Loss of function *ABCG2 c.421C>A* (rs2231142) polymorphism increases steady-state exposure to mycophenolic acid in stable renal transplant recipients: exploratory matched cohort study

Vladimir Trkulja[1]

[1]Affiliation not available

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Ana Borić-Bilušić A[1], Nada Božina[2], Zdenka Lalić[3], Mila Lovrić[3], Sandra Nađ-Škegro[4], Luka Penezić[4], Karmela Barišić[5], Vladimir Trkulja[2]

Running title: *ABCG2 c421C>A* (rs2231142) and mycophenolic acid

[1]Agency for Medicinal Products and Medical Devices of Croatia, Zagreb, Croatia

[2] Department of Pharmacology, Zagreb University School of

Medicine, Zagreb, Croatia

[3] Department of Laboratory Diagnostics, Analytical Toxicology and

Pharmacology Division, University Hospital Center Zagreb, Zagreb, Croatia

[4] Department of Urology, University Hospital Center Zagreb, Zagreb, Croatia

[5] Department of Medical Biochemistry and Hematology, Faculty of Pharmacy and Biochemistry, Zagreb University, Zagreb, Croatia.

Corresponding author: Vladimir Trkulja

Department of Pharmacology

Zagreb University School of Medicine

Šalata 11, 10000 Zagreb, Croatia

e-mail: vladimir.trkulja@mef.hr

Abstract

Introduction. Polymorphism *ABCG2 c.421C>A*(rs2231142) results in a reduced activity of the important drug efflux transporter breast cancer resistance protein (BCRP/ABCG2). One study suggested that it may affect enterohepatic recirculation of mycophenolic acid (MPA). We evaluated the effect rs2231142 on steady-state exposure to MPA in renal transplant recipients.

Methods . Consecutive, stable adult (age [?]16 years) renal transplant recipients on standard MPA-based immunosuppressant protocols (N=68, 43 co-treated with cyclosporine, 25 with tacrolimus) underwent routine therapeutic drug monitoring after a week of initial treatment, and were genotyped for *ABCG2 c.421C>A* and 11 polymorphisms in genes encoding enzymes and transporters implicated in MPA pharmacokinetics. *ABCG2 c.421C>A* variant vs. wild-type (wt) patients were matched in respect to demographic, biopharmaceutic and genetic variables (full optimal combined with exact matching) and compared for dose-adjusted

steady-state MPA pharmacokinetics (frequentist and Bayes [skeptical neutral prior] estimates of geometric means ratios, GMR).

Results . Raw data (12 variant vs. 56 wt patients) indicated by around 40% higher total exposure (frequentist GMR=1.45, 95%CI 1.10-1.91; Bayes = 1.38, 95%CrI 1.07-1.81) and by around 30% lower total body clearance (frequentist GMR=0.66, 0.58-0.90; Bayes=0.71, 0.53-0.95) in variant carriers than in wt controls. The estimates were similar in matched data (11 variant vs. 43 wt patients): exposure GMR=1.41 (1.11-1.79) frequentist, 1.39 (1.15-1.81) Bayes, with 90.7% and 85.5% probability of GMR >1.20, respectively; clearance GMR=0.73 (0.58-0.93) frequentist, 0.71 (0.54-0.95) Bayes. Sensitivity analysis indicated high unsusceptibility of the estimates to unmeasured confounding.

Conclusions . Loss-off-function polymorphism *ABCG2 c.421C>A* increases steady-state exposure to MPA in stable renal transplant patients.

Key words : mycophenolic acid, renal transplant, breast cancer resistance protein, polymorphism

Key summary points

Why carry this study?

- ABCG2 is an efflux transporter important in pharmacokinetics of various drugs
- Polymorphism *ABCG2 c.421C>A* results in reduced transporter activity
- One study suggested that this polymorphism could affect enterohepatic recirculation of mycophenolic acid

What was learned from the study?

