Risk of diarrhoea in a long-term cohort of renal transplant patients given mycophenolate mofetil: the significant role of the *UGT1A8**2 variant allele

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Mycophenolate mofetil (MMF), the most widely used drug in allograft transplantation, is subject to hepatic and intestinal glucuronidation and entero-hepatic cycling.
- Diarrhoea is its most frequent adverse event leading to non-compliance, treatment interruption and ultimately to an increased rate of acute rejection.
- Cyclosporin reduces the biliary excretion of mycophenolate metabolites, presumably by inhibiting the efflux transporter MRP2.
- When combined with MMF, cyclosporin reduces the incidence of diarrhoea, suggesting the role played by biliary excretion of mycophenolate glucuronides in this adverse event.

WHAT THIS STUDY ADDS

- In a long-term cohort of renal transplant patients on MMF, the two factors significantly associated with a reduced incidence of diarrhoea were the co-medication with cyclosporin (as opposed to tacrolimus or sirolimus) and the *2 variant allele of the intestinal *UGT1A8*.
- Polymorphisms in the other UDP-glucuronosyl-transferases and MRP2 were not significant.

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AIM

In renal transplant patients given mycophenolate mofetil (MMF), we investigated the relationship between the digestive adverse events and polymorphisms in the *UGT* genes involved in mycophenolic acid (MPA) intestinal metabolism and biliary excretion of its phase II metabolites.

METHODS

Clinical data and DNA from 256 patients transplanted between 1996 and 2006 and given MMF with cyclosporin (CsA, $n = 185$), tacrolimus (TAC, $n = 49$) or sirolimus (SIR, $n = 22$), were retrospectively analysed. The relationships between diarrhoea and polymorphisms in *UGT1A8* (*2; 518C>G, *3; 830G>A), *UGT1A7* (622C>T), *UGT1A9* (-275T>A), *UGT2B7* (-840G>A) and *ABCC2* (-24C>T, 3972C>T) or the co-administered immunosuppressant were investigated using the Cox proportional hazard model.

RESULTS

Multivariate analysis showed that patients on TAC or SIR had a 2.8 higher risk of diarrhoea than patients on CsA (HR = 2.809; 95%CI (1.730, 4.545); $P < 0.0001$) and that non-carriers of the *UGT1A8**2 allele (CC518 genotype) had a higher risk of diarrhoea than carriers (C518G and 518GG genotypes) (HR = 1.876; 95%CI (1.109, 3.175); $P = 0.0192$). When patients were divided according to the immunosuppressive co-treatment, a significant effect of *UGT1A8**2 was found in those co-treated with CsA (HR = 2.414; 95%CI (1.089, 5.354); $P = 0.0301$) but not TAC or SIR ($P = 0.4331$).

CONCLUSION

These results suggest that a possible inhibition of biliary excretion of MPA metabolites by CsA and a decreased intestinal production of these metabolites in *UGT1A8**2 carriers may be protective factors against MMF-induced diarrhoea.

Introduction

Mycophenolate mofetil (MMF), the prodrug of mycophenolic acid (MPA), is an immunosuppressive drug widely used in combination therapy with cyclosporin (CsA), tacrolimus (TAC) or sirolimus (SIR) for the prevention or the treatment of acute rejection following kidney, heart and liver allograft transplantation.

