Author’s response to reviews

Title: Effect of CYP3 A4, CYP3 A5 and ABCB1 gene polymorphisms on the clinical efficacy of tacrolimus in the treatment of nephrotic syndrome

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Dear editors.

Please find revised manuscript entitled “Effect of CYP3 A4, CYP3 A5 and ABCB1 gene polymorphisms on the clinical efficacy of tacrolimus in the treatment of nephrotic syndrome” that we would like you to consider for publication in BMC Pharmacology and Toxicology as original paper.

This manuscript ID was PHAT-D-17-00164. We thank you for your efforts for the paper review, and the reviewers for their positive comments and constructive suggestions, which have substantially improved the manuscript. We have made point-by-point responses to reviewer comments raised in your letter. These changes will not influence the content and framework of
the paper. We earnestly appreciate the editors and reviewers’ hard work, and hope that the correction will meet with approval.

Once again, thank you so much for your comments and suggestions.

Thank you for your consideration.

With best wishes,

Yours sincerely

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Response to Jiraganya Bhongsatiern, PhD (Reviewer 1)

Authors thank the reviewer for these comments and constructive suggestions. We would like to answer the questions you raised one by one below. We have numbered each point as comment 1 (C1), response 1 (R1), and so on.
(1) Tacrolimus clinical use

C1: Tacrolimus in clinical use is complicated by its high between-patient variability in pharmacokinetics as well as its narrow therapeutic index. The tacrolimus doses in this study were suggested by the 'formula' in the National Health and Family Planning Commission of People's Republic of China (Reference 18). However, I was not able to find the reference on PubMed and it could not be checked by the Journal system. The dosing management of tacrolimus which usually includes therapeutic drug monitoring system is very critical. I think it is necessary to include more information on this issue.

R1: Firstly, we thank the reviewer for this point very much. The full 'formula' is as follows.

Tacrolimus stable dose = 5.409–2.584*CYP3 A5GGa –1.732*CYP3 A5GAb +0.279*ABCB1 C1236Tc +0.205*ABCB1 G2677Td -0.163*donor typee–0.149*CCBf –0.140*infectiong – 0.197* Hypertensionh

a. CYP3 A5GG: AA=0, GG=1;

b. CYP3 A5AG: AA=0, AG=1;

c. ABCB1 C1236T: 0 for CC, 1 for CT or TT;

d. ABCB1 G2677T: 1 for GG or GT, 2 for TT;

e. Transplantation type: other =0, viable transplantation=1;

f. CCB: Merge using CCB=1, do not merge =0;

g. Infection: infection =1, no =0;

h. Hypertension: hypertension =1, no =0.

From the effect of “e”, the formula is suitable for patients with kidney transplantation. The document was published in the Practical Journal of Organ Transplantation (Electronic Version) in China, which was not found on PubMed. In our study, the dose of tacrolimus was not calculated according to this formula. We formulated the dose of tacrolimus on the basis of the
previous literature published in PubMed (References 11 and 19-21). TAC was given at the dose of 0.05 mg kg⁻¹ day⁻¹ for all patients, divided twice from oral application at intervals of 12 hours, which can be seen in Methods section, line 2-9, page 13.

Second, according to your suggestion, I have included more information on the issue of dosing management and therapeutic drug monitoring system. The pharmacokinetics of tacrolimus is variable among individuals. The safety range between effective dose and toxic dose is narrow, and it is easy to be affected by genetic polymorphism and other combined drugs. Therefore, it is very important to monitor the blood concentration of tacrolimus in clinic. After taking 3 days (taking the medicine 6 times), the blood concentration of tacrolimus can reach steady state (according to the drug specification). In clinical, the blood concentration of tacrolimus is usually measured after a week of medication. The commonly used methods is enzyme immunoassay enhancement (EMIT). The recommended concentration of TAC was 5-10 ng/ml. In clinical practice, most experts believe that the blood concentration of tacrolimus in 4ng/ml has also achieved good therapeutic effect(References19, 21).