ABCG2 c.421C>A variant allele increases steady-state exposure to mycophenolic acid in stable renal transplant recipients

Introduction

Mycophenolic acid (MPA) is a standard component of immunosuppressant protocols in organ transplantation. Considerable variability of MPA pharmacokinetics has attracted much attention and has been comprehensively reviewed a number of times [e.g., 1-5]. A range of "classical" factors interfere with exposure to MPA, including age, body mass index (BMI), renal function, changes of gut microbiota, reduced albumin levels, interactions with food, drug-drug interactions at different levels (in particular with calcineurin inhibitors [CNI] cyclosporine A [CsA] and tacrolimus, but also with other drugs) and MPA formulation (immediate-release tablets of mycophenolate mofetil [IR MMF] or enteric-coated [acid-resistant] tablets containing MPA sodium salt [EC-MPS]) [1-5]. Orally administered MPA undergoes complex processes that include prodrug activation (in the case of MMF; by carboxylesterases, CES) in the intestinal cells and in the liver; extensive biotransformation (around 90% of bioavailable fraction) to an inactive 7-O-glucuronide (MPAG) mainly by the uridine 5'-diphospho-glucornosyltransferase (UGT) 1A9 in the liver (less so in the kidney) with a minor contribution of other UGTs; less extensive biotransformation by UGT2B7 (intestine, liver) to a biologically active acyl-glucuronide (AcMPAG); minor biotransformation by cytochrome P450 enzymes CPY3A4 and CY3A5 (liver) to inactive 6-O-desmethyl MPA; extensive albumin binding (in competition with MPAG); entero-hepatic recirculation and, to a minor extent, active renal secretion of MPA and MPAG [1-5]. MPA is a substrate of the efflux transporter multidrug resistance protein 1 (MDR-1, encoded by *ABCB1*) (intestine), while MPAG and AcMPAG are substrates to efflux transporter multidrug resistance-associated protein 2 (MRP-2, encoded by *ABCC2*) and influx organic anion transporter polypeptides, primarily OATP1B1 and 1B3 – these proteins move MPAG/AcMPAG in and out of the hepatocytes and renal tubular cells [1-5].

A recent systematic review [4] identified 38 studies with different designs, sampling populations and sample sizes, measured outcomes and control of confounding, mainly in renal transplant recipients, assessing relationship between several tens of single nucleotide polymorphisms (SNPs) in 10 enzyme (*UGT* , *CYP* , *CES* families) and 6 transporter genes (*ABCB1* , *ABCC2* , *SLCO1B1* ,*SLCO1B3, SLCO2B1, ABCG2*) and exposure to- or occurrence of MPA-related adverse event. Those with at least 2 consistent reports

(*in vivo* or *in vitro/in vivo*) about association with MPA exposure/clinical effects and regardless of the number of "negative" studies include: i) *UGT1A9 c.-275T>A*(rs6714486) and *c.-2152C>T* (rs17868320) variants (in complete linkage disequilibrium, LD) result in increased enzyme activity and lower exposure to MPA; ii) *UGT2B7 802C>T*(rs7439366) variants (or loci that are in LD) may also be relevant for MPA clearance; iii) *ABCB1 2677G>T/A* (rs2032582),*3435C>T* (rs1045642) or *1236C>T*(rs1128503) variant alleles (or haplotypes/diplotypes, since in LD) increase the risk of adverse events; iv) *in vitro* , OATP1B1 with the *SLCO1B1*5 c.521T>C* (rs4149056) polymorphism shows reduced MPAG/AcMPAG uptake into hepatocytes. This might reduce enterohepatic recirculation, and in one study, this SNP was associated with a lower risk of MPA adverse events (no association in 6 other studies, and further 5 failed to associate this SNP with MPA levels); v)*SLCO1B3 c.334T>G* (rs4149117) is in complete LD with*SLCO1B3 c.699G>A* (rs7311358). OATP1B3 with the variant haplotype shows reduced MPAG up-take *in vitro* . In one study, *c.334T>G* TT/TG patients had somewhat higher MPA exposure vs. GG subjects (not observed in three further studies, and one indicated just the opposite); vi) *UGT1A9*3*(*c.98T>C* , rs72551330) SNP results in reduced enzyme activity. Prevalence of variant carriers is very low ([?]3% in most of the studies) [4,5]. In two studies, it was suggested that variant carriers had lower exposure to MPA than wt subjects, but no association between this SNP and MPA exposure/clearance was found in several other studies [4,5]. vii) *ABCC2 c.-24C>T* (rs717620) was reported associated with somewhat higher exposure to MPA, but the opposite has also been reported; viii) so far, donors' SNPs in renal transplantation were rarely investigated – one study associated donor's*ABCC2 1249G>A* (rs2273697) with increased MPA clearance [4].