The main adverse events (AE) reported for MPA are gastrointestinal (GI) disorders (in particular diarrhoea), bone marrow suppression and anaemia [1, 2]. MMF would be discontinued in 20% of the patients because of such adverse events [3]. It was first hypothesized that the digestive adverse events of MMF could be related to MMF dose and/or to MPA plasma concentrations [4, 5] but this was not confirmed by a further study [6]. Other hypotheses include the possible predisposition of patients to MMF diarrhoea in relation to MPA metabolism, and drug–drug interactions. The metabolism of MPA is mainly by conjugation of its phenol group to give the inactive MPA-phenyl-glucuronide (MPAG) [7] which involves UGT1A9, and to a lesser extent UGT1A7, 1A8 and 1A10 [8, 9]. The conjugation of MPA carboxylic acid moiety leads to a second glucuronide, namely MPA-acyl-glucuronide (AcMPAG) [10], which is mainly produced by UGT2B7 in the liver and, to a lesser extent, in other tissues including the intestine and the kidneys [8]. MMF induces a particular type of diarrhoea, the exact mechanism of which remains unknown. Several authors reported that the normal villous structure of the small bowel was lost [11–13]. Owing to the reactivity of AcMPAG [14, 15], it was suggested that AcMPAG could be involved in this adverse event through a secondary immunological mechanism [16]. However, neither MPAG nor AcMPAG plasma exposures were associated with diarrhoea in a study in kidney transplants patients [6], where the only significant factor found was the calcineurin inhibitor associated with MMF: a lower incidence of diarrhoea was observed in patients co-treated with CsA than in those co-treated with TAC. As CsA inhibits the multidrug resistance protein 2 (MRP2)-mediated excretion of MPA metabolites into the bile [17], it suggests that biliary excretion of MPA metabolites, hence intestinal exposure to these metabolites would be more closely linked with diarrhoea than systemic exposure.

The aim of this study was to investigate in a long-term cohort of renal transplant patients on MMF the influence on digestive adverse events of i) polymorphisms of the genes encoding the UGTs involved in MPA intestinal metabolism (*UGT1A7*, *UGT1A8*, *UGT1A9* and *UGT2B7*), ii) polymorphisms of the gene encoding the efflux transporter involved in the biliary excretion of MPA metabolites (*ABCC2*) and iii) co-administered immunosuppressants.

Methods

Patients

The clinical data-on-file and banked DNA samples from patients transplanted between 1996 and 2006, routinely followed as outpatients at Limoges University Hospital were retrospectively studied. The ethics committee of Limoges Hospital approved the protocol. Informed consent was obtained from each living patient, while the French Health Authorities waived the requirement for consent for deceased patients. The following inclusion criteria were used: recipient age >18 years, functioning graft after more than 1 year post-transplantation, kidney graft from a cadaveric donor, constitutional DNA available, deceased patient or signed informed consent. Exclusion criteria were as follows: patient age <18 years, pregnancy, graft survival <1 year and kidney and pancreas, heart or liver combined transplantation. For each patient, the following clinical data were recorded from the medical file by the same nephrologist (JPR): date of birth, sex, HLA mismatches between donor and recipient, duration of cold ischaemia, induction therapy, immunosuppressive drug regimens and gastrointestinal adverse events (GI AEs), with their starting and ending dates. The GI AEs were classified as diarrhoea, abdominal pain, nausea/vomiting and anorexia. In order to reduce the number of statistical tests and because of the low frequencies of abdominal pain (11.0%), nausea/vomiting (5.5%) and anorexia (7.0%), only diarrhoea (27.7%) and the global incidence of all GI AEs (35.1%) were finally analysed. Clinical data collection was performed before the initiation of the genetic study to avoid any bias. Diarrhoea was taken into consideration when sufficient and convincing data were available in the clinical file, in particular regarding the duration, severity and resolution of the diarrhoea episode, and when it most likely fulfilled the following definition: more than two loose, watery stools per day persisting for more than 5 days, without fever or inflammatory disease, or any other patent aetiology (positive viral or cytobacterial or parasitological examination of the stools when available and treatments known to provoke diarrhoea, other than immunosuppressive therapy) and/or when the episode stopped after MMF dose reduction or discontinuation. Patients were treated following the medical practice at that time in Limoges University Hospital.

Genomic DNA bank

DNA collection and conservation was performed by the immunogenetic laboratory of Limoges University Hospital. Genomic DNA was extracted from EDTA-treated blood using a previously described manual method [18].

