, Tac level did not correlate with ALAT.On the other hand, liver dysfunction or elevated aminotransferases are described as common side effects of Tac therapy. In the case of severe liver dysfunction it is recommended to reduce the dosage of this drug. Thus, it is possible that M-I/Tac ratio is a marker of hepatotoxicity. It is less likely that metabolite M-I itself leeds to deterioration of liver function because it has very little immunosupperssive activity. Iwasaki et al. found that M-I had 6,4% immunosuppressive activity of the parent drug [3].

We did not observe any relations between Tac metabolites or Tac concentration and kidney function. Gonschior et al in kidney transplant recipients with impaired kidney function found lower Tac levels and no difference in metabolites’ concentrations in comparison with patients with good kidney function [11].

Additionally, we have found a negative correlation between M-I/Tac and hemoglobin. The significance of this relation remains unclear.In the blood Tac is largely bound to erythrocytes and proteins. Factors such as low hematocrit lead to an increase in unboud Tac fraction, that can result in increased clearance of the drug. In the study of 25 liver transplant recipients authors concluded, that hematocrit had a significant effect on Tac level [**13**]. On the other hand, among common side effects of Tac is anemia, leucopenia, and thrombocytopenia. In some patients pure red cell aplasia (PRCA) can even occur [**14**]. Probably we underestimate the anemia as a side effect of Tac therapy. In the case of anemia in transplant patients we are linking this side effect rather with mycophenolates regimen or kidney failure, than with Tac treatment. It seems interesting in our study that concentrations of Tac did not correlate with hemoglobin.Thus, probably M-I/Tac ratio could be a marker of myelotoxicity.

Hypercholesterolaemiaand hypertriglycerydaemia are common side effects of Tac therapy. Our study confirmed that patients with hiperlipidemia have higher Tac levels. These results are consistent with other studies. Kim et al. in the study of 129 kidney transplant recipients found that trough levels of Tac were associated with post-transplantation hyperlipidaemia [**15**].

**Conlusions**

In conclusion, our data indicate that anemia change Tac metabolism or Tac metabolite M-I may be a marker of myelotoxicity. Liver dysunction leads to M-I Tac metabolite acumulation. Additionally, it is possible that this metabolite is linked to hepatotoxicity. Higher Tac concentrations result in higher incidence of dyslipidemia. However, further studies are needed to confirm if monitoring of M-I could minimize adverse effects such as hepatotoxicity and anemia. Then it would prove a potential use of Tac metabolite for the individualization of drug therapy.

**Acknowledgements**

The authors thank Astellas Pharma for kindly donating standards of tacrolimus metabolites.

**Reference**

[1] Kahan BD, Keown P, Levy GA, Johnston A. Therapeutic drug monitoring of immunosuppressant drugs in clinical practice. Clin Ther 2002; 24(3):330-50. 1

[2] Christians U, Braun F, Kosian N, Schmidt M, Schiebel HM, Ernst L, et al. High performance liquid chromatography/mass spectrometry of FK 506 and its metabolites in blood, bile and urine of liver grafted patients. Transplant Proc 1991; 23:2741-4. 2

[3] Iwasaki K, Shiraga T, Matsuda H, Nagase K, Tokuma Y, Hata T, et al. Further metabolism of FK506 (tacrolimus). Identification and biological activities of the metabolites oxidized at multiple sites of FK506. Drug Metab Dispos 1995; 23:28-34. 3

[4] Iwasaki K, Shiraga T, Nagase K, Matsuda H, , Tokuma Y, Hata T, et al. Isolation, identification, and biological activities of oxidative metabolites of FK506, a potent immunosuppressive macrolide lactone. Drug Metab Dispos 1993; 21:971-7. 4\*

[5] Dubbelboer IR, Pohanka A, Said R, Rosenborg S, Beck O. Quantification of tacrolimus and three demethylated metabolites in human whole blood using LC-ESI-MS/MS. Ther Drug Monit 2012; 34:134-42. 4

[6] Levey AS, Bosch JP, Lewis JB, et al. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. Ann Intern Med 1999; 16:461-70. 5

[7] Tszyrsznic W, Borowiec A, Pawlowska E, Jazwiec R, Zochowska D, Bartlomiejczyk I, et al. Two rapid ultra performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) methods with common sample pretreatment for therapeutic drug monitoring of immunosuppressants compared to immunoassay. Journal of Chromatography B 2013; 928:9– 15. 6

[8] Zegarska J, Hryniewiecka E, Zochowska D, Samborowska E, Jazwiec R, Borowiec A, Tszyrsznic W, Chmura A, Nazarewski S, Dadlez M, Paczek L. Tacrolimus metabolite M-III may have nephrotoxic and myelotoxic effects and increase the incidence of infections in kidney transplant recipients. Transplant Proc. 2016;48(5):1539-42. 16

[7] Chen YL, Hirabayashi H, Akhtar S, Pelzer M, Kobayashi M. Simultaneous determination of three isomeric metabolites of tacrolimus (FK506) in human whole blood and plasma using high performance liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2006; 830(2):330-41. 7

[8] Gonschior AK, Christians U, Winkler M, Schiebel HM, Linck A, Sewing KF. Simplified high-performance liquid chromatography-mass spectrometry assay for measurement of tacrolimus and its metabolites and cross-validation with microparticle enzyme immunoassay. Ther Drug Monit. 1995; 17(5):504-10. 8

[9] Davis DL, Murthy JN, Gallant-Haidner H, Yatscoff RW, Soldin SJ. Minor immunophilin binding of tacrolimus and sirolimus metabolites. Clin Biochem. 2000; 33(1):1-6. 9 **present various immunosuppressive activity of M-I, M-II, M-III and M-IV (6,4%, 100%, 0%, 3,5%, respectively**

[10] Murthy JN, Davis DL, Yatscoff RW, Soldin SJ. Tacrolimus metabolite cross-reactivity in different tacrolimus assays. Clin Biochem. 1998; 31(8):613-7. 10 **present various immunosuppressive activity of M-I, M-II, M-III and M-IV (6,4%, 100%, 0%, 3,5%, respectively**

[11] Gonschior AK, Christians U., Winkler M et al. Tacrolimus (FK506) metabolite patterns in blood from liver and kidney transplant patients. Clinical Chemistry. 1996; 42:1426-32. 11

[12] Kuypers DR, de Jonge H, Naesens M, Lerut E, Verbeke K, Vanrenterghem Y. CYP3A5 and CYP3A4 but not MDR1 single-nucleotide polymorphisms determine long-term tacrolimus disposition and drug-related nephrotoxicity in renal recipients. Clin Pharmacol Ther. 2007; 82(6):711-25. 12

**13** Limsrichamrern S, Chanapul C, Mahawithitwong P, Sirivatanauksorn Y, Kositamongkol P, Asavakarn S, Tovikkai C, Dumronggittigule W. Correlation of hematocrit and tacrolimus level in liver transplant recipients. Transplant Proc. 2016;48(4):1176-8.

**14** Patil MR1, Choudhury AR1, Chohwanglim M1, Divyaveer S1, Mahajan C1, Pandey R1. Post renal transplant pure red cell aplasia-is tacrolimus a culprit? Clin Kidney J. 2016;9(4):603-5.

**15** Kim IW, Noh H, Ji E, Han N, Hong SH, Ha J, Burckart GJ, Oh JM. Identification of factors affecting tacrolimus level and 5-year clinical outcome in kidney transplant patients. Basic Clin Pharmacol Toxicol. 2012;111(4):217-23.