In the present analysis we aimed to assess potential effect of an SNP in the gene encoding breast cancer resistance protein (BCRP, ABCG2)*ABCG2 c.421C>A* (rs2231142; p.Q141K) on steady-state exposure to MPA in stable renal transplant recipients. As reviewed [4], four studies have failed to detect associations between this SNP and exposure to MPA. Our motivation was based on the following: i) ABCG2 is important for transmembrane transport of numerous drugs in the intestine, liver and the kidney [6-9] and*c.421C>A* SNP results in reduced transporter activity due to increased proteosomal degradation [9, 10]; ii) one study in Japanese renal transplant patients suggested that ABCG2 participated in pharmacokinetics of MPAG [11], and this may reflect on exposure to MPA.

Patients and Methods

Study outline

We included consecutive adult and adolescent (age [?]16 years) *de novo* renal transplant recipients submitted to routine therapeutic drug monitoring (TDM) of immunosuppressants after completion of the initial week of treatment. All participants provided signed informed consent for genotyping of pharmacogenes. Clinical and bioanalytical procedures were described in detail previously [12, 13]. Briefly, patients on standard immunosuppressant protocols including MPA (IR MMF or EC-MPS), CNI (CsA [microemulsion] or tacrolimus) and glucocorticoids were closely monitored over 5-7 post-transplant days; on the subsequent day (steady-states of MPA, CsA/tacrolimus achieved), after overnight fast, at 08:00 hours blood samples were taken for quantification of MPA and CsA/tacrolimus, treatments were administered and 6 blood samples were taken over the 12-hour dosing interval (at 0.5, 1, 2, 3, 8 and 12 hours post-dose) for quantification of MPA. They were included in the present analysis if: 1) clinical status was considered stable during the observed period based on (i) lack of surgical complications and signs of graft dysfunction or rejection; (ii) no severe comorbidity (cardiovascular, hepatic, metabolic, infectious, gastrointestinal); (iii) low immunological risk, (iv) stably improving renal function (serum creatinine [?]300 μmol/L and by at least 1/3 lower than on the 1st postoperative day, with stable diuresis at around 60 mL/hour); (v) serum albumin >31 g/L; 2) were not treated with drugs that affect exposure to MPA (proton pump inhibitors, antacids, phosphate binders, oral iron, magnesium or calcium, rifampicin or any antibiotics) during the prestudy and study days. Patients were genotyped for the *ABCG2 c.421C>A* (rs2231142) and further SNPs suggested (although not unambiguously) to be associated with MPA pharmacokinetics: *UGT1A9 -275T>A*(rs6714486) and *-2152C>T* (rs17868320); *UGT2B7 -161C>T* (rs7668258) [in complete LD with *UGT2B7 802C>T* (rs7439366)] [14]; *ABCB1 2677G>T/A* (rs2032582), *3435C>T*(rs1045642) and *1236C>T* (rs1128503); *SLCO1B1 c.521T>C*

(rs4149065) [in complete LD with c.388A>G (rs2306283)] [4]; *CYP3A4*22*(rs35599367) and *CYP3A5*3* (rs776746); *ABCC2 -24C>T* (rs717620) and *1249G>A*(rs2273697) (both recipients and donors). To estimate the effect of the*ABCG2 c.421C>A* SNP on exposure to MPA at steady-state, patients were classified as *c.421C>A*variant carriers ("treated") and wild-type (wt) subjects ("controls"), and we used matching to achieve conditional exchangeability. We followed the principles introduced by Pearl [15] with operational development [16, 17] and implementation in package*daggity* [18] in R [19] [see Electronic supplementary material (ESM) – Supplemental Methods A, for details].

