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Research Article

Plasma Versus Saliva Therapeutic Drug Monitoring of Mycophenolate in Jordanian Patients with Nephrotic Syndrome

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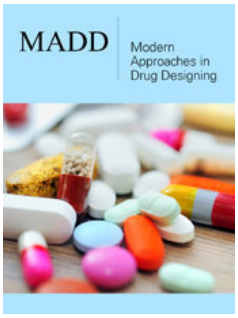

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Abstract

Mycophenolate Mofetil (MF) is an ester prodrug of the immunosuppressant Mycophenolic Acid (MPA) and is commonly used for maintenance immunosuppressive therapy in solid organ and stem-cell transplantation, as well as immunological kidney disorders such as Nephrotic syndrome. MPA exerts a specific and reversible cytostatic effect on lymphocytes. The aim of this study is to examine the robustness of employing a non-invasive saliva sample method as a surrogate for Therapeutic Drug Monitoring (TDM) of MF was used to treat nephrotic syndrome in Jordanian individuals. Trough salivary and plasma samples of Mycophenolate mofetil were collected for comparison. Vlidated (LC-MS/MS) method was used to measure MF & MPA levels. Statistical analyses were performed using the ANOVA test. The results of this study showed that saliva MF and MPA levels were less than that of plasma and there is no significant correlation between saliva and plasma levels of MMF and MPA (P>0.05). MF is classified as class three in the salivary excretion classification system.

Keywords: Mycophenolate mofetil; Therapeutic drug monitoring; Salivary excretion classification system; Pk-sim

Introduction

Nephritic Syndrome (NS) is one of the most common renal conditions in the developing countries. Nephritic syndrome is a group of symptoms with four defining features of proteinuria, edema, hypoalbuminemia, and hyperlipidemia. Minimal change nephrotic syndrome, membranous nephropathy, and focal segmental glomerulosclerosis were recognized as distinct histologic forms of NS [1-3]. Steroid therapy is the initial treatment for idiopathic nephrotic syndrome, but many patients experience relapses, leading to Steroid-Dependent Nephrotic Syndrome (SDNS) or Frequently Relapsing Steroid-Sensitive Nephrotic Syndrome (FR-SSNS) [4,5]. Therefore, alternatives to steroids are needed in order to avoid these clinical problems. Cyclophosphamide, cyclosporine and tacrolimus are the most frequent alternatives according to the Kidney Disease: Improving Global Outcomes (KDIGO) 2012 guidelines [6]. Mycophenolate (MF) therapy was added to the KDIGO 2012 recommendations for cases of relapsing INS showing intolerance or toxicity to classic immunosuppressive drugs. MF has been used as an immunosuppressive agent in renal transplantation, employed in the treatment of a variety of immunologic diseases, including systemic lupus erythematosus and Antineutrophil Cytoplasmatic Antibody (ANCA)-mediated vasculitis [7-11]. Mycophenolate

(MF) is available in two formulations, Mofetil Mycophenolate (MMF) and Sodium Mycophenolate (SMF). Mycophenolate Mofetil (MMF) is the ester prodrug of the active Immunosuppressant Mycophenolic Acid (MPA) It is transformed after its absorption into active metabolite, mycophenolic acid, which exerts a specific and reversible cytostatic effect on lymphocytes; furthermore, it displays a low toxicity profile [12,13].

Therapeutic Monitoring (TDM) of Mycophenolic Acid (MPA) has the potential to improve drug efficacy and reduce toxicities in kidney transplantation. MPA has a narrow therapeutic window and shows a large inter-individual variability in Pharmacokinetic (PK), resulting in an over 10-fold range of variability. In addition, there is a clear association between MPA exposure and efficacy has been reported [14,15] Therapeutic drug monitoring that based on blood sampling is invasive and cumbersome for both patients and health services. The use of saliva as an alternative biological fluid for therapeutic drug monitoring is less invasive and more convenient, which may facilitate fuller pharmacokinetic profiling. Furthermore, since only free drug enters the saliva. Therefore, saliva concentrations may better reflect the unbound and pharmacologically active drug level in the circulation [16]. This may be very important in cases of hypoalbuminemia or severely impaired kidney function, which alter MPA binding to plasma proteins thereby making interpretation of total drug concentrations difficult [17]. A significant correlation between blood and saliva concentrations has been found for a number of drugs [18-20]. However, data regarding the correlation between plasma and saliva concentrations of mycophenolate from previous studies are conflicting [21-24].