Identification of genotypes

Genomic DNA was used to characterize the genotypes of each patient for SNPs in the *UGT1A7*, *UGT1A8*, *UGT1A9*,

Table 1

Frequency and distribution of the polymorphisms studied

Gene	Polymorphism	Frequency of the variant allele	Genotype wt/wt§	wt/m	m/m
UGT	622C>T (<i>UGT1A7</i>)	0.627	37/251*	113/251	101/251
	518C>G (<i>UGT1A8</i> *2)	0.226	151/256	94/256	11/256
	830G>A (<i>UGT1A8</i> *3)	0.017	247/256	9/256	0/256
	-275T>A (<i>UGT1A9</i>)	0.055	228/256	28/256	0/256
	-840G>A (<i>UGT2B7</i>)	0.482	73/256	117/256	65/256
ABCC2	-24C>T	0.235	148/251*	88/251	15/251
	3972C>T	0.394	82/251*	140/251	29/251

*Five patients remained undetermined for *UGT1A7* and *ABCC2* genotypes. §wt, wild type; m, variant.

UGT2B7 and *ABCC2* (*MRP2*) genes (Table 1). Genotypes were determined using Real-Time Quantitative Polymerase Chain Reaction (ABI PRISM 7000 Sequence Detection System; Applied Biosystems, Courtaboeuf, France) and validated allelic discrimination assays (TaqMan Custom or Drug Metabolism Genotyping assays®, Applied Biosystems).

Briefly, 1–20 ng of genomic DNA were mixed with each assay and PCR universal master mix (Applied Biosystems, Foster City, CA USA) in a total volume of 14 µl. Thermal cycler parameters included 10 min at 95°C and 40 cycles of denaturation at 92°C for 15 s and annealing/extension at 60°C for 1 min, except for *UGT1A8* and *UGT1A7* assays which required 1.5 min elongation steps and 45 PCR cycles.

Statistical analysis

Deviations from the Hardy-Weinberg equilibrium were studied using the Fisher exact test. The effect of the polymorphisms (SNPs or haplotypes) on phenotypes was investigated using the Cox proportional hazard model, considering successively all GI AEs and diarrhoea only. *P* values less than 0.05 were considered significant and 95% confidence intervals provided when relevant. For SNP and haplotype association analyses, the most frequent allele was considered as the reference. When the frequency of variant homozygous patients was lower than 5%, these patients were gathered with the heterozygotes. For multivariate analysis, the significance of variables in the final model was tested by a backward stepwise process using the likelihood ratio to evaluate the effect of omitting variables. After studying the effect of each polymorphism independently, the association of haplotypes with GI AEs was analysed using the THESIAS program (<http://genecanvas.ecgene.net>) [19] when appropriate.

The *ABCC2* polymorphism was investigated in two subgroups of treatment independently: CsA and TAC/SIR because of the hypothesis of *MRP2* biliary inhibition by CsA.

In order to investigate the effect of MMF dose on the incidence of diarrhoea, the dose at the time of the first

episode of diarrhoea in 'case' patients was compared with the dose collected at a similar time after initiation of MMF in 'control' patients paired on follow-up duration while on MMF. Dose was classified into four groups (≤ 750 , 1000, 1500 or ≥ 2000 mg) and compared using the Fisher exact test, then in two groups (< 2000 mg or ≥ 2000 mg) to be analysed using the Cox model. For significant covariates, time-to-event data (first episode) were estimated using Kaplan-Meier analysis for patients with or without the factor of interest, and groups were compared by the log-rank test.

Except when stated otherwise, all statistical analyses were performed using Statview 5.0 (SAS Institute Inc, Cary, NC, USA).

Results

Clinical data

The clinical and demographic characteristics of the 256 patients who fulfilled the inclusion and exclusion criteria (out of 386 patients transplanted at Limoges University Hospital over the period 1996–2006) are described in Table 2. Patients' follow-up was 41.0 months on average (ranging from 0.6 to 115.0 months). Each patient was taken into consideration as from the initiation of MMF treatment, which corresponded to the first days post-transplantation in 222 patients (86.7%). The 34 others were switched from azathioprine to MMF in the stable post-transplantation period. During the study period, a total of 194 episodes of gastrointestinal adverse events (GI AEs) were observed in 90 patients (35.1%), including 118 episodes of diarrhoea in 71 patients (27.7%). Twenty-five patients had more than two episodes of diarrhoea. The mean MMF dose at the time of the episode was 1750 ± 699 mg in the group with diarrhoea (cases) and 1768 ± 443 mg in the group without (controls) (NS). Among patients with diarrhoea, there was a significant difference in MMF dose between patients co-treated with TAC (1278 ± 521 mg) and those co-treated with either SIR (1889 ± 333 mg) or CsA (1927 ± 738 mg) ($P = 0.0026$).