Study was approved by the Ethics Committee of the University Hospital Center Zagreb (approval No. 8.1-17/242-2 02/21, January 30, 2018). All procedures performed in the study were in accordance with the 1964 Declaration of Helsinki and its later amendments. All patients included in the present analysis underwent standard routine therapeutic drug monitoring in their post-transplant period. Those meeting inclusion criteria were included only if they signed an informed consent for genotyping of pharmacogenes for research purposes.

Bioanalytical procedures and genotyping

Whole blood cyclosporine and tacrolimus were determined by a validated affinity chrome-mediated immunoassay (ACMIA, Siemens, Germany). Total plasma MPA was determined by high pressure liquid chromatography (HPLC) with UV/VIS spectrophotometric detection (at 215 nm, 25°C, workflow 1 mL/min) using a commercially available HPLC kit for MPA in plasma (Chromsystems, Germany). All analytes were included in the external proficiency testing schemes (RfB and Instand).

Creatinine clearance was estimated (Cockroft-Gault) based on serum creatinine quantified by an enzymatic assay on an automated analyzer (Cobas c 501; Roche, Germany) validated by isotope dilution mass spectrometry.

Genomic DNA was isolated from whole blood using BioSprint 15 DNA Blood Kit (Qiagen, Hilden, Germany) on KingFisher mL System (Thermo Labsystems, Vantaa, Finland). Genotyping was performed on an Applied Biosystems 7500 Real Time PCR System, according to manufacturer's instructions (Applied Biosystems, CA, USA by) using a validated TaqMan® Drug Metabolism Genotyping Assays (Life Technologies, Carlsbad, CA, USA) for the following polymorphisms: *ABCG2 c.421C > A* (rs2231142, ID C_15854163_70); *ABCC2-24C>T* (rs717620, ID C_2814642_10) and*1249G>A* (rs2273697; ID C_22272980_20);*SLCO1B1 c.521T > C* (rs4149065, ID C_30633906_10); *UGT2B7 −161C>T* (rs7668258, ID C_27827970_40);*UGT1A9-275 T>A* (rs6714486, ID C__27843087_10) and-*2152C>T* (rs17868320, ID C__34418857_10);*ABCB1 3435C>T* (rs1045642, ID C___7586657_20) and*1236C>T* (rs1128503, ID C___7586662_10)*; CYP3A4*22* (rs35599367, ID C__59013445_10) and *CYP3A5*3*(rs776746, ID C__26201809_30). Genotyping of *ABCB1 c.2677G> T/A* (rs2032582) was performed by real-time PCR genotyping on the LightCycler® instrument (Roche Diagnostics, Mannheim, Germany).

Pharmacokinetic indicators

Standard MPA steady-state measures [peak exposure ($C_{max,ss}$ mg/L), area under the concentration-time curve over the dosing interval of 12 hours ($AUC_{\tau,\sigma\varsigma}$ mg × h/L), morning and evening pre-dose concentrations (C_0, C_{12}, mg/L), apparent total body clearance ($CL_{T/F,ss}$ mL/min/kg)] were determined by the non-compartmental method (Kinetica 4.1, InnaPhase Corp., USA). We calculated also the $C_{max}/AUC_{\tau,\sigma\varsigma}$ (1/h) ratio as an indicator of the absorption rate [20]. The analysis was based on dose-normalized concentrations (per 1000 mg) accounting for the fact that 1000 mg of MMF corresponded to 739 mg of MPA and 1000 mg of EC-MPS corresponded to 936 mg of MPA.

