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# Development of a Formula to Correct Particle-Enhanced Turbidimetric Inhibition Immunoassay Values so That it More Precisely Reflects High-Performance Liquid Chromatography Values for Mycophenolic Acid

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**Background.** Mycophenolic acid (MPA) concentration measured by homogeneous particle-enhanced turbidimetric inhibition immunoassay (PETINA) may be overestimated due to its cross-reactivity with pharmacologically inactive MPA glucuronide (MPAG), as well as other minor metabolites, accumulated with renal function impairment or co-administered cyclosporine A. In contrast, high-performance liquid chromatography (HPLC) is precise because it can exclude the cross-reactivity. In this study, we assumed HPLC values for MPA (HPLC-MPA) as a reference and aimed to develop a formula correcting PETINA values for MPA (PETINA-MPA) to more precisely reflect HPLC-MPA. **Methods.** MPA trough concentrations were measured both by HPLC-UV and PETINA in 39 samples issued from 39 solid-organ transplant recipients. MPAG concentrations were also measured using HPLC UV assay. We determined the impacts of renal function and coadministered calcineurin inhibitor on concentrations of MPA and MPAG measured by HPLC. Then, we evaluated the difference between PETINA-MPA and HPLC-MPA. Finally, we develop a formula to reflect HPLC-MPA by using multilinear regression analysis. **Results.** MPAG concentration was negatively correlated with estimated glomerular filtration rate (eGFR) ($R^2$ = 0.376, $P$ < 0.001), although MPA was not correlated with eGFR. There were no significant differences in MPA or MPAG concentrations per dose between the patients who were co-administered tacrolimus versus cyclosporine A. Finally, we developed the formulas to reflect HPLC-MPA: Formula 1: Estimated MPA concentration = 0.048 + 0.798 × PETINA-MPA Formula 2: Estimated MPA concentration = – 0.059 + 0.800 × PETINA-MPA + 0.002 × eGFR However, there was no significant improvement in the coefficient of determination with addition of eGFR in the formula, suggesting that HPLC-MPA can be well predicted by only 1 variable, PETINA-MPA. **Conclusions.** This study developed a formula so that PETINA-MPA can be corrected to more precisely reflect HPLC-MPA.

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Mycophenolate mofetil (MMF) is an immunosuppressive drug widely used in patients after solid-organ transplantation.[1] MMF is rapidly hydrolyzed into the pharmacologically active metabolite mycophenolic acid (MPA). MPA is further metabolized primarily to the pharmacologically inactive MPA-glucuronide (MPAG) as well as the other minor metabolites, acyl glucuronide and phenolic glucoside.[2-4] MPAG is then excreted into the bile and hydrolyzed into MPA. It has been reported that enterohepatic recycling contributes approximately 10% to 60% to MPA exposure.[5] Moreover, MPAG is usually present at a 20- to 100-fold higher concentration than that observed for MPA. In plasma,

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*K.N. and T.Y. participated in the measuring of MPA and MPAG. K.Y., H.S., and K.K. gave advice on the research.*

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MPA as well as MPAG bind to albumin, with intracellular MPA, which is only 3% of total MPA and responsible for the immunosuppressive effect.[2,6]

Pharmacokinetics of MPA is affected by a number of variables, including sex, renal and hepatic function, time after organ transplantation, and coadministered medication.[7,8] The complex pharmacokinetics of MPA has been reported to be responsible for the intrapatient and interpatient variabilities of the concentrations that are noted for MPA. Thus, to obtain optimal immunosuppressive effects in solid organ transplantation, stem cell transplantation and autoimmune disorders, precise therapeutic drug monitoring of MPA concentration is required. High-performance liquid chromatography (HPLC) methods are routinely used to determine plasma MPA.[9,10] Moreover, immunological methods, such as the enzyme-multiplied immunoassay technique and homogeneous particle-enhanced turbidimetric inhibition immunoassay (PETINA), have been widely used because of their simplicity of use.[11-14] These assays are automatized to measure MPA concentrations based on an immunoassay. However, there is a concern about cross-reactions with MPA metabolites in these immunoassays, as Shipkova et al[12,13] report that enzyme-multiplied immunoassay technique values were overestimated compared with those determined by HPLC.