Table 2

Patients' characteristics according to the immunosuppressant associated with MMF

	CsA (n = 185)	SIR (n = 22)	TAC (n = 49)
Male/Female	129/56	9/13	24/25
Age (years)	48.7 ± 14.0	55.3 ± 13.9	47.8 ± 13.0
(min/max)	(17.5/74.3)	(20.6/72.7)	(21.8/70.5)
Follow-up (months)	48.7 ± 2.5	19.1 ± 4.7	21.9 ± 3.2
(min/max)	(0.7/114.6)	(0.6/95.0)	(0.7/115.1)
Number of patients (%) with ≥1 episode of diarrhoea	33 (17.8%)	12 (54.5%)	26 (53.1%)

Parameters are expressed as mean ± SD, and the frequency is given for the number of patients with adverse events; CsA, cyclosporin; TAC, tacrolimus; SIR, sirolimus.

Linkage disequilibrium study

All the genotype distributions were in conformity with the Hardy-Weinberg equilibrium and similar to those reported in the literature (Table 1). A strong linkage disequilibrium (LD) was observed between the *ABCC2*-24C>T and the 3972C>T single nucleotide polymorphisms (SNPs) (LD parameters: $D' = 0.93$, $r^2 = 0.41$). Four haplotypes were found: -24C/3972C (59.6%), -24T/3972T (22.5%), -24C/3972T (16.9%) and -24T/3972C (1.0%). No LD was observed between the UGT SNPs.

Effects of co-administered immunosuppressant and MMF dose

The percentage of patients with diarrhoea was 17.8%, 54.5% and 53.1% in the CsA, SIR and TAC subgroups, respectively (Table 2). There was no difference in the incidence of diarrhoea between patients on TAC or SIR (Cox model: $P = 0.5789$) (Figure 1). Consequently, due to the rather small number of patients in these subgroups ($n = 49$ and 22, respectively), they were combined for comparison with CsA co-treated patients. Univariate analysis using the Cox model showed a highly significant association of the co-administered immunosuppressant with diarrhoea (TAC/SIR vs. CsA: Hazard ratio (HR) = 4.251; 95% CI 2.637, 6.853; $P < 0.0001$) (Table 3), as well as with the GI AEs studied globally (TAC/SIR vs. CsA: HR = 3.788; 95% CI 2.457, 5.848; $P < 0.0001$).

No significant association was found between GI AEs and MMF dose at the time of the event, whether the dose was considered in four groups (≤ 750 , 1000, 1500, ≥ 2000 mg; Fisher exact test $P = 0.095$) (Figure 2), or in two groups (< 2000 and ≥ 2000 mg: HR 1.146; 95% CI 0.702, 1.869; $P = 0.5861$) (Table 3).

Survival analysis

For patients with more than one episode of diarrhoea, only the first episode was taken into account. Kaplan Meier analysis of the time to the first episode of diarrhoea dem-

Figure 1

Kaplan-Meier analysis of the time until the first episode of diarrhoea in patients on either CsA/MMF, SIR/MMF or TAC/MMF (TAC: tacrolimus, SIR: sirolimus, CsA: cyclosporin). CsA co-treated patients ($n = 185$) (---); SIR co-treated patients ($n = 22$) (---); TAC co-treated patients ($n = 49$) (—)

onstrated a significantly higher incidence in carriers of at least one *UGT1A8**2 variant allele (Figure 3A; $P = 0.0101$). Similar results (Figure 3B; $P = 0.0352$) were obtained when only the patients who started MMF in the first days post-transplantation ($n = 222$) were considered, while a similar trend, with a seemingly even larger difference (Figure 3C; $P = 0.1448$) was observed in the others, who started MMF later after transplantation ($n = 34$). The Kaplan Meier analysis of the time to the first episode of diarrhoea shows a significantly higher incidence in patients co-treated with TAC/SIR than in patients co-treated with CsA (Figure 1; $P < 0.0001$). Similar results were obtained for the GI AEs studied globally (data not shown, $P < 0.0001$). No difference in the Kaplan Meier analysis of the time to graft loss was found between patients with and without diarrhoea ($P = 0.3016$), nor with and without GI AEs studied globally ($P = 0.1641$).