C2: The authors informed the same tacrolimus doses were given by weight for both children and adults and BMI was one of the potential covariates in the regression analyses. The authors should be careful. Children typically require higher tacrolimus dosages (mg/kg) than adult patients, most likely reflecting the higher mean total body clearance and volume of distribution in children. I think the authors may want to at least report percentage of children (and any dose changes?), or reanalyze the children group separately from the adult group, or compare and address pharmacokinetic parameters such as trough concentrations of tacrolimus in both children and adults whether they are similar.

R2: We thank the reviewer for this question. First, we explain why the same tacrolimus doses were given by weight for both children and adults. Shanshan Liu et al. (References19) had done a forward-looking cohort study, and the enrolled patients were older than 18 years. tacrolimus was initially administered at 0.05 mg/kg daily, divided into 2 doses over 12-hour intervals in the treatment of Idiopathic membranous nephropathy (IMN). So, according to this literature, the dose of tacrolimus was 0.05 mg/kg/d for adults in the present study. What’s more, Lavjay et al.
(References 20) points out that the initial tacrolimus dose was 0.05 - 0.2 mg/kg/d for children. According to this range, we chose the minimum dose of tacrolimus for children in the study, namely 0.05 mg/kg/d. Finally, we selected 0.05 mg/kg/d as the initial dose of tacrolimus for both adults and children after a comprehensive consideration.

Second, we should report the percentage of children in this study. There were 10 children and 90 adults in this study. Among the children, 1 children were 6 years old and the rest were between 15 - 18 years old. The average age is 15.90 ± 3.54 years, and the average weight is 49.4 ± 11.7kg. It can be found in Results section, line 31-37, page 15.

(2) Results of logistic regression analysis and clinical efficacy evaluation

C3: Table 4, can the authors please include the results for all covariates (CYP3A4 and CYP3A5 genotypes, and CCB)? The significant results were already reported but I think it is important to show statistical parameters of all covariates. This study found no significant effect of the CYP3A5 genotype on tacrolimus clinical efficacy. The authors mentioned the study limitation of small sample size and the guideline (reference 18) in renal transplantation not nephrotic syndrome, which are somewhat reasonable. Nonetheless, the full table report of the regression analysis table is recommended, particularly when this CYP3A5 genotype was reported in previously published studies with regard to its significant effect of the genotype on tacrolimus concentration.

R3: We thank you for reminding us very much. The statistical method we used was forward likelihood ratio step by step (Forward:LR) before the article is repaired. Now, we use the enter method, that is, the full model method, and all variables are entered into the equation to screen meaningful variables. We found that CYP3A4, CYP3A5 and CCB does not directly affect the clinical efficacy of TAC (P = 0.125, P = 0.397, P = 0.357, respectively). We have put the results for all covariates in Table 5 according to your opinion. The details can be seen in Results section, line 1-27, page 19.
C4: The authors may consider providing a report of the genotype effects in multiple time points in the duration of 3 months. The clinical efficacy of tacrolimus can change owing to tacrolimus concentration that is associated with one or more gene polymorphisms in this study. Including an analysis at multiple time point or at least address the time points that the clinical efficacy data were collected is strongly suggested.

R4: We thank the reviewer for pointing out our deficiency very much. In this study, the patients were followed up for 3 months. We determined the relevant indicators every month, such as albumin, urine protein, renal function, and so on. The trend of the first two months is similar and there is no marked significance. The indicators of third months have been significantly improved compared with the previous 2 months. So we chose the indicators of the third months to judge the effect of clinical treatment.

C5: Clinical efficacy was classified into 4 categories (complete remission, partial remission, no remission, and recrudescence). There was no information mentioned anywhere in the study of how many patients were in each category. I think this information would be very helpful when interpreting the results of regression analysis.

R5: We appreciate the reviewer’s point very much. There were 35 patients (35%) with complete remission, 43 patients (43%) with partial remission, 22 patients (22%) with no remission, and no patients with recurrence. The overall rate of effective treatment was 78% (78/100). We have described the number of patients in each category according to your opinion. The details can be seen in Results section, line 23-31, page 16.

(3) Writing and conclusion

C6: Repetitive summary of genetic polymorphisms were written in the Background and Discussion sections. For example, Discussion section 'Relationship between ABCB1 gene polymorphism and clinical efficacy of tacrolimus', line 39, 'It can result in 839 amino
acid…protein expression [17]' is almost the same sentence as that of Background section page 2 line 56.