Matching and data analysis

ABCG2 c.421C>A variant carriers and wt controls were matched in combined exact and optimal full matching with Mahalanobis as a distance measure using package *MatchIT* [21] in R [19]. The procedure allows "one-to-many" variant to control matching (and*vice-versa*) and attains (exact) or approximates (Mahalanobis) balance achieved by fully blocked randomization (in respect to measured confounders) (see ESM

– Supplemental Methods B, for details) [21-23]. Since in substantially different ranges (70-341 vs. 3.4-37.4 μg/L), to be used in matching CsA and tacrolimus troughs were rescaled [ln(tacrolimus) troughs rescaled to ln(CsA troughs) range by linear transformation]. Inadequately matched covariates (standardized mean difference, d [?]0.1) were adjusted for in data analysis. The variant allele effect on (ln-transformed) pharmacokinetic outcomes was estimated in raw and matched/adjusted data in frequentist (maximum likelihood with Gauss-Hermite approximation for raw data; cluster robust variance estimator for matched data) and Bayesian (4 chains, 4000 iterations, 8000 samples of the posterior, highest posterior density [HPD] credible intervals) general linear models, and was expressed as geometric means ratio (GMR). In the latter, we defined a moderately informed skeptical prior for the effect of interest consistent with the *a priori* hypothesis of no effect: centered at 0 for ln(GMR) with a standard deviation of 0.355. It assigns with 95% probability to a GMR between 0.5 and 2.0, and 48% probability to a GMR within the "conventional" limits of equivalence (0.80 to 1.25). We used SAS 9.4 for Windows (SAS Inc., Cary, NC) to fit frequentist models and R package *nstanarm* [24] to fit Bayesian models. We used CubeX [25] to evaluate linkage disequilibrium (LD).

Sensitivity to unmeasured confounding

We calculated E-values (package *Evalue* [26] in R], and also bias-corrected estimates [27] (package *episensr* [28] in R) to account for bias arising from the fact that we did not control for the *SLCO1B3 c.334T>G* (rs4149117) and *UGT1A9 c.98T>C* (rs72551330) SNPs. For the latter purpose, we used literature data to generate meta-analytical estimates (package *meta* [29] in R) of strength of association (we used ratio of means, ROM [30], as an effect measure) between these SNPs and steady-state exposure to MPA (see ESM – Supplemental Methods C, for details).

Results

Patients

Of the 68 included patients, 12 (17.7%) were *ABCG2 c.421C>A* variant allele carriers and 56 were wt subjects (Table 1). Variants in *CYP3A4* and *CYP3A5* were rare (Table 1). The *UGT1A9 -275T>A* and *UGT1A9 -2152C>T* SNPs were in complete LD (Table 1). Consequently, patients were considered as having a wt or a variant diplotype. The three *ABCB1* SNPs (Table 1) were in a strong LD (pairwise D'=0.85-0.95, r^2=0.615-0.687). Therefore, patients were categorized in respect to the number of variant alleles : (i) all three genotypes are wt, or one is heterozygous (none or one variant allele); (ii) two to three variant alleles (any two or all three loci are heterozygous; or one variant homozygous and one heterozygous locus); (iii) four to six variant alleles. Variant carriers prevailed regarding *UGT2B7 -161 C>T* SNP, while wt homozygotes prevailed regarding *SLC01B1 521T>C* and *ABCC2 -24C>T* and *ABCC2 1249G>A* SNPs (Table 1). In respect to these SNPs, patients were categorized as variant carriers or as wt homozygotes.

Characteristics of *ABCG2 c.421C>T* variant carriers and wt controls are summarized in Table 2 and Figure 1A summarizes their (dose-adjusted) MPA concentration-time profiles over the dosing interval.