Pharmacogenetic association

Considering again the first episode of diarrhoea, univariate analysis showed non-carriers of *UGT1A8**2 had a significantly higher incidence of diarrhoea than heterozygous or homozygous carriers of the allele (HR = 1.968; 95% CI 1.163, 3.322; $P = 0.0117$). However, this SNP was not associated with GI AEs considered as a whole (HR = 0.803, 95% CI 0.559, 1.154; $P = 0.2365$). No significant associations were found between *UGT1A7* (622C>T), *UGT1A8**3 (830G>A), *UGT1A9* (-275T>A), *UGT2B7* (-840G>A) or *ABCC2* (-24C>T, 3972C>T) SNPs and all GI AEs (data not shown) or diarrhoea (Table 3). The influence of the main *ABCC2* haplotypes compared with the most frequent haplotype (-24C/3972C), called here CC, was not significant on diarrhoea

Table 3

Univariate analysis (Cox model) of the influence of the different variables studied on the incidence of diarrhoea

Variable	Category	Hazard ratio*	95% CI	P	Number of patients
MMF dose	<2000 mg vs. $\geq$ 2000 mg	1.146	0.702, 1.869	0.5861	68 vs. 68*
Co-administered immunosuppressant	TAC vs. CsA	3.817	2.262, 6.452	<0.0001	49 vs. 185
	SIR vs. CsA	4.808	2.463, 9.434	<0.0001	22 vs. 185
	SIR vs. TAC	1.214	0.612, 2.408	0.5789	22 vs. 49
	SIR/TAC vs. CsA	4.251	2.637, 6.853	<0.0001	71 vs. 185
UGT1A8*2 518C>G	CC vs. CG/GG	1.968	1.163, 3.322	0.0117	151 vs. 94/11
UGT1A8*3 830G>A	GA/AA vs. GG	1.190	0.374, 3.788	0.7679	9/0 vs. 247
UGT1A7 622C>T	CC vs. TT	0.962	0.465, 1.987	0.9161	37 vs. 101
	CT vs. TT	1.065	0.640, 1.772	0.8084	113 vs. 101
UGT1A9 -275T>A	TA/AA vs. TT	1.389	0.712, 2.712	0.3355	28/0 vs. 228
UGT2B7 -840G>A	AA vs. GG	1.104	0.569, 2.142	0.7700	65 vs. 73
	AG vs. GG	1.245	0.705, 2.200	0.4496	117 vs. 73
ABCC2 -24C>T	CC vs. CT/TT	1.085	0.673, 1.751	0.7376	148 vs. 88/15
ABCC2 3972C>T	CC vs. TT	0.765	0.353, 1.657	0.4964	82 vs. 29
	CT vs. TT	0.774	0.374, 1.602	0.4901	140 vs. 29
ABCC2 haplotype -24C>T/3972C>T	C-T vs. C-C	1.271	0.813, 1.989	0.2930	Haplotype frequency in 251 patients: CC: 0.47 CT: 0.30 TC: 0.14 TT: 0.09
	T-T vs. C-C	0.980	0.523, 1.544	0.9324	
	T-C vs. C-C	0.644	0.098, 4.215	0.6463	

*Case-control sub-study. NB in five patients with diarrhoea, the dose could not be taped off. For the most frequent category of the variable, taken as reference, HR = 1.

Figure 2MMF dose in patients with diarrhoea ($n=68$) and in controls ($n=68$). diarrhoea (□); non diarrhoea (▨)

(Table 3) or GI AEs as a whole (data not shown), neither when considering all patients together, nor when considering patients on CsA or patients on TAC or SIR separately (data not shown).