Renal function impairment may lead to an increased MPAG concentration because MPAG is primarily eliminated by renal excretion.[15-18] A previous study reported that increases in MPAG were followed by decreases in total MPA in patients and increases in total MPAG who were coadministered with cyclosporine A (CsA).[14] Because PETINA can cross-react with MPA metabolites, such as MPAG, this can lead to an overestimation of the true MPA value.

Based on these previous results, the aim of our current study were (i) to evaluate the influence of estimated glomerular filtration rate (eGFR) and calcineurin inhibitor (CNI) coadministration on MPAG values as measured by HPLC (HPLC-MPA) and (ii) from these observations, to develop a formula to correct PETINA values for MPA (PETINA-MPA) to reflect more precisely the concentrations of HPLC-MPA.

## MATERIALS AND METHODS

### Patients

This retrospective study enrolled 39 adult kidney or liver transplant patients receiving MMF in combination with tacrolimus (TAC) or CsA between December 2012 and May 2014. MMF dosage was adjusted according to the targeted MPA concentration in the immunosuppression protocol after each organ transplant, the target trough were 1 to 3 μg/mL in both organ transplant. Blood samples were collected from the recipients 12 hours after dosing MMF (ie, just before next dosing) as a trough level monthly. All determinations of MPA or MPAG concentrations and all of the biochemical examinations were performed at the same points. The study was conducted with 39 blood samples for measurement of MPA and MPAG, one from each patient. All patient information and laboratory data, such as aspartate aminotransferase and alanine aminotransferase, eGFR (calculated using the equation established for the Japanese population[19]), and serum creatinine (SCr), were retrospectively obtained from the medical records. All patients had stable graft function and no allograft rejection confirmed by graft biopsy. The present study was carried out in accordance with the guidelines for the care of humans in experimental studies, with the study protocol approved by the Ethics Committee of the Hokkaido University Hospital (study protocol no. 014-0175). Informed consent was obtained from all subjects.

### Measurement of Plasma MPA and MPAG

MPA and MPAG concentrations in the patient plasma samples were measured by HPLC UV assay as previously described with modifications.[20,21] HPLC separations of MPA and MPAG were performed independently. Briefly, the separation was achieved using an ERC ODS-1161 column (6 × 100 mm; Yokohamarika Co., Yokohama Japan). The mobile phase consisted of a 40:60 ratio of acetonitrile and phosphoric acid (60 mM) for MPA assay and a 23:77 ratio for MPAG assay. The internal standard used for MPA assay was naproxen, whereas β-naphthol was used for MPAG assay. The column was maintained at 55°C. HPLC analysis was performed using a Shimadzu LC-10ADLP system (Shimadzu, Kyoto, Japan). The compounds were quantified based on the peak-area ratio, with MPA assay using an absorbance of 215 nm and MPAG assay using an absorbance of 256 nm. Calibrations were performed through the use of standards during each run.

In addition to HPLC assay, MPA concentrations in the same samples were also measured by PETINA. PETINA assay was performed using the Flex reagent cartridge MPAT, which is based on the immunoassay of the Dimension Xpand Plus system (Siemens Healthcare Diagnostics, Tarrytown, NY). All analyses were performed in accordance with the manufacturer's instructions.

### Analysis Strategies

Using the above measured values, the following evaluations were performed.

(1) The influences of interval between transplantation and blood collection on the concentrations of MPA and MPAG measured by HPLC were analyzed.

(2) The correlation between the MMF dosage or renal function (eGFR) and the plasma trough concentration of MPA or MPAG was analyzed.

(3) The effect of coadministered CNI (TAC or CsA) on MPA and MPAG trough concentrations in the plasma was determined.

(4) HPLC-MPA and PETINA-MPA were compared.

(5) Finally, multilinear regression was used to predict HPLC-MPA. To see whether there is a consistent pattern to the direction of bias, Bland-Altman analyses were performed between PETINA-MPA or estimated MPA and HPLC-MPA. Corrected concentration values obtained with the formula will be referred to as "estimated values" in the article.

