Chapter

Limited Sampling Strategies to Monitoring Mycophenolic Acid Exposure in a Heterogeneous Population of Heart Transplant Recipients: A Pilot Study

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Abstract

Mycophenolate mofetil (MMF) represents a cornerstone in heart transplant (HTx) treatment. The area under the 12-hour concentration-time curve ($AUC_{0\text{-}12h}$) of mycophenolic acid (MPA) -MMF's active drug- is associated with treatment outcome. Nonetheless, therapeutic drug monitoring (TDM) of MPA $AUC_{0\text{-}12h}$ is impractical to assess in clinical practice and Limited Sampling Strategies (LSSs) represent a consolidated tool to estimate $AUC_{0\text{-}12h}$. Two LSSs were previously generated in a selected cohort of HTx recipients treated with MMF and cyclosporine (CsA). This pilot study aimed to test these LSSs in a cohort of non-selected HTx recipients treated with MMF combined with CsA or tacrolimus (TAC). Complete PK profile was performed in 40 adults HTx recipients. MPA-$AUC_{0\text{-}12h}$ was estimated by two algorithms, LSS3 and LSS4, based on 3 and 4 time-points. The evaluation was made through linear regression and Bland-Altman analyses. Both LSS3 and LSS4 tended to underestimate the value of MPA-$AUC_{0\text{-}12h}$ (mean percentage prediction error, MPE%: −6.0%; and −4.8%, respectively). Nonetheless, high correlations (*r*: 0.92 and 0.94, respectively) and goodness of fit of linear regression models (R^2: 0.84 and 0.88, respectively) emerged for both LSSs. A study with a wider and more homogenous sample size should be performed to support these results.

Keywords: heart transplantation, immunosuppressive treatment, therapeutic drug monitoring, treatment efficacy, rejection prevention

1. Introduction

Mycophenolate Mofetil MMF (CellCept; Roche, Basel, Switzerland) is a widely prescribed drug as part of maintenance immunosuppressive regimen after heart transplant (HTx) [1]. It is frequently administered in association with calcineurin inhibitors (CNIs) like cyclosporine (CsA), tacrolimus (TAC), and prednisone.

MMF is a pro-drug that, after oral administration, is rapidly hydrolyzed to its active form, mycophenolic acid (MPA), by esterases mainly in the gastrointestinal wall, blood, and liver, but also in other tissues [2]. MPA is a selective, potent and reversible inhibitor of inosine-5′-monophosphate dehydrogenase (IMPDH), a key enzyme of the *de novo* purine synthesis. This block causes the arrest of the proliferation of T- and B-cells [2]. In addition to this major immunosuppressive mechanism, MPA could cause the alteration of lymphocyte and monocyte recruitment, adhesion, and penetration. Furthermore, exposure to MPA could result in the apoptosis of activated human T lymphocytes, and the reduction of cytokine production. Moreover, it has been evidenced an antiproliferative effect on monocytes, fibroblasts, endothelial cells, mesangial cells, and smooth muscle cells. Nonetheless, MPA could inhibit mesangial matrix expansion, and alter the cytoskeletal organization [3, 4]. Some of these effects, including the reduction of important lymphocyte cell surface antigens expression, are independent from IMPDH inhibition [5, 6].

Generally, MMF is prescribed at a fixed dose, but there are several pharmacokinetic (PK) factors that could affect its efficacy. After MMF administration, MPA shows non-linear absorption kinetics, and a complex inter-patient and intra-patients PK variability [7], that could be attributable to MPA enterohepatic circulation (EHC), graft function, genetic factors, changes in plasma protein binding, and drug–drug interactions. MPA time to reach the plasma maximum concentration (T_{max}) occurs after 1–2 hours after dosage [8].

MPA presents a higher bioavailability, ranging from 80.7–94% [8]. In blood, MPA widely binds serum albumin, from 97–99% in patients with normal renal and hepatic function. Consistently, it has been evidenced that hypoalbuminemia could increase MPA free fraction *in vitro* [9] and *in vivo* [10]. In particular, an increase of 2.2-fold of MPA free fraction emerged *in vitro* when MPA albumin was reduced from 41.4 g/L to 20.7 g/L, and a further increase of 41-fold when albumin was reduced to 0.07 g/L [9]. In a study including 42 adult kidney transplant recipients, a relationship between low serum albumin and an increased MPA free fraction was reported [10]. The authors identified a threshold of 31 g/L below of which MPA free fraction was considered to be significantly elevated, suggesting that the Therapeutic Drug Monitoring (TDM) of MPA free fraction could be recommended in patients with this clinical condition [10].