R6: We thank the reviewer for this point. We have made some modifications in the two parts. The details can be seen in Background section, page 11 and in Discussion sections, line 48-54, page 21.

C7: Abstract line 22, the word 'Results' should be moved to line 24.

R7: We thank you for reminding us very much. We have made the corresponding modification according to your opinion. The details can be seen Abstract sections, line 28, page 9.

C8: Wrong spelling of 'Females' in Table 1.

R8: We thank you for reminding us very much. We've made a change in Table 1.

C9: Discussion section page 4 line 56-59, '…the effect of TAC (tacrolimus) in patients with 2677G>T/A mutation genotype is better (reference 13 and 18). In reference 13, Kurzawski et al 2014 reported that CYP3A5/A4 not ABCB1 polymorphisms affect tacrolimus trough concentrations. This result is opposite from what the authors found.

R9: We thank you for reminding us very much. Because of our negligence, we have made such a low error, and we have made some adjustments in the article. We've made a change in Discussion sections, line 34, page 22.

C10: Two repetitive sentences are detected in Conclusion section "Detecting the related genes…improve the curative effect." While I agree that individual drug guided by 'gene monitoring' can add more values in clinical work, I cannot agree with the authors based on results of this study that it is safer, more effective, more economical, and more reasonable.
R10: We thank you for this point very much. I have deleted the repeating part and made the corresponding changes. We agree with you and we have made the following modifications. To sum up, the results of this study reveal that the gene mutations of CYP3A4 and CYP3A5 and CCB do not directly affect the clinical efficacy of TAC. However, ABCB1 C1236T, ABCB1 G2677T/A genotype and BMI are the factors influencing the clinical efficacy of TAC in treating patients with NS. Therefore, detecting the related genes is helpful to optimize the drug administration program and improve the curative effect and should be encouraged in clinical work. The details can be seen in Conclusions section, line 3-20, page 24.

Response to Chun Shing Kwok (Reviewer 2):

C1: The title should include something about tacrolimus treatment.

R1: We really appreciate the reviewer’s pertinent comments very much. The revised title is “Effect of CYP3 A4, CYP3 A5 and ABCB1 gene polymorphisms on the clinical efficacy of tacrolimus in the treatment of nephrotic syndrome” in page 8.

C2: The abstract methods should include more in the methods to describe clearly the population (patients with nephrotic syndrome treated with tacrolimus, the exposure (genotype differences) and outcomes (response to treatment). The method of analysis (multivariable logistic regression) should be described.

R2: We thank you for reminding us very much. We made the following modifications. 100 patients with nephrotic syndrome were treated with tacrolimus and prednisone and followed up for 3 months. Genotype differences (CYP3 A4*1G, CYP3 A5*3, ABCB1 1236C>T and ABCB1 2677G>T/A) were detected by Sanger sequencing. The clinical efficacy was evaluated according to the 24 hour urinary protein quantitation, albumin, renal function and the degree of edema. Multivariable logistic regression was used to analyze the effect of gene polymorphism on the clinical efficacy of tacrolimus. The details can be seen in Abstract section, line 14-28, page 9.
C3: The abstract results should state the number of patients and number of patients with each allele type (not just percentages).

R3: we thank the reviewer for this constructive suggestion very much. For CYP3A4, there were 56, 42, and 2 patients with *1/*1, *1/*1G and *1G/*1G genotype, respectively. For CYP3A5, there were 8, 36 and 56 cases with *1/*1, *1/*3 and *3/*3 genotype, respectively. For ABCB1 C1236T, there were 10, 44, and 46 cases with 1236CC, 1236CT and 1236TT genotype, respectively. For ABCB1 G2677T/A, there were 13, 57, and 30 patients with GG genotype, GT/GA genotype and TT/AA/TA genotype, respectively. The mutant allele frequencies of CYP3A4*1G, CYP3A5*3, ABCB1 C1236T and ABCB1 G2677T/A were 23%, 74%, 68% and 58.5%, respectively. The details can be seen in Abstract section, line 28, page 9.