Effect of ABCG2 c.421C>A variant on exposure to MPA

In estimating the effect of *ABCG2 c.421C>A* variant, we controlled for a number of potentially interfering factors (Table 3). Before matching, variant and wt patients differed considerably in respect to most of the matching variables (Table 4), while total exposure ($AUC_{\tau,\sigma\varsigma}$) appeared higher and total body clearance ($CL_{T/F,ss}$) appeared lower in variant carriers than in wt controls (Table 4). Frequentist and Bayesian estimates suggested by 45% (95%CI 10-92) and by 38% (95%CrI 7-81) higher total exposure, respectively (Table 5), and by 34% (10-52) and 29% (5-47) lower total body clearance, respectively (Table 5) in variant carriers than in wt controls. Eventually, 11/12 variant carriers were matched to 43/56 wt controls with excellent balance regarding most matching covariates except (d [?]0.1) for the donors' *ABCC2 1249 G>A* genotype, body mass index and estimated creatinine clearance (Table 4): average concentration-time profiles were not much changed vs. raw data (Figure 1B), $AUC_{\tau,\sigma\varsigma}$ was still higher (d=0.824) and $CL_{T/F,ss}$ was lower (d=-0.559) in variant carriers than in wt controls (Table 4). With additional adjustment for suboptimally matched covariates, frequentist and Bayesian estimates indicated by 41% (95%CI 11-79) and by 39% (95%CrI

5-81), respectively, higher total exposure, and by 27% (7-42) and by 29% (5-46) lower total body clearance, respectively, in variant carriers than in wt controls (Table 5). Probability that the GMR for $AUC_{\tau,\sigma\varsigma}$ was >1.20 was 90.7% based on the frequentist analysis and it was 85.6% based on the Bayesian analysis (Figure 2).

Sensitivity to unmeasured confounding

Using published studies (see ESM –Supplemental results: sensitivity analysis, Figure S2, S3, S4), we estimated association between the*SLCO1B3 c.334T>G* TT/TG genotype (vs. GG genotype, total N=241 vs. 400) and steady-state MPA $AUC_{0\text{-}12}$ as ROM=1.136 (95%CI 0.949-1.361), and association between the *UGT1A9 c.98T>C* genotype TC (vs. TT, total N=25 vs. 593) and the outcome as ROM=1.098 (95%CI 0.548-2.198), with similar estimates in patients co-treated with CsA or tacrolimus/sirolimus in both cases; estimated prevalence of TT/TG (4 cohorts, total N=1192) and TC subjects (9 cohorts, total N=1827) was 31.1% and 3.9% respectively. Although there is no reason to expect higher prevalence of TT/TG (*SLCO1B3*) or TC (*UGT1A9*) patients among *ABCG2 c.421C>A*variant carriers than among wt controls, we assumed scenarios with high chance imbalances (see ESM – Supplemental results: sensitivity analysis, Table S1, Table S2) and TT/TG and TC effects much higher than estimated. Even under such conditions, bias-corrected estimate of the*ABCG2 c.421C>A* variant effect on exposure to MPA is still higher than the conventional limit of equivalent exposure (Figure 3). E-values suggested that the cumulative effect of unmeasured confounders would have to be strong, i.e., GMR=1.63 and GMR=1.59 (frequentist and Bayesian, respectively) in order to at least partly explain-away the observed, i.e., to "push" the observed GMR point-estimates (1.40) to 1.20.