Materials and Methods

The clinical part of the study was done at Albashir hospitals with ethical approval from Institutional Review Board (IRB) from Ministry of health. A total of 14 patients (7 Males and 7 Females). The Age ranges from 19 to 55 years, (Average 33.14). Weights and heights were taken as part of the computation for creatinine clearance. The range of participant weight is 64-90kg while the range of participant height is 155cm-174cm. All patients who participated in the study were on chronic mycophenolate therapy, were admitted to the hospital at the time of data collection and provided informed consent. Plasma and saliva samples from patients were collected just before the next dose to Measure Mycophenolate (MMF) and Mycophenolic Acid (MPA) concentrations. Other laboratory tests obtained from plasma samples included urea, creatinine, Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), and albumin. Creatine clearance was calculated as a factor of age, weight, and creatinine levels. In addition, medical files were used to obtain patient demographics such as age, gender, weight, and height (Table 1).

Table 1: The general patient characteristics in this investigation.

Age	Gender	Weight	Height	Creatinine	Clearance	ALT	AST	Urea	Albumin
34	M	64	170	80	1.17777778	57.8	28.9	5.4	39
44	F	72	169	34	2.4	24.8	18.9	13	31.28
23	F	60	155	50	1.6575	22	30	10	20.9
24	M	67	172	61	1.76958106	30	19.2	7.9	34.7
46	F	88	165	95	1.02795322	19.8	15	6	28.9
39	M	86	174	100	1.20638889	20.8	17.9	8	30.8
22	F	60	159	92	0.90851449	19.6	22	7.4	39.7
25	M	70	168	102	1.0961329	24.7	13.5	9	33.5
29	F	66	162	70	1.23553571	27.9	30.4	11	40.7
33	F	73	163	45	2.0491821	23.8	26.7	17.9	31
55	M	90	171	90	1.18055556	30.4	24.6	29.1	33.8
19	M	66	165	115	0.96449275	33.2	38.8	18.9	50
44	F	66	163	44	1.7	9.1	16.6	2.6	37
27	M	75	169	104	1.1318109	32.6	25.4	6.7	38

Method of analysis validation for MMF and MPA

Liquid Chromatography with Tandem Mass Spectrometry (LC-MS-MS) method was used in this investigation to analyze MF and MPA in patients saliva and plasma. LC-MS-MS (Agilent 1260 chromatographic system, API 3000 mass spectrometer with pump auto sampler Agilent 1260/1290 series HPLC instrument, MS/MS detector API 5500 (Applied Biosystems, MDS SCIEX), IKA Vibrax and Vortex Eppendorf centrifuge 5810 R were used. Acetonitrile (ACN) (HPLC grade), Methanol (HPLC grade), ammonium formate, MF, MPA, and MF-D4, purified water was the chemical used in this method. Chromatographic Conditions include a solution of acetonitrile and Ammonium formate 10mm and 0.5% formic acid [70%-30%] was used as the mobile phase. (Mix 700ml acetonitrile with 300ml Ammonium formate (NH_4HCO_2) 10mm+0.5% formic acid (HCO_2H) 500µl then shake well). The flow rate was 0.7ml/min. Analytical column (thermos, BDS, c18 50*4.6mm, 3.00µm) using an LC-MS/MS instrument (i.e., Agilent 1260 chromatographic system, API 3000 mass spectrometer), injection volume 10µl.

Sample preparation steps

The process involves adding 50µl of Mycophenolate mofetil-d4 (internal standard) to 300µl of subject plasma or saliva samples.

Next, 50µl of HCl (2% in water) is added to the samples, followed by vertexing for 15 seconds. Then, 4ml of Tert butyl methyl ether is added, and the sample is vortexed for approximately 3 minutes. Then, the samples are centrifuged at 4000rpm for about 5 minutes. The organic layer is then carefully poured off, and the remaining liquid is evaporated using a stream of compressed air at room temperature. The residue is reconstituted with 400µl of mobile phase, and finally, 200µl of the solution is transferred to an auto sampler vial insert.