### Statistics

The Wilcoxon signed rank test was performed to compare the difference between HPLC-MPA and PETINA-MPA. The Mann-Whitney *U* test was performed to compare the means for discrete covariates. Linear correlation analysis was used to assess correlations between each numeric variable. A univariate and simultaneous multivariate linear regression analyses were performed to develop the formula. Values of *P* less

than 0.05 were considered significant. Data analyses were performed with SPSS version 22 (SPSS Inc., Chicago, IL).

## RESULTS

### Correlation Between MMF Dosage or eGFR and MPA or MPAG Trough Concentrations Based on HPLC

Table 1 summarizes the clinical characteristics of the patients. Thirteen patients received kidney transplants and 26 received liver transplants, respectively. Median age at evaluation and at transplantation was 51 and 49 years, respectively. Median Scr and eGFR was 1.04 mg/dL and 52.5 mL/min per 1.73 $m^2$, respectively. Median MPA and MPAG per MMF dose was 0.09 and 2.27 μg/mL per mg/kg, respectively. In this study cohort, the period of sample collection after transplantation varied from 0.1 to 182.0 months. The linear correlation test demonstrated that the time after transplantation was not significantly correlated with MPA ($P = 0.07$) or MPAG ($P = 0.81$) concentration (Figure 1).

As shown in Figure 2A, there was no significant correlation between MPA plasma concentrations and MMF dosage per body weight (BW) (mg/kg per day) ($R^2 = 0.066$, $P = 0.76$). In contrast, MPAG plasma concentrations were positively correlated with MMF dosage per BW (mg/kg per day) ($R^2 = 0.248$, $P < 0.001$). Moreover, although MPAG concentration was negatively correlated with eGFR ($R^2 = 0.376$, $P < 0.001$), there was no correlation between MPA concentrations and eGFR ($R^2 = 0.081$, $P = 0.10$) (Figure 2B).

### Comparison of the Impact of Coadministered CNI on Plasma Trough Concentrations of MPA and MPAG Based on HPLC

Table 2 shows comparison of characteristics between the patients coadministered with TAC and CsA. There were more patients who received kidney in the CsA group (kidney recipients were 87.5% in CsA group and 19.3% in TAC group, $P = 0.002$), the time of blood collection after organ transplant was significantly longer in TAC group (41.2 months in TAC group and 4.8 months in CsA group, $P = 0.044$), and BW

**TABLE 1.**

**Clinical characteristics**

| | All, N = 39[a] |
|---|---|
| Co-administered CNI (TAC:CsA) | 31:8 |
| Transplantation (kidney:liver) | 13:26 |
| Age at evaluation, y | 51 (20-70) |
| Age at transplantation, y | 49 (5-70) |
| Time after transplantation, mo | 25.6 (0.1-182.0) |
| Sex (male:female) | 19:20 |
| BW, kg | 54.8 (38.1-89.0) |
| MMF dose, mg/d | 1000 (500-3500) |
| MMF dose per BW, mg/kg per day | 18.0 (8.1-70.7) |
| SCr, mg/dL | 1.04 (0.40-2.59) |
| eGFR, mL/min per 1.73 $m^2$ | 52.5 (16.3-126.4) |
| ALT, U/L | 18 (5-81) |
| AST/ALT, U/L | 1.00 (0.57-2.50) |
| MPA per MMF dose, μg/mL per mg/kg per day | 0.09 (0.01-0.33) |
| MPAG per MMF dose, μg/mL per mg/kg per day | 2.27 (0.53-7.69) |

[a] Data are expressed as number or median (range).

ALT, alanine aminotransferase; AST, aspartate aminotransferase.