MPA is mainly metabolized in liver, kidney, and gastrointestinal tract by uridine 5′-diphospho-glucuronosyltransferases (UGTs). The major metabolite of MPA, 7-O-MPA-glucuronide (MPAG), is inactive but it is present in the plasma at higher concentrations than MPA. MPAG is excreted into the urine via active tubular secretion and into the bile by multi-drug resistance protein 2 (MRP-2), and at the gastrointestinal level MPAG could be de-conjugated back to MPA by gastrointestinal flora and then reabsorbed in the colon, resulting in a secondary plasma peak between 6 and 12 hours after oral administration. This may contribute to the 30–40% of MPA exposure. Severe renal impairment, liver disease, and hypoalbuminemia could affect MPA exposure [11]. The co-administration of CsA, by inhibiting the MRP-2 mainly in the gastrointestinal tract, causes a reduction of MPA EHC, resulting in an approximately 30–40% lower MPA exposure than when MMF is administered in combination with TAC [2, 8, 12]. Furthermore, it has been evidenced that CsA administration could affect MPA Clearance (Cl) [13]. Moreover, corticosteroids may reduce the exposure of MPA by inducing the expression of UGTs [8].

For these reasons, the execution of TDM could be an effective strategy to maximize the efficacy of the treatment also reduce the risk of toxicity. Several studies have suggested the importance of MPA TDM in renal and heart transplants

recipients [14–16]. The best PK parameter correlating with the efficacy of treatment is represented by MPA's area under the plasma concentration-time curve from 0 to 12 hours (MPA $AUC_{0\text{-}12h}$) [11, 17] and several studies show that MPA plasma levels correlate to risk of rejection [18, 19]. The therapeutic range has been well determined in renal transplant recipients (30–60 mg × h/L) [20], and some authors suggested similar therapeutic thresholds on MPA-$AUC_{0\text{-}12h}$ also in HTx [21, 22].

The entire MPA $AUC_{0\text{-}12h}$ is difficult to calculate in clinical practice, due to its costly and laborious assessment. On the contrary, the single time-point measurement is the easiest for sampling, but it is not sufficiently predictive of patient outcome [20], taking also in consideration that MPA is characterized by >10-fold range variation in MPA $AUC_{0\text{-}12h}$ dose-normalized among patients undergoing heart or renal transplantation [23, 24].

Limited Sampling Strategies (LSSs) represent the most relevant assessment in solid organ transplantation for dosage individualization, that could overcome this problem [20]. LSSs are algorithm-based strategies able to predict the entire $AUC_{0\text{-}12h}$ without the necessity of sampling all the time-point concentrations after drug administration, but limiting the sampling to a reduced number of measurements, usually three time-points or even fewer. They can be developed by two main methods represented by multiple linear regression (MLR) or by with maximum a posteriori Bayesian estimation (MAP-BE).

MLR represents the simplest technique to develop an LSS. It requires statistical knowledge and the main strength of this approach is the adhesion to the sampling time.

On the other hand, developing an LSSs by maximum a posteriori Bayesian estimation (MAP-BE) is more complex because specialized PK modeling software knowledge is required.

From a methodological point of view, LSS should be generated on a cohort of patients (*training group*) and then validated in the second cohort of patients (*validation group*) to be used in clinical practice [25]. In the case of MLR LSSs, the relationship between the observed $AUC_{0\text{–}12h}$ and the estimated blood concentration-time points must be determined in the *training group* through linear regression, considering $AUC_{0\text{–}12h}$ as the dependent variable and the blood concentrations at each time point as the independent variables.

To exclude biased results, the LSS performance should be assessed in the *validation group* evaluating the mean prediction error or bias and the root mean squared prediction error or precision, as well as the median prediction error and the median absolute prediction error [26]. These same figures can be also calculated based on percentage prediction error, and expressed in percentages, to be more easily interpretable in the clinical contest as suggested by Baraldo et al. [25]. In both cases, the values of these parameters are inversely and proportionally linked to the LSS prediction. In the end, the correlation coefficient (r) and the coefficient of determination (R^2) between the estimated and the observed $AUC_{0\text{-}12h}$ must be assessed.

Recently, Baraldo et al. reviewed the state of the art of MPA LSSs in HTx recipients [25]. In the last few years, the immunosuppression therapy after HTx has changed, with the massive use of TAC compared to CsA, in combination with MMF and corticosteroids.

This pilot study aimed to test, in a heterogeneous cohort of patients treated with MMF and CSA or TAC, two algorithms of LSSs previously generated by Baraldo et al. [27, 28] in a selected cohort of HTx recipients treated with MMF and CSA. These algorithms were selected due to their good performance [28] and given the hypothesis

that the LSSs sampling time point schedule was able to determinate MPA $AUC_{0\text{-}12h}$ even when MMF was administrated combined with TAC.

If this pilot study reports positive results, the generation of new LSS in a population of HTx treated with MMF and TAC would not be required.

2. Methods

2.1 Study characteristics

This is a pilot observational, retrospective, cohort study. The study was performed at the University Hospital of Udine, in Italy. The study was approved by the Internal Review Board (I.R.B.) of the Commission for the Experimentation and Protection of Human Subjects of the Department of Medical Area of the University of Udine with the protocol number: 036/2020_IRB.

The study included 40 HTx recipients previously treated as per standard clinical practice with MMF and CsA or MMF and TAC, and prednisone, at the University Hospital of Udine, and routinely monitored for MPA quantification in the period starting from the 01st/01/2011 up to the 31st/12/2019. The patients included in the study were HTx recipients, aged 18 years old or more, and treated with MMF and either CsA or TAC and prednisone. Patients treated with immunosuppression drugs other than MMF, CsA and TAC, or with the absence of necessary information for the study in the clinical records or with the absence of informed consent for clinical, epidemiological research, training and study of pathologies were excluded from the study. All consecutive HTx recipients in the study period who met inclusion/exclusion criteria were included in the analysis.