Bioanalytical method validation in saliva

The mycophenolate mofetil calibration curve in saliva was prepared by including 8 standard points: (0.025, 0.050, 0.100, 0.250, 0.800, 1.500, 2.500, and 3.500 (ng/mL)). The mycophenolic acid calibration curve in Saliva was prepared by including 8 standard points: 150.0, 300.0, 1000.0, 3000.0, 7000.0, 10000.0, 14000.0, and 20000.0 (ng/mL).

Accuracy and precision

Intra-day precision and accuracy were calculated at LLOQ of MF (0.025ng/mL), low quality-control (0.075ng/mL), medium quality-control (1.400ng/mL), and high quality-control (2.800ng/mL) with six replicates for each level. Intra-day precision and accuracy were calculated at LLOQ of MPA (150.0ng/mL), low quality-control (450.0ng/mL), medium quality-control (8000.0ng/mL), and high quality-control (15000.0ng/mL) with six replicates for each level.

Linearity of MMF and MPA

Calibration curves were created by plotting the instrument's analytical response. (Area ratio; Analyte area/IS area) versus Mycophenolate mofetil and Mycophenolic acid concentration. A linear relationship (R≥0.98) between the analytical response and concentrations of Mycophenolate mofetil and Mycophenolic acid was obtained over the concentration range (0.025-3.50)ng/ml for Mycophenolate mofetil and (150.00-20000.00ng/ml) for Mycophenolic acid.

Selectivity and specificity

To confirm the absence of interfering substances around the retention time of analyst; blank saliva samples were analyzed. The endogenous components were well isolated from MMF and MPA (internal standard). At the retention time of both MMF and the internal standard, there were no interferences. The peaks were in good shape, completely resolved from the saliva components. The matrix peak was less than 5% of the internal standard's peak area, which is acceptable according to US FDA guidelines.

Lower limit of quantitation (LLOQ)

The analytical method was developed and validated by the USFDA bioanalytical method validation guidance to measure the lowest concentration that can be detected with appropriate precision and accuracy. These limits had a precision of less than 20%., with the LLOQ in Saliva of MMF being 0.025ng/ml. And the LLOQ in the Saliva of MPA is 150.0ng/ml.

Bioanalytical Method Validation in Plasma

Calibration plasma

The mycophenolate mofetil calibration curve in Plasma was prepared using human plasma spiked with eight standered concentrations of MF(0.025, 0.050, 0.100, 0.250, 0.800, 1.500, 2.500, and 3.500(ng/mL)). MPA calibration curve in human plasma spiked with eight standard concentrations of MPA (150.0, 300.0, 1000.0, 3000.0, 7000.0, 10000.0, 14000.0, and 20000.0(ng/mL)).

Accuracy and precision

Intra-day precision and accuracy were calculated at LLOQ of MMF (0.025ng/mL), low quality-control (0.075ng/mL), medium quality-control (1.400ng/mL), and high-quality control (2.800ng/mL) with six replicates for each level. Intra-day precision and accuracy were calculated at LLOQ of MPA (150.0ng/mL), low quality-control (450.0ng/mL), medium quality-control (8000.0ng/mL), and high-quality control (15000.0ng/mL) with six replicates for each level.

Selectivity and specificity

To confirm the absence of interfering substances around the retention time of the analysts, blank samples were analyzed. Mycophenolic acid was well separated from the endogenous components. At the retention time of Mycophenolic acid and the internal standard, there were no interferences. The peaks were in good shape and were resolved from the plasma components. The peak area of the matrix was less than 5% of the peak area of the internal standard. It is acceptable according to US FDA guidelines.

Lower limit of quantitation (LLOQ)

The analytical method was developed and validated by the USFDA bioanalytical method validation guidance to measure the lowest concentration that can be detected with appropriate precision and accuracy. These limits had a precision of less than 20%., with the LLOQ in Plasma of MMF being 0.025ng/ml, and the LLOQ in Plasma of MPA being 150.0ng/ml.