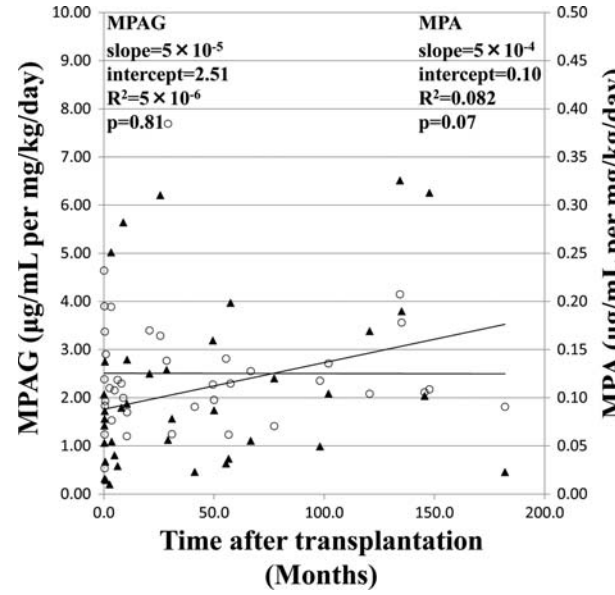


**FIGURE 1.** MPAG or MPA trough concentrations are not affected by the time after transplantation. Correlation between the time after transplantation and MPAG or MPA. Results are expressed as μg/mL per mg/kg per day. Open circles represent MPAG and solid triangles represent MPA.

was significantly higher in TAC group (55.3 kg in TAC group and 49.0 kg in CsA group, $P = 0.010$). The other characteristics were comparable between TAC and CsA groups. There were also no significant differences for the concentration of MPA per MMF dose (TAC; 0.09 μg/mL per mg/kg per day vs CsA; 0.08 μg/mL per mg/kg per day, $P = 0.825$) and MPAG per MMF dose (TAC; 2.20 μg/mL per mg/kg per day vs CSA; 2.84 μg/mL per mg/kg per day, $P = 0.184$) between the 2 groups. Although there was a trend for the patients coadministered CsA to have higher MPAG concentrations than those who were coadministered TAC, this difference did not reach significance.

### Comparison of Concentrations Between HPLC-MPA and PETINA-MPA

The median and ranges of HPLC-MPA and PETINA-MPA was 0.09 (0.01-0.33) μg/mL per mg/kg per day and 0.11 (0.01-0.42) μg/mL per mg/kg per day, respectively. PETINA-MPA was significantly higher than HPLC-MPA ($P < 0.001$).

### Development of a Formula to Correct the PETINA-MPA Using the Clinical Factor So That the Values More Precisely Reflect the HPLC-MPA

The results of the univariate and multivariate linear regression analyses are listed in Tables 3 and 4. Formulas used PETINA-MPA with or without eGFR as the independent predictors. The multiple regression equations for the formulae are as follow:

Formula 1: Estimated MPA concentration = 0.048 + 0.798 × PETINA-MPA

Formula 2: Estimated MPA concentration = – 0.059 + 0.800 × PETINA-MPA + 0.002 × eGFR

Both estimated MPA with formulae 1 (Figure 3A) and 2 (Figure 3B) demonstrated a good correlation with HPLC-MPA. There was very small improvement in coefficient of determination in formula 2 ($R^2 = 0.984$) compared with