Discussion

Present data strongly suggest that the variant *ABCG2 c.421C>A* (rs2231142) allele increases $AUC_{\tau,\sigma\varsigma}$ of MPA in stable renal transplant patients (by around 40%, with a high probability that the effect is >20%) in agreement with proportionally reduced $CL_{T/F,ss}$. The estimates are consistent based on raw data (patients free of relevant interfering comorbidities and co-medication) and in matched/adjusted analysis, where a number of further potential confounders, "classical" and pharmacogenetic, were controlled for. Considering the latter, we did not account for the *SLCO1B3 c.334T>G* (rs4149117) and *UGT1A9*3*(*c.98T>C* , rs72551330) SNPs. OATP1B3 mediates MPAG uptake, and variant *SLCO1B3 c.334T>* G shows around 40% reduced activity *in vitro* [33]. We identified 4 studies (two in European patients [31, 33], and one each in Chinese [34] and Japanese [35] patients) reporting crude mean±SD dose-adjusted MPA $AUC_{\tau,\sigma\varsigma}$ in TT/TG vs. GG patients on IR MMF co-treated with CsA (3 cohorts) or macrolactam immunosuppressants (3 cohorts): pooled TT/TG vs. GG differences in the co-treatment subgroups (consistently) and overall suggested a slight tendency of higher exposure (by some 10-15%) in TT/TG subjects (see Figure S2). The most compelling individual study findings were those [31] suggesting by around 24% higher (crude) AUC in 56 TT/TG vs. 111 GG patients co-treated with CsA, and around 18% higher AUC in 54 TT/TG vs. 107 GG patients co-treated with macrolactams. The *UGT1A9 c.98T>C* SNP results in reduced enzyme activity *in vitro* [36]. We identified 3 studies (European patients) [32, 33, 37] reporting crude $AUC_{\tau,\sigma\varsigma}$ in TC vs. TT patients on IR MMF co-treated with CsA (2 cohorts) or with macrolactams (3 cohorts): pooled TC vs. TT differences consistently suggested a mild tendency of higher (by 10%) exposure in TC subjects (see Figure S3). The most compelling individual study findings were those [32] reporting around 50% higher AUC (time-averaged estimate of 6 measurements over 1 year) in 5 TC vs. 170 TT patients co-treated with CsA and in 5 TC vs. 158 TT patients co-treated with tacrolimus. The present sensitivity analysis (Figure 3) demonstrates: even with a marked simultaneous imbalance between *ABCG2 c.421C>A* variant and wt patients regarding both TT/TG (*SLCO1B3*) and TC (*UGT1A9*) genotypes and assuming their maximum reported effects, bias-adjusted estimate of the *ABCG2 c.421C>A* variant allele effect would still be >1.25 (i.e., above the conventional upper limit of equivalent exposure). However, it is not very likely that the present estimate was biased by these two SNPs to such an extent: (i) all the reported values were crude, unadjusted values; ii) there is no biologically plausible reason to expect such a huge simultaneous imbalance in prevalence of the two genotypes between *ABCG2*variant and wt subjects; (iii) *UGT1A9*