All HTx recipients received a standard triple immunosuppressive therapy: MMF in combination with CsA or TAC and prednisone. The posology regimen of MMF varied from 1000 to 3500 mg/day, with a mean of 1785.5 mg/day (± 553.4). While the mean CsA dose was 3.0 mg/kg/day (± 1.3) p.o. in 2 divided doses, mean TAC dose was 0.1 mg/kg/day (±0.06). Patients treated with prokinetic drugs, resins or other drugs known to interfere with MPA PK, other than prednisone, were excluded from the analysis.

2.2 PK profiles of mycophenolate mofetil

A complete PK profile was available for the 40 HTx recipients included in the present analysis. Patients had been asked to take their usual morning dose of MMF after having a standard meal. Patients had not changed the therapeutic regimen for 30 days and had been at a steady state for MMF. Eight venous samples had been collected for the analysis of MPA plasma concentrations. For MPA assays, blood samples had been collected in EDTA tubes at 0 (pre-dose), 0.5, 1.25, 2, 4, 6, 8, and 12 hours after the morning dose. Separation of plasma was performed immediately in a centrifuge at 4°C. Plasma MPA concentration was measured using validated High Pressure Liquid Chromatography with UV Detector (HPLC/UV) method [23], that ensure to achieve an analytical precision and accuracy that fulfill the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) recommendations [20]. The laboratory reported the following parameters for the HPLC/UV method used for MPA quantification: limit of detection, 0.1 μg/mL; linearity, 0.1–40 μg/mL (R^2: 0.9988); intrabatch imprecision (CV), 3.15%, 1.55%, and 1.76%

at MPA plasma concentrations of 1.5, 5.0, 15.0 μg/mL, respectively; interbatch imprecision (CV), 3.41%, 3.21%, and 1.92% at MPA plasma concentrations of 1.5, 5.0, 15.0 μg/mL, respectively; overall inaccuracy (% Bias) of the procedure, ranged from 8.7% to 13.6%. MPA AUC_{0-12h} had been calculated by the linear trapezoidal rule.

2.3 Algorithms evaluation

The two algorithms used for MPA $AUC_{0\text{-}12h}$ evaluation were the followings:

$$\boldsymbol{LSS3 : MPA\ AUC_{0-12h} = 5.568 + 0.902 \times C_{1.25h} + 2.022 \times C_{2h} + 4.594 \times C_{6h}}$$

$$\boldsymbol{LSS4 : MPA\ AUC_{0-12h} = 3.800 + 1.015 \times C_{1.25h} + 1.819 \times C_{2h} + 1.566 \times C_{4h} + 3.479 \times C_{6h}}$$

According to Sheiner and Beal, to assess the bias of the LSSs, we calculated Mean Percentage Prediction Error (MPE%) and the Median Percentage Prediction Error (MPPE%) [26]. To assess precision was calculated Root Mean Squared Percentage Prediction Error (RMSE%) and the Median Absolute Percentage Prediction Error (MAPE%) [26].

The MPE%, MPPE%, RMSE% and MAPE% were calculated as follows:

Bias:

$$MPE\% = mean\left(\frac{predicted\ AUC_{0-12h} - measured\ AUC_{0-12h}}{measured\ AUC_{0-12h}} \times 100\%\right) \quad (1)$$

$$MPPE\% = median\left(\frac{predicted\ AUC_{0-12h} - measured\ AUC_{0-12h}}{measured\ AUC_{0-12h}} \times 100\%\right) \quad (2)$$

Imprecision:

$$RMSE\% = \sqrt{mean\left(\frac{predicted\ AUC_{0-12h} - measured\ AUC_{0-12h}}{measured\ AUC_{0-12h}} \times 100\%\right)^2} \quad (3)$$

$$MAPE\% = median\left(\frac{|predicted\ AUC_{0-12h} - measured\ AUC_{0-12h}|}{measured\ AUC_{0-12h}} \times 100\%\right) \quad (4)$$

For bias, we set the limit of 15%, while for imprecision the limit was set at 20%. The percentage of estimated $AUC_{0\text{-}12h}$ between 75–125% of the observed $AUC_{0\text{-}12h}$ was also calculated.

To compare our results to an already validated algorithm, we tested one other LSS equation developed in HTx by Kaczmareck et al. [29]:

$$\boldsymbol{LSS_{Kazmareck} : MPA\ AUC_{0-12h} = 1.65 \times C_{0.5h} + 4.74 \times C_{2h}}$$

2.4 Statistical consideration

Descriptive statistical analyses were conducted for all the study variables, reporting position and variability indexes (e.g., mean and standard deviation, SD) for quantitative variables. Differences between groups were evaluated using the Fisher's exact test for nominal variables and the Student's T-test for quantitative variables, and considering as statistically significant a p-value <0.05.