Pharmacokinetic calculations

The theoretical ratio between saliva and plasma concentrations can also be calculated using the below formula.

$$S/P = \frac{1+10^{(pHs-pKa)} \times f_p}{1+10^{(php-pka)} \times f_s}$$

$$S/P = \frac{1+10^{(7-8)} \times 0.11}{1+10^{(7.38-8)} \times 1} = 0.097659$$

Ph. saliva=7, Ph. plasma=7.38, F_p=0.11, pka 8, F_s=1. Where FP is a free fraction in plasma, Fs is a free fraction in saliva. The creatinine clearance was calculated using the Cockcroft-Gault equation without adjustment for ideal body weight.

((140–age (yr))*weight (kg))/((72*serum creatinine (mg/dL))*(0.85 female))

Statistical calculations

The results of this study were achieved by using Excel for the calculation of the mean, standard deviation, the coefficient of variance, distributive statistics, and ratio statistics. Systat version 5 was used for the ANOVA and Statistica version 4.5 was used for the correlation analysis and the calculation of the r and p values.

Result and Discussion

Plasma concentrations of MMF and MPA

The plasma concentrations of MMF and MPA were measured for 14 patients who participated in the study. Trough concentrations of MMF ranged from 26 to 175.38ng/ml while concentrations of MPA ranged from 1.9 to 11ng/ml. Mean values for MMF trough concentrations were 99.17ng/ml (SD=68.75) while mean values for MPA concentrations were 5.967ng/ml (SD=2.939). Table 2 shows the trough plasma concentrations of MMF and MPA for all participants.

Table 2: Trough plasma concentrations of MMF and MPA for all participants.

Number	Plasma MMF	Plasma MPA
1	273.06	3.84
2	175.38	1.9
3	42.85	4.32
4	26.00	5.53
5	135.33	10.53
6	70.56	10.37
7	69.30	6.44
8	54.00	5.76
9	116.54	3.53
10	155.12	7.45
11	33.54	6.22
12	111.01	11
13	88.33	2.87
14	37.35	3.78
Mean	99.17ng/ml	5.967ng/ml
SD	68.75	2.939

Saliva concentrations of MMF and MPA

The saliva concentrations of MMF and MPA were measured for 14 patients who participated in the study. The ranges of saliva concentrations of MMF were from 2.65 to 27.86ng/ml while ranges of MPA saliva concentrations were from 0.19 to 1.053ng/ml. The mean values of MMF saliva concentrations were 11.39 ng/ml (SD=7.48) while mean values of MPA saliva concentrations were 0.5765ng/ml (SD=0.2746). Table 3 shows the saliva concentrations of MMF and MPA

Table 3: Table shows the saliva concentrations of MMF and MPA.

Number	Saliva MMF	Saliva MPA
1	4.37	1.037
2	11.89	0.384
3	17.90	0.432
4	5.51	0.644
5	7.07	0.553
6	13.81	0.576
7	7.20	0.19
8	27.86	0.353
9	2.65	1.053
10	12.58	0.35
11	6.54	0.89
12	21.05	0.71
13	17.29	0.25
14	3.74	0.65
Mean	11.39ng/ml	0.5765ng/ml
SD	7.48	0.2746

Relationship between MMF and MPA concentrations in plasma and saliva

Data of plasma and saliva levels were log-transformed to compensate for the non-normality of the sample distribution Afterward, correlations between plasma and saliva concentrations were measured using Pearson's r. First, there was a weak, non-significant correlation between both trough saliva and plasma concentrations of MMF (r=0.1679, p=0.760) and for MPA (r=0.09, p=0.83) (Figure 1 & 2).

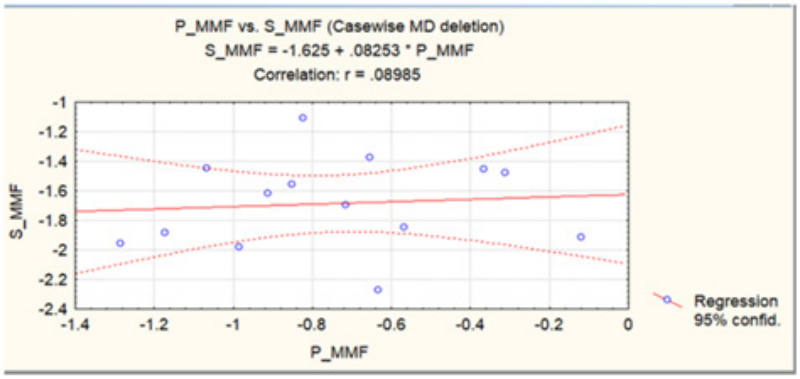

Figure 1: Plots of trough saliva versus plasma concentrations of MMF after log transformation.