A multivariate Cox model taking into consideration in the same model the co-administered immunosuppressant and the *UGT1A8* genotype showed that the patients on tacrolimus or sirolimus had a 2.8-fold higher risk of diarrhoea than the patients on CsA (HR = 2.809; 95% CI 1.730, 4.545; $P < 0.0001$), while non-carriers of *UGT1A8*2* had a

1.9-fold higher risk of diarrhoea as compared with homozygous or heterozygous carriers (C518G or 518GG genotypes) (HR = 1.876; 95% CI 1.109, 3.175; $P = 0.0192$).

In order to evaluate the respective roles of the *UGT1A8*2* allele and the immunosuppressive co-treatment over time, four groups were set up and further compared using the Cox model: the patients co-treated with CsA and carrying at least one *UGT1A8*2* allele had the lowest incidence of diarrhoea, followed in increasing order of incidence by patients with: CsA and no *UGT1A8*2* allele (HR = 2.414; 95% CI 1.089, 5.354; $P = 0.0301$); TAC or SIR and one or two *UGT1A8*2* allele (HR = 6.287; 95% CI 2.503, 15.792; $P < 0.0001$); and TAC or SIR and no *UGT1A8*2* allele (HR = 8.332; 95% CI 3.769, 18.417; $P < 0.0001$) (Figure 4). However, there was no significant association between the risk of diarrhoea and *UGT1A8*2* in patients co-treated with TAC or SIR (*UGT1A8*2* carriers vs. non-carriers: HR = 0.755; 95% CI 0.373, 1.526; $P = 0.4331$).

Discussion

Based on the data collected retrospectively in 256 renal transplant patients receiving MMF, we found that patients given TAC or SIR or non-carriers of *UGT1A8*2* had a higher incidence of diarrhoea than those given CsA or carrying the *UGT1A8*2* allele, respectively. Several studies investigated the relations between gene polymorphisms of the UGTs or efflux transporters and interindividual variability of MMF exposure, but only a few have focused on the direct association of these polymorphisms with MMF-related AEs. Here we studied the potential link of these

Figure 3

Kaplan-Meier analysis of the time until the first episode of diarrhoea according to *UGT1A8* 518C>G genotype (*UGT1A8**2). 3A: all patients ($n=256$); *UGT1A8**2 carriers (C518G and 518GG genotypes) ($n=105$) (—); *UGT1A8**2 non-carriers (CC518 genotype) ($n=151$) (····); 3B: patients who started MMF in the first days post-transplantation ($n=222$); *UGT1A8**2 carriers (C518G and 518GG genotypes) ($n=93$) (—); *UGT1A8**2 non-carriers (CC518 genotype) ($n=129$) (····); 3C: patients who were switched from azathioprine to MMF in the stable post-transplantation period ($n=34$). *UGT1A8**2 carriers (C518G and 518GG genotypes) ($n=12$) (—); *UGT1A8**2 non-carriers (CC518 genotype) ($n=22$) (····)

Figure 4

Kaplan-Meier analysis of the time until the first episode of diarrhoea according to the *UGT1A8* genotypic groups and immunosuppressive co-treatments (TAC, tacrolimus; SIR, sirolimus; CsA, cyclosporin). Cox proportional hazard models showed significant differences between: i) CsA co-treated/*UGT1A8**2 carriers and CsA co-treated/*UGT1A8**2 non-carriers (* $P=0.03$), ii) CsA co-treated/*UGT1A8**2 carriers and TAC or SIR co-treated/*UGT1A8**2 carriers or non-carriers (\$ $P<0.0001$) and iii) CsA co-treated/*UGT1A8**2 non-carriers and TAC or SIR co-treated/*UGT1A8**2 carriers or non-carriers (# $P<0.01$). In patients co-treated with TAC or SIR, no significant difference was found between *UGT1A8**2 carriers and non-carriers. CsA/*UGT1A8**2 carriers ($n=78$) (---); CsA/*UGT1A8**2 non-carriers ($n=107$) (····); TAC/SIR/*UGT1A8**2 carriers ($n=27$) (-·-·-); TAC/SIR/*UGT1A8**2 non-carriers ($n=44$) (—)

genes with the occurrence of MMF-related diarrhoea episodes. The low numbers of other kinds of AEs prevented us from performing statistical analyses for each of them.