The two methods of LSS were validated by using both linear regression and Bland–Altman analysis, as recommended by the literature [26, 30]. All the analyses were

performed with Medcalc Software version 19.7.2 ® (Med-Calc Software, Ostend, Belgium®). Pearson's linear correlation coefficient (r) was calculated using linear regression (considering the following categories for the absolute value $|r|$: <0.50 weak correlation, 0.50–0.80 moderate correlation; >0.80 strong correlation). The determination coefficient (R^2) was also reported to assess the goodness of fit of the linear models. Bland–Altman analysis was used to evaluate the agreement between the predicted $AUC_{0–12h}$ and the measured $AUC_{0–12h}$.

3. Results

3.1 Patients characteristics

The main characteristics of study patients are reported in **Table 1**.

All patients were Caucasian and most of the analyzed patients shown normal renal and hepatic functionality. Patients treated to CsA- or TAC-based maintenance immunosuppression were comparable for most of the baseline characteristics, including age, body mass index (BMI), MMF administered dose, renal and hepatic function, except for sex, bilirubin, post transplantation time, MPA $AUC_{0\text{-}12h}$ and MPA C_0. A number of 15 acute cell rejections occurred after a median time of 8.95 months from transplantation, especially in the patients group treated with MMF-CsA than in the MMF-TAC group (87% vs. 13%, respectively). According to the International Society for Heart and Lung Transplantation, the overall rejections were classified as follows: 8 GRADE 1R (55%), 5 GRADE 2R (33%) and 2 GRADE 3R (13%) [31]. No patients reported any episodes of diarrhea.

3.2 Method results

In the whole cohort of patients, a low tendency to underestimation of the value of MPA $AUC_{0\text{-}12h}$ by both LSS3 and LSS4 emerged evaluating MPE% for mean values (−6.0% and − 4.8%, respectively) and MPPE% for median values (−3.8% and − 1.1%, respectively). The precision of LSS3 and LSS4 was acceptable, by evaluating RMSE% for mean values (19.6% and 16.2%, respectively) and MAPE% for median values (13.5% and 11.0%, respectively). The percentages of MPA $AUC_{0\text{-}12h}$ predicted by LSS3 and LSS4 within the 25% of the MPA $AUC_{0\text{-}12h}$ full value was 73% and 80%, for LSS3 and LSS4, respectively.

Linear regression and Bland–Altman analyses evidenced that both LSS3 and LSS4 methods can effectively predict the values of MPA $AUC_{0\text{-}12h}$. The value of r stated for both LSSs methods a strong correlation between the measured MPA $AUC_{0\text{-}12h}$ and the $AUC_{0\text{-}12h}$ predicted by both LSSs methods (r: 0.92 and 0.94 for LSS3 and LSS4, respectively). Finally, the R^2 (0.84; 0.88, for LSS3 and LSS4, respectively) indicates high goodness of fit of the regression line for both methods. The results are shown in **Figure 1a** and **b**. The Bland–Altman plots (**Figure 2a** and **b**) showed that the data were arranged almost totally within the range mean +/−1.96*SD. The visual inspection of the plots does not reveal any particular pattern, thus excluding other types of bias. This was also assessed by analyzing the linear dependence of the dots in the Bland Altman plot using linear regression, reporting the following results for LSS3 and LSS4 respectively (r = 0.51 and 0.55; R^2: 0.26 and 0.30). These results do not indicate linear dependence.

A subgroup analysis was also conducted stratifying the patients for the co- treatment.

	Total	MMF + CsA	MMF + TAC	*p*-value[a]
No. pts.	40	28	12	
Sex (No. males, % males)	30 (75%)	25 (89%)	5 (42%)	0.003
Age (years)	56.1 ± 12.1	58.4 ± 10.8	57.5 ± 13.7	0.10
MMF Dose (mg/day)	1785.7 ± 553.4	1785.7 ± 551.6	1791.7 ± 582.3	0.98
MMF Dose (mg/kg/day)	24.4 ± 8.1	23.1 ± 6.9	24.3 ± 4.6	0.22
Post-Transpl. time (months)	34.7 ± 52.5	45.1 ± 59.4	10.6 ± 14	0.01
BMI (Kg/m^2)	25.9 ± 5.4	26.5 ± 5.7	24.3 ± 4.6	0.21
ALT (IU/L)	26.5 ± 19.5	28.6 ± 20.9	25.9 ± 15.5	0.24
AST (IU/L)	22.9 ± 16.6	24.5 ± 18.6	19.0 ± 9.9	0.23
Bilirubin (mg/dL)	0.9 ± 0.7	1.1 ± 0.7	0.5 ± 0.2	<0.001
RBCs ($x10^6/\mu L$)	4.1 ± 0.6	4.2 ± 0.7	3.9 ± 0.5	0.08
Hb (g/dL)	12 ± 1.9	12.3 ± 1.9	11.4 ± 1.6	0.10
WBCs ($x10^3/\mu L$)	7.7 ± 2.8	8.1 ± 2.8	6.9 ± 2.8	0.25
Neutro ($x10^3/\mu L$)	5.7 ± 2.7	6.1 ± 2.8	5 ± 2.3	0.20
Lymph ($x10^3/\mu L$)	1.2 ± 0.6	1.2 ± 0.5	1.1 ± 0.5	0.73
Mono ($x10^3/\mu L$)	0.86 ± 1.3	0.9 ± 0.2	0.7 ± 0.2	0.41
Eos ($x10^3/\mu L$)	0.09 ± 0.08	0.09 ± 0.1	0.1 ± 0.1	0.84
Bas ($x10^3/\mu L$)	0.04 ± 0.03	0.04 ± 0	0.05 ± 0	0.74
CrCl (mL/min)[b]	62.0 ± 26.3	59.7 ± 25.4	67.4 ± 28.7	0.4
GFR ($ml/min/1.73m^2$)[c]	59.0 ± 23.4	56.4 ± 24.8	64.4 ± 19.9	0.3
MPA $AUC_{0\text{-}12h}$ (mg × h/L)	47.2 ± 24.7	36.4 ± 13.0	72.3 ± 27.6	0.001
MPA C_0 (ug/ml)	2.4 ± 2.0	1.6 ± 1.0	4.1 ± 2.6	<0.001
Prednisone (mg/day)	12.8 ± 9.4	11.9 ± 9.6	15.4 ± 8.8	0.24
Prednisone (mg/kg/day)	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.10
CsA Dose(mg/day)	—	179.6 ± 75.4	—	
CsA Dose (mg/kg/day)	—	3.0 ± 1.3	—	
CsA C_0 (ng/mL)		177.1 ± 64.9		
TAC Dose (mg/day)	—	—	6.0 ± 5.0	
TAC Dose (mg/kg/day)	—	—	0.1 ± 0.06	
Tac C_0 (ng/mL)			10.6 ± 4.25	