C4: The abstract results should also state not just odds ratios and p-values but 95% confidence intervals.

R4: We thank the reviewer for this reminding very much. According to your request, We added 95% confidence intervals to the abstract results. For ABCB1 C1236T, TT genotype can increase the effectiveness 12.085 times compared with CC and CT genotype (P = 0.018, OR = 12.085, 95%CI 1.535-95.148). For ABCB1 G2677T/A, the clinical efficacy of patients with mutant genotype was 8.683 times than that of wild-type and heterozygous patients (P = 0.042, OR =8.683, 95%CI 1.080-69.819). Overweight patients can improve the clinical efficacy by 15.838 times (P = 0.020, OR = 15.838, 95%CI1.550-161.788). We've made a change in in Abstract section, line 1-20, page 10.

C5: The abstract results contain repetition as both the odds ratio/p-values and text description. I would keep the text description and put the odds ratios, 95% confidence intervals and p-values in parentheses after each numerical result.

R5: We really appreciate the reviewer’s point. My article has deleted repetition as both the odds ratio/p-values and text description and kept the text description and put the odds ratios, 95% confidence intervals and p-values in parentheses after each numerical result. For ABCB1
C1236T, TT genotype can increase the effectiveness 12.085 times compared with CC and CT genotype (P = 0.018, OR = 12.085, 95% CI 1.535-95.148). For ABCB1 G2677T/A, the clinical efficacy of patients with mutant genotype was 8.683 times than that of wild-type and heterozygous patients (P = 0.042, OR = 8.683, 95% CI 1.080-69.819). Overweight patients can improve the clinical efficacy by 15.838 times (P = 0.020, OR = 15.838, 95% CI 1.550-161.788). The details can be seen in Abstract section, line 1-20, page 10.

C6: Tacrolimus is not a novel agent as it has been around for a long time but its application in nephrotic syndrome.

R6: We thank you for reminding us very much. Tacrolimus (TAC), an inhibitor of calcium phosphatase, also named FK506, is used in the treatment of nephrotic syndrome in recent years. We've made a change in Background section, line 38, page 10.

C7: A key issue with the study is that nephrotic syndrome is relatively non-specific term. The cause of nephrotic syndrome is important whether it was mainly minimal change disease, focal segmental glomerulosclerosis, membranous, etc. This should be specified in the study.

R7: We thank the reviewer for this point very much. According to your suggestion, we described the cause of nephrotic syndrome, specified the number and proportion of each cause, and also made an analysis of the outcome of every disease (Table 3). Nephrotic syndrome can be divided into a variety of different pathological types. The population of this study included membranous nephropathy (MN) (36 cases, 36%), mesangial proliferative glomerulonephritis (MsPGN) (11 cases, 11%), minimal change nephropathy (MCN) (15 cases, 15%), focal segmental glomerulosclerosis (FSGS) (5 cases, 5%), systemic lupus erythematosus (SLE) (16 cases, 16%) and Henoch - Schonlein purpura nephritis (HSPN) (7 cases, 7%). The details can be seen in Table 3 and Results section, page 16.
C8: First paragraph of Background can be shortened. It should explain why nephrotic syndrome is important. It should explain that tacrolimus can be used to treat it. It should explain the relevance of genetic polymorphisms on efficacy (Is this the first study to look at this?). The second paragraph should explain what is known in the current literature about gene polymorphisms and response to tacrolimus treatment in nephrotic syndrome and it currently talks about transplant patients.

R8: We thank the reviewer for point very much. I have been modified according to relevant requirements raised by you and the other reviewer. The detailed responses are listed in Background section, page 10.

C9: Please explain how nephrotic syndrome aetiology was determined. Was renal biopsy performed?

R9: We thank the reviewer for this point very much. Nephrotic syndrome aetiology was determined by renal biopsy. We’ve made a change in Methods section, line 20, page 12.

C10: Please explain how the sample size was determined. A power calculation should be performed. Considering the large confidence intervals the study looks underpowered.