The co-administered calcineurin was also taken into consideration, since CsA is known to influence MMF pharmacokinetics through drug–drug interactions and TAC to induce diarrhoea. To the best of our knowledge, this is the first pharmacogenetic study of MMF-related toxicity in a long-term cohort of renal transplant patients. The number of patients studied over this extended period (1996–2006) represents a large sample of the renal transplant population in our center.

MMF-related diarrhoea was the major digestive AE reported in the patients' files.

Diarrhoea was previously described as the main digestive AE of MMF [20], with frequencies close to those observed herein (15% when associated with CsA and 38% with TAC) [6]. The role of MMF in diarrhoea episodes occurring in transplant patients is difficult to ascertain since numerous aetiologies could result in similar symptoms. In this study, all the clinical files were retrospectively screened by one individual nephrologist, allowing a homogenous definition and report of this AE. It excluded clinically

evident infectious diarrhoeas. Moreover, CMV antigen or PCR were negative at the time of diarrhoea in all patients. Because examination of the stools or extensive biological work-up was not systematically performed, we cannot exclude misclassification in some cases as suggested by the study of Maes *et al.* [13]. However, diarrhoeas of infectious origin usually do not disappear after MMF dose reduction. Furthermore, misclassification in the present study, if any, must have been similar in the different genotypic groups and would only result in a loss of statistical power and not in a statistical bias. Similarly, the environmental factors which can also be associated with diarrhoea (not investigated herein) may also have resulted in a loss of power but in no bias, which can only emphasize our findings.

Before MMF release, it had already been reported that patients receiving TAC had a higher incidence of diarrhoea than patients taking CsA: in a multicentre randomized trial comparing TAC vs. CsA in association with azathioprine and steroids, the 1 year incidence of diarrhoea was twice as much in TAC patients (21.8% vs. 10.3%; $P < 0.005$) [21]. In comparison, the incidence here was higher, at 53.1%, 54.5% and 17.8% for TAC, SIR and CsA, respectively, suggesting that MMF represents an independent risk factor of diarrhoea. This study showed that the associated immunosuppressant was the main factor associated with diarrhoea in patients on MMF: CsA was associated with approximately a 2.8-fold lower risk of diarrhoea as compared with SIR or TAC. Similar results were previously reported by Heller *et al.* who found that renal transplant patients receiving MMF in combination with TAC have a 2.4 higher incidence of diarrhoea than those on CsA ($n = 110$). It was also previously demonstrated that patients receiving MMF in combination with SIR or TAC were exposed to higher plasma concentrations of MPA than those with CsA [22–24]. CsA presumably decreases MPAG biliary excretion by inhibiting MRP2, as suggested by data from mutant rats not expressing MRP2 [17]. Consequently, less MPAG is subject to deconjugation by the intestinal flora, resulting in decreased re-circulation of MPA, impacting its plasma concentrations. However, several studies failed to demonstrate any direct association between plasma concentrations of MPA or MPA metabolites and MMF related AEs [6], including the Apomygre trial where the incidence of MMF related GI AEs was identical in the two protocol arms despite significantly higher MPA exposure over the first 3 months post-transplantation in the concentration-controlled group [25]. This suggests that the decreased risk of MMF-related diarrhoea in patients receiving CsA as compared with SIR or TAC would be more related to a local mechanism. CsA might decrease intestinal exposure to MPAG/AcMPAG, as well as to MPA (derived from intestinal hydrolysis of these metabolites), which could result in a lower risk of diarrhoea by a yet unknown mechanism. A potential limitation of the present work is that we did not investigate MPA exposure as a covariate in the multivariate

analysis, due to the fact that, apart from patients included in clinical trials, the determination of MPA concentrations was not regularly performed in this retrospective cohort, in particular before 2002. Another limitation is that no detailed information was available about the diarrhoea intensity and its evolution after treatment modification (if any).