[a]*p-values of 2-sided Fisher's exact test for nominal variables or T- test for quantitative variables.*

[b]*Evaluated by Cockcroft-Gault adjusted for body weight.*

[c]*Evaluated by CKD-EPI Equation.*
Data are reported as mean ± standard deviation, if not otherwise specified.
$AUC_{0\text{-}12h}$: Area under the plasma concentration-time curve from zero to 12 h; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; Bas: Basophils; BMI: Body Mass Index; C_0: pre-dose measurement; CsA: Cyclosporine; CrCl: Creatinine Clearance; Eos: Eosinophils; GFR: Glomerular Filtration Rate; Hb: Hemoglobin level; Lymph: Lymphocytes; Mono: Monocytes; MMF: Mycophenolate Mofetil; MPA: Mycophenolic Acid; Neutro: Neutrophils; RBCs: Red Blood Cells; TAC: Tacrolimus; WBCs: White Blood Cells.

Table 1.
Patients baseline demographical and clinical data, overall and according to the type of treatment.

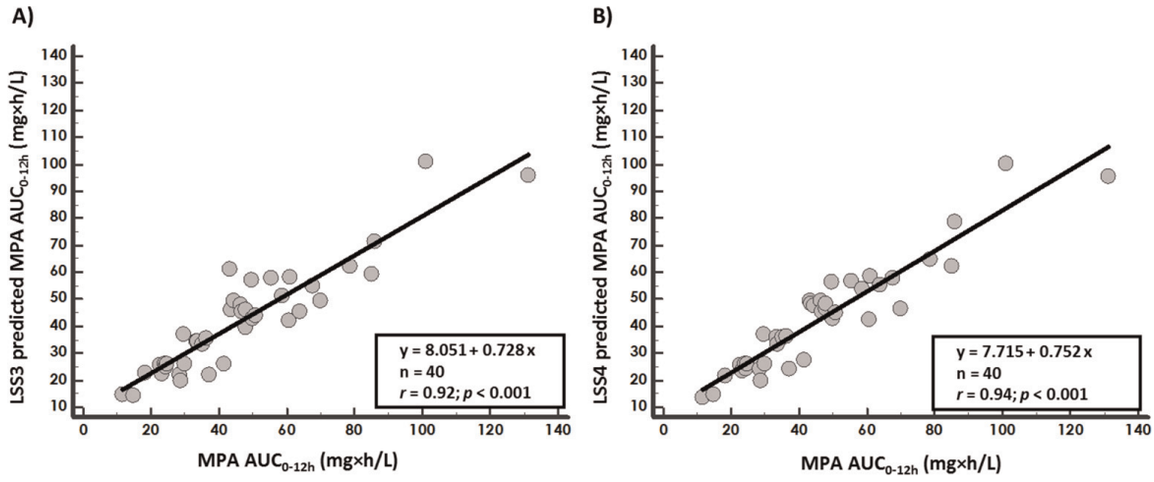

Figure 1.
Linear regression scatters plot of MPA AUC_{0-12h} predicted versus MPA AUC_{0-12h} measured, when MPA AUC_{0-12h} predicted was calculated with LSS3 (A) and LSS4 (B) (n = 40 PK profile).