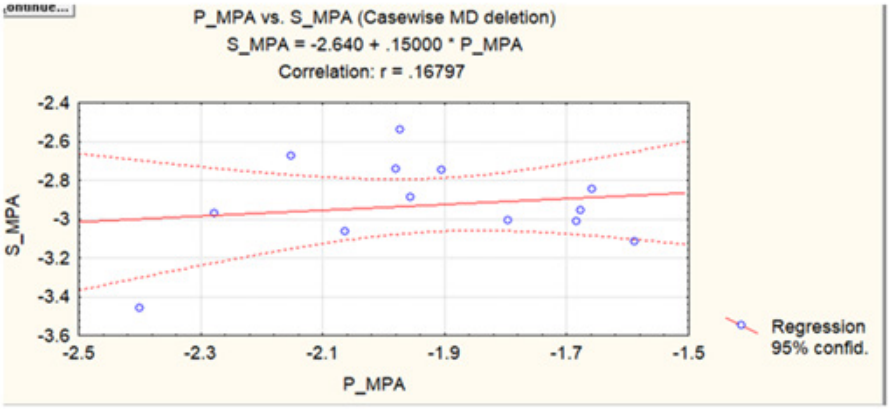

Figure 2: Plots of trough saliva versus plasma concentrations of MPA after log transformation.

Section of ratio analysis

The ratio of trough MMF & MPA saliva level to plasma level was calculated and presented in tables (Table 4 & 5). The therapeutic range of MMF in saliva can be calculated by multiplying the plasma. The therapeutic range of MMF is by the average ratio of minimum and maximum. The saliva therapeutic range is equal tos 4.2486 to 12.1388mcg/ml. The plasma normal range is 35 to 100mcg/ml. The average of the S/P ratio of the concentration was MMF 0.1689 while the average of the s/p ratio for MPA concentration was 0.1214, while the average of the actual s/p ratio for the minimum concentration was 0.12. Therefore, actual and theoretical ratios are very close (Table 6).

Table 4: Ratios of Saliva to plasma levels of MMF (n=14). S/D: Saliva/Dose. P/D: Plasma/Dose, Log P/D: Log Plasma/Dose, Log S/D: Log Saliva/Dose, S/P: Saliva/plasma.

Saliva MMF	Plasma MMF	DOSE	S/D	P/D	LOG P/D	LOG S/D	S/P
4.37	273.06	360	0.0121468	0.7584944	-0.12005	-1.91554	0.016014
11.89	175.38	360	0.0330326	0.4871528	-0.31233	-1.48106	0.067807
17.90	42.85	500	0.0357908	0.085708	-1.06698	-1.44623	0.41759
5.51	26.00	500	0.0110204	0.052	-1.284	-1.9578	0.211931
7.07	135.33	500	0.0141437	0.27065	-0.56759	-1.84944	0.052258
13.81	70.56	500	0.0276173	0.141114	-0.85043	-1.55882	0.195709
7.20	69.30	360	0.0199991	0.1925111	-0.71554	-1.69899	0.103886
27.86	54.00	360	0.0773974	0.15	-0.82391	-1.11127	0.515983
2.65	116.54	500	0.0053061	0.233078	-0.6325	-2.27522	0.022765
12.58	155.12	360	0.0349444	0.4308889	-0.36563	-1.45662	0.081099
6.54	33.54	500	0.01308	0.06708	-1.17341	-1.88339	0.194991
21.05	111.01	500	0.0421	0.22202	-0.65361	-1.37572	0.189623
17.29	88.33	720	0.0240139	0.1226806	-0.91122	-1.61954	0.195743
3.74	37.35	360	0.0103889	0.10375	-0.98401	-1.98343	0.100134

Table 5: Ratios of Saliva to plasma levels of MPA (n=14)