It was of note that the sex ratio was different between the three groups of associated immunosuppressants ($P = 0.01$). This difference may be explained by the physicians' prescription habits as they usually prefer to prescribe TAC instead of CsA to female patients because of the well-known risk of hypertrichosis associated with the latter. However, there was no difference in the incidence of diarrhoea between males and females.

We observed a longer follow-up period for patients co-treated with CsA as compared with those co-treated with TAC or SIR, which is due to the fact that CsA was more often prescribed than TAC or SIR over the first years of the follow-up period.

The daily dose of MMF itself does not seem to influence the risk of diarrhoea, although in clinical practice, a dose decrement is sometimes used to stop or reduce it. The relative risk of the MMF dose, estimated from 121 renal transplants by Borrows *et al.* was very low (1.17 per 1 g increase of MMF dose) [5]. In this study, we compared the dose received before the adverse events in patients with diarrhoea to that of paired patients without. Although the pairing strategy did not allow for more than one control per case, resulting in a loss of statistical power, the effect of MMF dose was not significant in this sub-group of 136 patients, which is consistent with another study where patients who suffered from diarrhoea had not received a significantly different MMF daily dose from those who did not [6]. Moreover, we found that patients on tacrolimus had the highest incidence of diarrhoea although they received a lower MMF dose at the time of diarrhoea than patients on SIR or CsA. The high reactivity of AcMPAG could possibly contribute to MMF GI AEs. Alternatively, the amount of MPA produced in the gut from the hydrolysis of MPAG during MPA enterohepatic cycling could also possibly trigger local inflammation, although MMF is already partly hydrolyzed to MPA in the gut lumen or in intestinal epithelial cells, due to the ubiquity of esterases in the body. In this study we investigated the major polymorphisms of the isoforms thought to be involved in MPA intestinal metabolism or metabolites excretion and found that patients carrying the *UGT1A8*2* allele had a lower risk of diarrhoea than homozygous wild-type carriers. Bernard *et al.* found that *UGT1A8* produces both MPAG and AcMPAG using stably-expressed enzyme in HEK-293 cells. In their study, cells transfected with *UGT1A8*2* had a decreased capability to produce AcMPAG as compared with *UGT1A8*1* ($V_{\max}$ and CL_{int} values were divided by 2) but similar activity for MPAG formation [26]. Thus, the lower incidence of MMF-related diarrhoea found here in patients

carrying the *UGT1A8*2* allele could possibly be linked to a lower local production of AcMPAG, which would prevent its toxicity on the intestinal mucosa. The relative risk of diarrhoea linked with the co-administered immunosuppressant is greater than that of the *UGT1A8* polymorphism, but the UGT activity might be an important factor in CsA treated patients (whose biliary excretion of metabolites is reduced) contrary to patients receiving SIR or TAC, as suggested by the ranking of their combined effects (Figure 4).

We observed no effect of *UGT1A8*2* when GI AEs were studied as a whole, which included diarrhoea, abdominal pain, nausea/vomiting and anorexia. It has already been suggested that MMF diarrhoea could be due to a local mechanism involving MPA glucuronidation. This hypothesis is probably less likely for the other GI AEs. This can explain why the results were not similar when studying diarrhoea alone or together with the other GI AEs. We hypothesized that GI AEs as a whole represented a too heterogeneous phenotype, masking the association between diarrhoea and the genotype.

ABCC2 was another good candidate gene in line with our hypothesis, but we did not find any significant effect of its SNPs or haplotype on the risk of diarrhoea in the whole population, or when considering separately patients on CsA and patients on TAC/SIR. This last result showed that this absence of genotypic effect was not due to a masking of MPR2 inhibition, which has been reported for CsA [17] but not for TAC or SIR.

In conclusion, the inhibition of the biliary excretion of MPA metabolites by CsA and the local production of these metabolites depending on the activity of *UGT1A8* may be important risk factors of MMF-related diarrhoea. The exact mechanisms underlying these findings deserve further investigation.

Competing interests

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