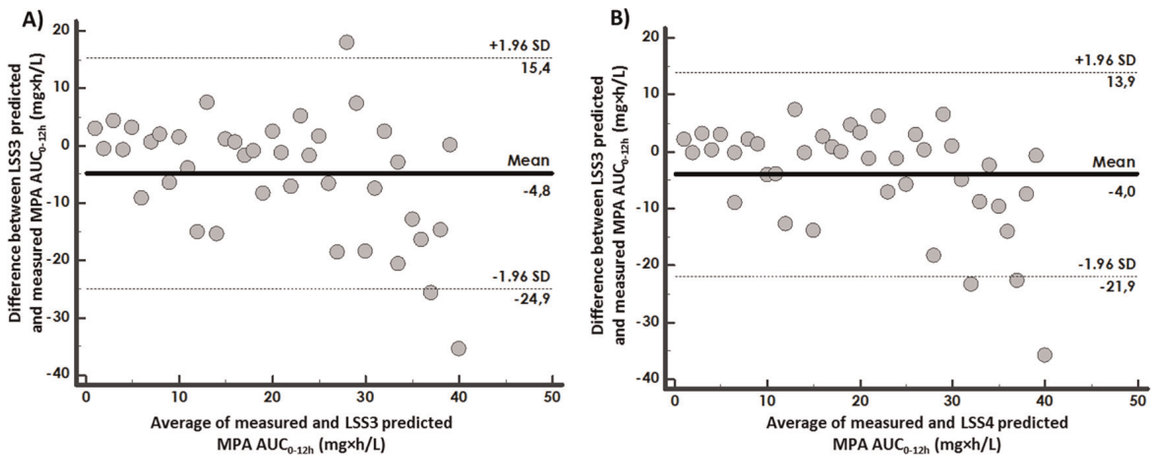

Figure 2.
Bland–Altman plots comparing MPA AUC_{0-12h} predicted – MPA AUC_{0-12h} measured and the average of MPA AUC_{0-12h} predicted and MPA AUC_{0-12h} measured, when MPA AUC_{0-12h} predicted was calculated by LSS3 (A) and LSS4 (B) respectively (n = 40 PK profile).

Among 28 patients treated with MMF and CsA, the bias was acceptable, evaluating MPE% for mean values (−0.5% and − 0.3%) and MPPE% for median values (2.3% and 0.7%) for LSS3 and LSS4, respectively. Analogously, the precision was acceptable evaluating RMSE% (18.6% and 14.8%) and MAPE% (12.4% and 9.7%), for LSS3 and LSS4, respectively. The percentages of MPA AUC_{0-12h} estimated by LSS3 and LSS4 within the 25% of the MPA AUC_{0-12h} full value were 79% and 86%, respectively.

Finally, in the sub-group of 12 patients treated with MMF and TAC, these same features were the followings: MPE% = −18.9% and − 15.3%; MPPE% = −19.9% and − 14.0%; RMSE% = 21.7% and 19.2%; MAPE% = 19.0% and 14.0%, for LLS3 and LSS4, respectively.

The percentage of MPA AUC_{0-12h} predicted within the 25% of the measured MPA AUC_{0-12h} full value: 58% and 67%, for LSS3 and LSS4 respectively.

Despite the very low number of patients, also the linear regression analyses executed on the two subgroups of patients evidenced good results.

In the MMF and CsA group the results were the followings: r = 0.83 and 0.89; R^2 = 0.70 and 0.79, for LSS3 and LSS4 respectively; while in the MMF and TAC group

Population	Algorithm	MPE (%)	MPPE (%)	RMSE (%)	MAPE (%)	% within 75–125% of full $AUC_{0\text{-}12h}$	R^2
Overall (N = 40)	LSS3	−6.0	−3.8	19.6	13.5	73	0.84
	LSS4	−4.8	−1.1	16.2	11.0	80	0.88
MMF and CsA group (N = 28)	LSS3	−0.5	2.3	18.6	12.4	79	0.70
	LSS4	−0.3	0.7	14.8	9.7	86	0.79
MMF and TAC group (N = 12)	LSS3	−18.9	−19.9	21.7	19.0	58	0.87
	LSS4	−15.3	−14.0	19.2	14.0	67	0.86

$AUC_{0\text{-}12h}$: Area under the plasma concentration-time curve from zero to 12 h; CsA: Cyclosporine; LSS3: Limited Sampling Strategy based on 3 concentration sampling points; LSS4: Limited Sampling Strategy based on 4 concentration sampling points; MAPE%: Median Absolute Percentage Prediction Error; MMF: Mycophenolate Mofetil; MPA: Mycophenolic Acid; MPE%: Mean Percentage Prediction Error; MPPE%: Median Percentage Prediction Error; RMSE%: Root Mean Squared Percentage Prediction Error; R^2: coefficient of determination; TAC: Tacrolimus.

Table 2.
Predictive performance of LSS3 and LSS4 in the estimation of the observed MPA $AUC_{0\text{-}12h}$.

these were the results: r = 0.93 and 0.93; R^2 = 0.87 and 0.86, for LSS3 and LSS4 respectively. All these results are summarized on **Table 2.**

The analysis of Kaczamarek LSSs applied to our patient's data reports the following results: r = 0.70; R^2 = 0.49; MPE% = 11.4% and RMSE% = 66.1% in the overall population. By applying these LSSs in the TAC subgroup of patients, we evidenced the following results: r = 0.69; R^2 = 0.48; MPE% = −6.2% and RMSE% = 32.1%.