R10: We appreciate the reviewer’s point very much. There are 6 variables in this study and they are CYP3A4, CYP3A5, ABCB1 C1236T, ABCB1 G2677T/A, CCB and BMI, respectively. Because the number of samples is generally 10 times of the number of independent variables, there should be at least 60 examples in theory. In our current study, 100 patients were included, which meets the requirement.

C11: Please state in the methods the approvals that were sought for the conduct of the study and the ethical approval. Also state how informed consent was obtained.
R11: We thank the reviewer for this point. Firstly, we made an application to the Medical Ethics Committee of the 88th Hospital of PLA for the conduct of the study and the ethical approval. The hospital ethics committees think that the design and program of the experimental take into the security and fairness principles in this study. The study will not cause harm and risk to participants and the rights and interests of subjects are adequately protected. The participants were informed about the details of the study, and signed their names to participate in this experiment. Participants under 18 years of age are signed by their parents on their behalf. They were totally voluntary and could withdraw from the study at any time. We added the ethical consideration in Methods section, line 34-59, page 12.

C12: Please explain how the statistical analysis as performed and what factors were adjusted for in the logistic regression.

R12: We thank the reviewer for this point very much. The statistical method is multivariable logistic regression. The covariates include CYP3A4, CYP3A5, ABCB1 C1236T, ABCB1 G2677T/A, CCB and BMI. The assignment description is shown in Table 3. Clinical efficacy was used as dependent variable.

Evaluation criteria are divided into complete remission (CR), partial remission (PR), no remission (NR) and recrudescence[4,9]. (1) CR: Urine protein < 0.3 g/d, ALB > 35 g/L, edema disappeared and stable renal function. (2) PR: Urine protein 0.3 ~ 3.5 g/d or decreased > 50%, ALB > 30 g/L, edema disappeared and stable renal function. (3) NR: Urine protein > 3.5 g/d and ALB < 30 g/L with edema or deterioration of renal function. (4) Recrudescence: When reaching CR or PR, proteinuria > 3.5 g/d and ALB < 30 g/L, accompanied by edema or deterioration of renal function appear again. CR + PR are considered to be effective and NR + recurrence are treated as ineffective. Logistic regression analysis was used to compare the effects of CYP3 A4, CYP3 A5, ABCB1 C1236T, ABCB1 G2677T/A, CCB and BMI on the clinical efficacy of patients with nephrotic syndrome. The details can be seen in Methods section, line 46, page 13.
C13: What is the definition of effective treatment? This should be stated in the methods. The evaluation of safety should also be stated in the methods.

R13: We thank the reviewer for the question. First, the effective treatment include complete remission (CR) and partial remission (PR). CR was defined as urine protein < 0.3 g/d, ALB > 35 g/L, edema disappeared and stable renal function. Whereas PR was defined as urine protein 0.3 ~ 3.5 g/d or decreased > 50%, ALB > 30 g/L, edema disappeared and stable renal function. Second, the safety of the tacrolimus was evaluated in the study. Referring to the relevant literatures, the main side effects of tacrolimus were closely observed in the present study, including abnormality of glucose tolerance, infection, renal toxicity, gastrointestinal adverse reactions, elevated blood pressure and liver toxicity, etc. Once the uncontrollable adverse reactions are found, we should stop tacrolimus immediately. The detailed responses are listed in Methods section, line 20-34 and line 46-53, page 13.

C14: The results should not contain explanation of what was done. That should be in the methods.

R14: We really appreciate the reviewer’s point. We have been modified according to relevant requirements in Methods section, line 7-22, page 15.

C15: The discussion first paragraph should state what the main findings of the study are and the clinical implications. The second paragraph should state how it compares to existing literature. The next paragraph should talk about the mechanisms of the findings. Other interesting findings can be described in other paragraphs like mutation frequency differing from general population, how does cause of nephrotic syndrome influence clinical efficacy and clinical efficacy of tacrolimus in other settings. There should also be a paragraph about strengths and limitations about the study. The study should finally have a conclusion paragraph.

R15: We thank the reviewer for this point very much. We have made the corresponding modification in the Discussion section.
C16: I think a native English speaker should go through the text. Words like for researches are generally not used.

R16: We thank you for your suggestion. A native English speaker has help me to revise the article.