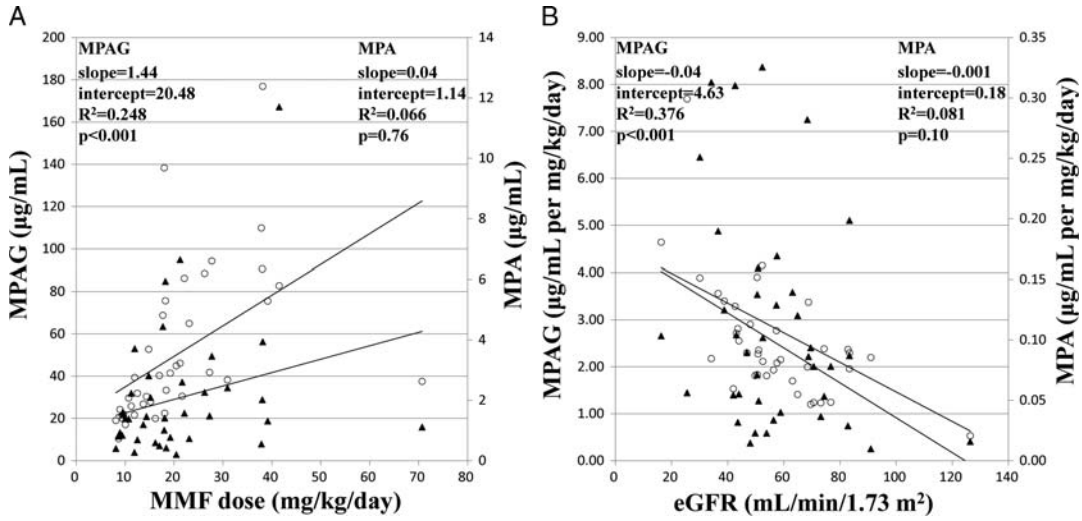


**FIGURE 2.** MPAG concentrations are affected by renal dysfunction while MPA concentrations are not. A, Correlation between the MMF dose and MPAG or MPA concentration. Results are expressed as μg/mL. B, Correlation between eGFR and MPAG or MPA. Results are expressed as μg/mL per mg/kg per day. Open circle represents MPAG while solid triangle represents MPA.

formula 1 ($R^2$ = 0.981). Moreover, in Bland-Altman analysis, there was no significant pattern to the direction of bias between the estimated with formula 1 (Figure 3C) or formula 2 (Figure 3D) and HPLC values.

## DISCUSSION

We first analyzed the relationship between the time after transplantation and their concentrations. There was no correlation between the time after transplantation and MPA or MPAG concentration (Figure 1). In this study, we enrolled patients whose time from transplantation to sample collection were highly different from one another.

This study demonstrated that there was no correlation between MPA concentrations and eGFR, while MPAG concentration was negatively correlated with eGFR (Figure 2B).

**TABLE 2.**

**Comparison of characteristics between the patient groups receiving co-administration of TAC and CsA**

| | TAC n = 31[a] | CsA n = 8[a] | *P* |
|---|---|---|---|
| Transplantation (kidney:liver) | 6:25 | 7:1 | 0.002 |
| Age at evaluation, y | 52 (20-70) | 40 (23–64) | 0.328 |
| Age at transplantation, y | 49 (5-70) | 40 (23-64) | 0.621 |
| Time after transplantation, mo | 41.2 (0.1-182.0) | 4.8 (0.2-29.1) | 0.044 |
| Sex (male:female) | 16:15 | 3:5 | 0.550 |
| BW, kg | 55.3 (41.8-89.0) | 49.0 (38.1-66.1) | 0.010 |
| MMF dose, mg/d | 1000 (500-3500) | 1000 (500-2500) | 0.597 |
| MMF dose per BW, mg/kg per day | 17.0 (8.1-70.7) | 24.2 (10.0-41.5) | 0.142 |
| SCr, mg/dL | 1.05 (0.40-2.59) | 0.95 (0.77-2.21) | 0.798 |
| eGFR, mL/min per 1.73 m$^2$ | 52.3 (16.3-126.4) | 56.8 (25.5-66.8) | 0.986 |
| ALT, U/L | 19 (6-81) | 15 (5-32) | 0.184 |
| AST/ALT, U/L | 1.00 (0.57-2.50) | 1.10 (0.79-2.20) | 0.670 |
| MPA per MMF dose, μg/mL per mg/kg per day | 0.09 (0.01-0.33) | 0.08 (0.01-0.28) | 0.825 |
| MPAG per MMF dose, μg/mL per mg/kg per day | 2.20 (0.53-4.64) | 2.84 (1.70-7.69) | 0.184 |

[a] Data are expressed as number or median (range).

These findings indicate that MPAG accumulated in conjunction with the worsening of the renal function, although MPA concentration was not affected by renal function. Thus, it is difficult to predict MPA concentration based on the MMF dosage or renal function, which highlights the importance of the precise monitoring of MPA concentrations.