4. Discussion

The importance of MPA TDM for renal transplant patients is known, but its execution on HTx patients in clinical practice is still debated [17]. Specific large prospective randomized trials should be conducted, but the considerable inter- and intra-patient variability of MPA after organ transplantation suggest MPA TDM to optimize MPA exposure.

The systematic review regarding MPA TDM in HTx reported by Zuk et al. suggests that the relationship between MPA levels and the efficacy of the treatment in terms of allograft rejection in HTx patients is not defined, but LSS may be a better assessment strategy to prevent rejection than a single-time point model [32]. An LSS can be generated using two main methods: MAP-BE method and MLR analysis.

In the first case, any recorded patient sample is compared with data derived from the population PK study, and the covariates can be continually improved by updating the PK population data. The main advantage of the first approach is represented by the flexibility in the timing of the samples as recently demonstrated by Woillard et al. [22]. The main limit of this approach is represented by the employment of complex and specific software, requiring skilled professionals.

On the contrary, multiple regression analysis is simpler, but adherence to the sampling time is mandatory to apply the algorithms in clinical practice. To our knowledge, up to now, few LSSs were developed in HTx, and most of them were generated in patients treated with MMF and CsA [25]. Only three studies focused on LSSs in HTx recipients treated with MMF and TAC [29, 33, 34].

Xiang et al. [33] generated an LSS for the estimation of MMF dispersible tablets combined with TAC in 30 Chinese HTx patients. The comparison of MPA PK among MMF dispersible tablets and MMF did not show significant differences. The LSS with the best performance was the following: MPA $AUC_{0\text{-}12h}$ = 8.424 + 0.781 × $C_{0.5h}$ + 1.263 × C_{2h} + 1.660 × C_{4h} + 3.022 × C_{6h} (R^2 = 0.844). The performance of this LSS can be considered comparable with our algorithms and both contain the C_{6h} sample timing point improving the MPA $AUC_{0\text{-}12h}$ estimation thanks to the inclusion of the typical secondary peak of MPA, minimizing the risk of MPA $AUC_{0\text{-}12h}$ underestimation. Nevertheless, this LSS was developed in Chinese patients so it could not properly fit the Caucasian population, although literature does not suggest this hypothesis [35]. Moreover, these LSSs were developed analyzing the plasma timing point by Liquid Chromatography with tandem mass spectrometry (LC/MS–MS), so they cannot be easily transferred in that laboratories which employ HPLC/UV methods.

Kaczmarek et al. [29] generated different LSSs in 28 HTx recipients treated with MMF and TAC. The best LSS was obtained using 4 sampling points: MPA-$AUC_{0\text{-}12\text{-}h}$ = 1.25 × C_{1h} + 5.29 × C_{4h} + 2.90 × C_{8h} + 3.61 × C_{10h} (R^2 = 0.95). The studied population is comparable to our population. Also, in this case, it can be seen that by sampling the timing point after several hours from MMF administration, a better MPA-$AUC_{0\text{-}12h}$ estimation can be achieved. These LSSs show an optimal performance, but it is based on a demanding sampling schedule that can be applied only on hospitalized patients, thus excluding the outpatient settings.

For this reason, authors proposed two different and more practical LSSs represented by: MPA $AUC_{0\text{-}12h}$ = 1.09 × $C_{0.5}$ + 1.19 × C_{1h} + 3.60 × C_{2h} (R^2 = 0.84) and MPA $AUC_{0\text{-}12h}$ = 1.65 × $C_{0.5h}$ + 4.74 × C_{2h} (R^2 = 0.75). Due to the missing data about the C_{1h} in our population, we test the second LSS. The performance was not acceptable for the use in clinical practice as compared to our algorithms. This could be due to the absence of the C_{6h} sampling time point, resulting in MPA $AUC_{0\text{-}12h}$ underestimation.

Wada et al. [34] generated an LSS in 11 Chinese HTx recipients treated MMF and TAC approximately 9 months after transplantation. In this case, the author used the same analytical method, pharmacokinetic and statistical approaches.

They generated a 3-point model LSS based on C_{1h}, C_{2h} and C_{4h}: MPA $AUC_{0\text{-}12h}$ = 23.56 + 1.05 × C_{1h} + 1.25 × C_{2h} + 2.53 × C_{4h} (R^2 = 0.73), with an MPE% of 2.73%. The results of Wada's study should be taken with caution because of the limited number of enrolled patients and the ethnic difference that could influence MPA PK.

On the other hand, Pawinski et al. proposed an accurate LSS in HTx patients treated with MMF (and CsA) [36] is based on 3 sampling time-points 2 hours after drug administration. The LSSs developed was the following: MPA $AUC_{0\text{-}12h}$ = 9.69 + 0.63 × $C_{0.5h}$ + 0.61 × C_{1h} + 2.20 × C_{2h}. It showed a good performance (R^2 = 0.84), and for its sampling schedule it can be applied in the outpatient setting. Nevertheless, this LSS was generated on the patient in combination therapy with MMF and CsA. For this reason, this algorithm could be acceptable in patients co-treated with CsA because of its effect on reducing the typical MPA secondary peak, affecting MPA EHC [2]. Moreover, the authors developed an algorithm including the C_{6h} blood sample. It presented a similar R^2 and can be considered more predictive of the entire $AUC_{0\text{-}12h}$ because it can describe the typical MPA secondary peak that occurs approximately 6 to 12 hours after MMF oral dose administration, thus affecting global MPA exposure.