Saliva MPA	Plasma MPA	DOSE	S/D (MPA)	P/D (MPA)	LOG(P/D)	LOG(S/D)	S/P
1.037	3.84	360	0.0028806	0.0106667	-1.97197	-2.54052	0.270052
0.384	1.9	360	0.0010667	0.0052778	-2.27755	-2.97197	0.202105
0.432	4.32	500	0.000864	0.00864	-2.06349	-3.06349	0.1
0.644	5.53	500	0.001288	0.01106	-1.95624	-2.89008	0.116456
0.553	10.53	500	0.001106	0.02106	-1.67654	-2.95624	0.052517
0.576	10.37	500	0.001152	0.02074	-1.68319	-2.93855	0.055545
0.19	6.44	250	0.00076	0.02576	-1.58905	-3.11919	0.029503
0.353	5.76	360	0.0009806	0.016	-1.79588	-3.00853	0.061285

1.053	3.53	500	0.002106	0.00706	-2.1512	-2.67654	0.2983
0.35	7.45	360	0.0009722	0.0206944	-1.68415	-3.01223	0.04698
0.89	6.22	500	0.00178	0.01244	-1.90518	-2.74958	0.143087
0.71	11	500	0.00142	0.022	-1.65758	-2.84771	0.064545
0.25	2.87	720	0.0003472	0.0039861	-2.39945	-3.45939	0.087108
0.65	3.78	360	0.0018056	0.0105	-1.97881	-2.74339	0.171958

Table 6: MMF & MPA levels in saliva and plasma.

	Age	Gender	WT	HT	ALT	AST	CRTN	Urea	Albumin
Plasma MMF	0.656	0.839	0.526	0.826	0.307	0.875	0.696	0.571	0.561
Plasma MPA	0.686	0.703	0.771	0.551	0.472	0.703	0.655	0.823	0.979
Saliva MMF	0.918	0.793	0.534	0.958	0.414	0.424	0.748	0.498	0.889
Saliva MPA	0.436	0.893	0.51	0.769	0.974	0.95	0.898	0.918	0.882

Effect of the demographic factor on the Mycophenolate concentration in saliva and plasma

Most of the p-value are insignificant (>0.05) which indicated no effects observed for age, gender, and weight on MMF & MPA levels in saliva and plasma shown in table.

Conclusion

The use of TDM necessitates a multidisciplinary approach that includes pharmacologic, pharmacokinetic, and pharmacodynamics approaches and analyses. TDM takes more than a simple understanding. A patient's blood medication concentration is measured and compared to a desired range. TDM, on the other hand, plays a vital role in the creation of safe and effective therapeutic drugs, as well as the individualization of these treatments. TDM can also assist in identifying medication compliance issues in noncompliant patient instances. Factors to consider when interpreting drug concentration readings include the sampling time of the dose, the dosage history, the patient's response, and the anticipated therapeutic targets. This data can be used to determine the best dosing regimen for achieving the best response with the least amount of toxicity.

Measurement of MPA concentration in saliva cannot currently replace plasma measurement for therapeutic drug monitoring of MPA following EC-MS administration. Additional studies are required to examine the relationship between MPA saliva concentrations and patient outcomes. suggests that saliva is a poor direct marker of plasma MPA concentrations and therefore should not be used for MPA TDM. Therapeutic Monitoring (TDM) of Mycophenolic Acid (MPA) has the potential to improve drug inefficacy and toxicities in kidney transplantation. However, measurement of plasma MPA concentrations is laborious and invasive. This study examined the utility of saliva compared with plasma based TDM of MPA. Paired blood and saliva samples were collected from 47 adult kidney transplant recipients pre- and at 1-, 2-, and 4-hours post mycophenolate mofetil administration. No relationship was observed between saliva MPA concentrations and either total or free plasma MPA concentrations (p>0.05). This suggests that saliva is a poor direct marker of plasma MPA concentrations and therefore should not be used for MPA TDM. Based on a limited sampling strategy, MPA saliva and plasma concentrations were found in good agreement with each other. We suggest that the described method is suitable to analyze saliva and plasma samples of small volumes for therapeutic drug monitoring (TDM) and pharmacokinetic studies in pediatric patients. Correlated well with total (r=0.909) and unbound (r=0.910) MPA concentrations in plasma. In conclusion, a simple, sensitive, and specific method was developed and validated for quantification of MPA in saliva. Additional clinical studies are required to establish the usefulness of this specimen in the clinical management of organ transplant recipients.

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