MPAG concentrations can be affected by a variety of clinical factors. Because a portion of MPAG can be detected as MPA due to a cross-reaction in PETINA, this makes it more difficult to monitor MPA values when using PETINA versus HPLC. This overestimation has been well documented in previous studies.[12,13] The current study attempted to evaluate how the renal function and the coadministration of CNI affected PETINA-MPA as compared with HPLC-MPA. The primary aim of our study was to develop a formula that can correct PETINA-MPA to more precisely reflect HPLC-MPA.

The linear correlation test suggested that eGFR was not significantly correlated with HPLC-MPA, whereas PETINA-MPA was correlated with HPLC-MPA in univariate analysis. The fact that the improvement in coefficient of determination from formula 2 ($R^2$ = 0.984) to formula 1 ($R^2$ = 0.981) was very small indicated that eGFR in the formula have no clinical influence on PETINA-MPA prediction. These results might suggest that MPA accumulated along with MPAG dependent on the decrease of eGFR. In other words, MPA might accumulate according to the impairment of renal function, although

**TABLE 3.**

**Univariate analysis between HPLC-MPA and PETINA-MPA or eGFR**

| Independent variables | Standardized regression coefficient | 95 % confidence intervals | *P* |
|---|---|---|---|
| PETINA-MPA | 0.992 | 0.764-0.831 | <0.001[a] |
| eGFR | −0.182 | −0.054-0.016 | 0.443[a] |

[a] Linear correlation tests were performed.

**TABLE 4.**

**Simultaneous multivariate linear regression model of variables associated with HPLC values for MPA**

| Independent variable | Standardized regression coefficient | *P* | 95 % confidence intervals | Residual variance | Multiple correlation coefficient | *P* |
|---|---|---|---|---|---|---|
| PETINA-MPA | 0.995 | <0.001 | 0.766-0.835 | 0.077 | 0.992 | <0.001 |
| eGFR | 0.017 | 0.443 | –0.003 to 0.006 | | | |

our data did not show significant correlation between eGFR and HPLC-MPA, which is similar to previously published data.[17] A small sample size in the study may have resulted in no correlation between eGFR and HPLC-MPA. These results indicate that HPLC-MPA can be well predicted from PETINA-MPA, without taking into account eGFR. To best of our knowledge, this is the first time a formula has been able to correct and change the PETINA-MPA to more precisely reflect the HPLC-MPA.

Kamińska et al[17] examined the differences between patients with renal impairment (creatinine clearance, < 60 mL/min) and patients with a normal renal function (creatinine clearance, > 60 mL/min) and reported finding a significantly increased area under the curve and predose MPAG for the renal impaired group. However, they divided their patients into 2 groups with a single cutoff value of renal function. Therefore, an aim of our current study was to more precisely evaluate the relationship between the renal function and MPAG concentration. In contrast to this previous study, we used eGFR to precisely evaluate the relationship between MPAG concentration and the renal function. In line with the previous report, we did find that MPAG concentration was negatively correlated with eGFR ($R^2$ = 0.376, $P$ < 0.001). In contrast, we also found that MPA concentration was not correlated with eGFR. These results indicate that while the renal function affects MPAG concentration, it does not have an effect on MPA. Other studies have shown a negative association between MPA and the renal function due to an accumulation of both MPA and MPAG.[22,23] Although the association between the accumulation of MPA and changes in the renal function remains controversial, it was obvious in our current study that MPAG accumulated in conjunction with a worsening of the renal function.

A previous study reported that MPAG was increased in patients who were coadministered CsA versus TAC.[16] This can be explained by the fact that CsA inhibits the activity of the organic anion transporting polypeptides OATP1B1 and OATP1B3, which then reduces the uptake of MPA reabsorption.[24-26] In contrast to the previous report, the differences for MPAG concentrations between the 2 groups did not reach significance. The reason why patients administered with CsA do not have higher MPAG compared with those