In our study the two evaluated LSSs reveal to be sufficiently precise and accurate for the estimation of the entire MPA $AUC_{0\text{-}12h}$ **Figure 1**. The major thesis that allows the application of these LSSs in this population is the presence of C_{6h} that offers the

opportunity to estimate MPA PK accurately in both immunosuppressive regimens, even if it is not easy to apply in the outpatient setting.

This study has several limitations: 1) the whole study group was mainly composed by men, whereas, the small subgroup of patients treated with TAC included a high percentage of women. However, it has been demonstrated that MPA PK is not influenced by sex in solid organ recipients [8, 37], even if Tornatore et al. [38] showed differences in MPA and MPAG PK related to sex among stable renal transplant recipients receiving enteric-coated mycophenolate sodium combined with TAC.; 2) in this pilot study, the sample size of the MMF and TAC group was smaller than MMF and CsA group; 3) MMF and TAC group presented a higher C_0 and MPA $AUC_{0\text{-}12h}$. However, exposure to MPA when MMF is in combination therapy with CsA is approximately 30–40% lower than when given in monotherapy or with TAC [8, 39]; 4) the MMF and TAC group presented a lower level of bilirubin. Bilirubin could displace MPA from albumin binding sites, affecting MPA exposure [40]. However, this effect is limited to only patients presenting hyperbilirubinemia, and could be detected only when the free drug is measured [40]; 5) TDM was not planned to be executed at the same time for all enrolled patients but it was executed by clinical decision. This can be a source of bias, because it is known that the exposition of MPA $AUC_{0\text{-}12h}$ could vary extensively after HTx [11]; 6) furthermore, co-medications commonly used in clinical practice could alter MPA exposure [8, 11] . However, as shown in **Table 1**, the major clinical parameter, including age, BMI, liver and renal function between the two treatment groups were statistically comparable.

5. Conclusion

In this pilot study, two LSSs resulted to be sufficiently precise and accurate to predict MPA $AUC_{0\text{-}12h}$ in a heterogeneous cohort of HTx patients. This study confirmed that the two LSSs, generated in HTx recipients treated with MMF and CsA could be used also in patients treated with MMF and TAC, in particular on in hospitalized patients in the first period after HTx and in outpatients with suspected toxicity or at high risk of organ rejection with considerable social, healthcare and economic advantages.

These results suggest to confirm this hypothesis in a prospective study with a wider cohort of HTx recipients, treated mainly with MMF and TAC, and with a pre-planned TDM.

Acknowledgements

The authors thank the healthcare staff of the Clinical Pharmacology and Toxicology Institute “University Hospital Santa Maria della Misericordia”, Udine, Italy, for the collection of blood samples and the analyses performed.

Conflict of interest

The authors declare no conflict of interest.

The authors received no specific funding from any organization for this work.

Thanks

The authors thank Miss. Enza Pincente for the English revision of the Manuscript.

Nomenclature

$AUC_{0\text{-}12h}$	Area under the plasma concentration-time curve from zero to 12 h;
ALT	Alanine Aminotransferase;
AST	Aspartate Aminotransferase;
BMI	Body Mass Index;
C_0	pre-dose measurement;
Cl	Clearance;
CsA	Cyclosporine;
CrCl	Creatinine Clearance;
EDTA	Ethylenediaminetetraacetic acid;
EHC	enterohepatic circulation;
GFR	Glomerular Filtration Rate;
HPLC/UV	High Pressure Liquid Chromatography with UV Detector
IATDMCT	International Association of Therapeutic Drug Monitoring and Clinical Toxicology
IMPDH	inosine-5′-monophosphate dehydrogenase;
I.R.B.	Internal Review Board;
LC/MS–MS	Liquid Chromatography with tandem mass spectrometry;
LSS	Limited Sampling Strategy;
LSS3	Limited Sampling Strategy based on 3 concentration sampling point;
LSS4	Limited Sampling Strategy based on 4 concentration sampling point;
MAPE%	Median Absolute Percentage Predictive Error;
MPE%	Mean Percentage Prediction Error;
MMF	Mycophenolate Mofetil;
MRP-2	multi-drug resistance protein 2;
MPA	Mycophenolic Acid;
MPAG	7-O-MPA-glucuronide;
MPPE%	Median Percentage Predictive Error;
PK	Pharmacokinetics;
r	Pearson's linear correlation coefficient;
R^2	coefficient of determination;
RMSE%	Root Mean Squared Percentage Prediction Error
T_{max}	time to reach the plasma maximum concentration;
TAC	Tacrolimus;
TDM	Therapeutic Drug Monitoring;
UGTs	uridine 5′-diphospho-glucuronosyltransferases.

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