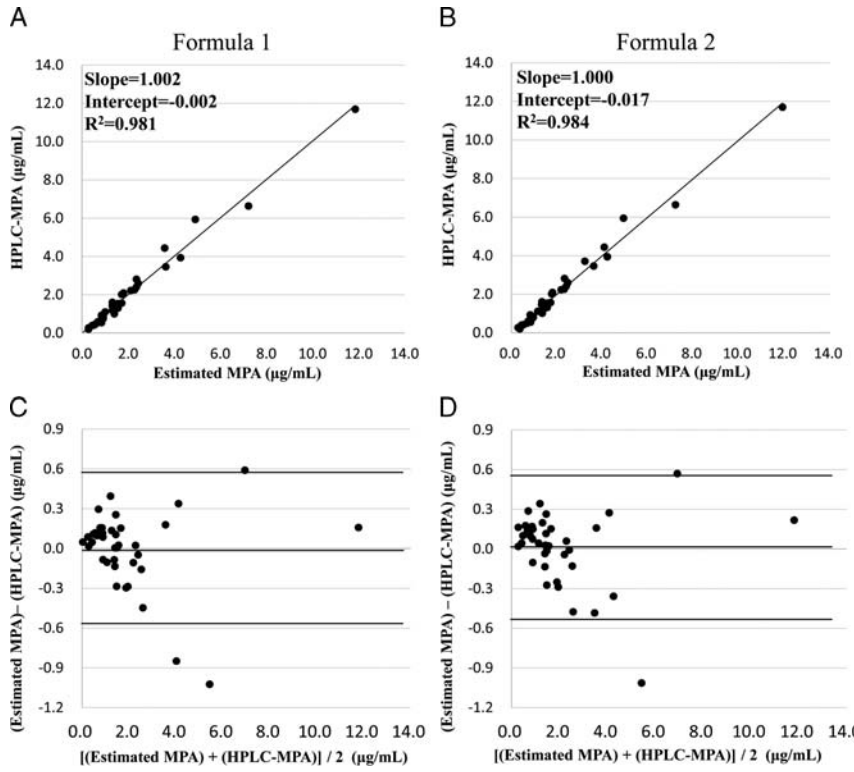


**FIGURE 3.** Development of the formulas so that PETINA values more precisely reflect HPLC values for MPA. A, Correlation between HPLC-MPA and estimated MPA values (Formula 1: Estimated MPA concentration = 0.048 + 0.798 × PETINA-MPA). B, Correlation between HPLC-MPA and estimated MPA values when taking into account eGFR (Formula 2: Estimated MPA concentration = –0.059 + 0.800 × PETINA-MPA + 0.002 × eGFR). Bland-Altman plots showed there was no consistent pattern to the direction of bias in formula 1 (C) and formula 2 (D). Solid line and dashed line represent the mean difference and the 1.96 standard deviation limits.

with TAC may be explained by the trend the patients with CsA have higher eGFR, but not significantly. The better renal function may have offset the increase of MPAG concentrations in CsA recipients. They might be associated with the differences in the patients' clinical characteristics, such as the type of transplanted organ (liver or kidney) or the very small number of patients coadministered CsA.

Our study has several limitations. The first is that it is a retrospective study with a small number of patients. We recognize that these factors increase the risk of errors. Further study enrolling lager number of patients may be needed to assess the formula. The second limitation is that MPA or MPAG concentrations may have been influenced by the CNIs concentrations and the liver function. Although we understand that these could have had an impact on our results, we could not analyze these effects due to the small sample size. There have been many other studies that have demonstrated the influence of coadministered CNI on the metabolism and the concentrations of MPA and MPAG.

In conclusion, this study evaluated the influence of eGFR and co-administered CNI on MPAG values as measured by HPLC-UV and from these observations, developed a formula to correct PETINA values to reflect more precisely the concentrations of MPA alone as measured by HLPC. Although MPAG accumulates in conjunction with the worsening of the renal function, MPA concentration is not affected by the renal function. By being able to successfully develop a formula that excluded the effects of cross-reactions with the accumulated MPA metabolites, it was possible to correct PETINA-MPA to values that more precisely reflected HPLC-MPA. This newly developed formula can help to ensure that PETINA-MPA will be as precise as HPLC-MPA.

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