c.98T>C SNP is rare, and a reasonably expected number of TC subjects in the present sample is 2-3; (iv) population pharmacokinetic models in French [38] and Chinese patients [39] found no association between these two SNPs and MPA clearance. Also, it does not seem likely that other enzyme/transporter SNPs could explain the present observations. Three *UGT1A9* promoter SNPs [beyond *-275T>A*(rs6714486) and *-2152C>T* (rs17868320) that we controlled for] are associated with increased UGT1A9 levels in the liver: - 440C>T (rs2741045), -331T>C (rs2741046) and -665C>T (rs10176426) [4,5,40]. However, studies have failed to provide consistent signals about association of any of these SNPs and exposure to MPA; moreover, rs6714486 and rs17868320 are in complete LD with these SNPs and form two haplotypes (*UGT1A9*1l* and **1n*) [40]. Therefore, by controlling for rs6714486 and rs17868320, one controls also for several SNPs that were not directly genotyped. No consistent signal of association with MPA exposure has been found for several other *UGT1A9* SNPs (rs6731242, rs13418420, rs3832043, rs2741049, rs13418420, rs17868323) [4,5,39,41]. Moreover, rs6714486 and rs17868320 are in LD with some of them (haplotypes *UGT1A91v* and **1w*) [40]. Apart from *UGT2B7 802C>T* (rs7439366), here "represented" by rs7668258 (since in complete LD), studies have consistently failed to yield a clear, reproducible signal of association of any other *UGT2B7* SNP and exposure to MPA. The same applies for a number of evaluated *UGT1A1, 1A7* and *1A8* SNPs [4,5,39,41]. In the present analysis, we evaluated the effect of one of the *ABCG2*polymorphisms (rs2231142). Reduced transporter function has been reported associated with three further SNPs (rs34783571, rs192169062 and rs34264773), for three SNPs no effect on function is reported and for the rest functional consequences are unknown [8]. The estimated global cumulative minor allele prevalence of all "reduced function" SNPs is 0.68%, and for combined "unknown" and "reduced" it is 1.3% [8] – this implies that at most one of the present patients should be reasonably expected to carry any of these SNPs, and it is highly unlikely that this possibility affected the present estimates. Similarly, the three *ABCB1* (linked) SNPs controlled for are by far the most prevalent (among Caucasians) coding *ABCB1* variants. Cumulative prevalence of other six coding *ABCB1* SNPs in Caucasians is around 10% [42], suggesting that at most 6-7 patients in the current sample might have harbored any of those SNPs. In order to be accountable to any relevant part of the present observations, all such (hypothetical) SNPs should have had marked and synergistic effects – not a likely scenario: as recently reviewed [43], most of them have no practical relevance in drug pharmacokinetics. The same is applicable to the *ABCC2* SNPs (beyond those controlled for in the present study) and a wide range of investigated *ABCC1* and*ABCC3* SNPs [43]. Specifically, in respect to MPA, apart from*ABCC2 1249G>A* (rs2273697) and-*24C>T* (rs71762) controlled for in the present analysis, studies have consistently failed to identify a relevant signal of association between MPA exposure and a range of investigated*ABCC2* SNPs (rs3740066, rs8187710, rs1885301, rs7910642, rs113646094, rs8187694, rs17222723, rs3740066, rs2804402) and*ABCC3* SNPs (rs4793665, rs2277624) [4,5,39,41]. Finally, (apart from the *SLCO1B1 c.521T>C* , in LD with*c.388A>G* , controlled in the present study, and already discussed *SLCO1B3 c334T>G*), no consistent signal of association between a range of *SLCO1B1* and *1B3*SNPs and MPA exposure has been detected across numerous individual studies [4,5,39,41]. To attribute the observed effect to these unmeasured but unlikely confounders, one needs to assume their simultaneous synergistic effects. The present sensitivity analysis suggests: even if it existed, and even if really marked (GMR=1.60), such a (hypothetical) cumulative confounding effect would not completely explain away the observed effect since GMR for the variant *ABCG2 c421C>A* allele vs. wild type would still be 1.20. Overall, it is justified to state that present data reasonably validly document an effect of the *ABCG2 c.421C>A* variant allele on steady-state exposure to MPA in renal transplant patients. Discrepancy between the present results and earlier studies not detecting associations between exposure to MPA and *ABCG2 c.421C>A* SNP might, at least in part, be due to methodological differences. A study in Chinese patients co-treated with CsA reported slightly higher crude dose-adjusted $AUC_{\tau,\sigma\varsigma}$in17 variant carriers than in 20 wt controls (30.9±13.0 vs. 27.7±10.7 mg × h/L) [44]. Our patients were co-treated with CsA or tacrolimus (and matched for CNI and CNI troughs). Neither CsA nor tacrolimus are ABCG2 substrates, but both are ABCG2 inhibitors, and their inhibitory effect might differ, particularly under *c.421* SNP (with reduced transporter numbers) [45-47]. Two larger studies (Chinese [48] and Brazilian [49] patients) reporting no association between the*c.421* SNP and MPA measured only trough concentrations while present data refer to $AUC_{\tau,\sigma\varsigma}$ (note: in the present analysis, dose-adjusted MPA troughs tended to be higher in variant carriers, but variability was high), while a Chinese population phar-

macokinetic model included only patients co-treated with tacrolimus [39]. Clearly, it is difficult to directly compare results from observational studies differing in methodology and design, sampling populations and sample sizes, outcomes and control of confounding – each should be evaluated on its own merit. We believe that the present analysis reasonably supports a conclusion that the observed difference in $AUC_{\tau,\sigma\varsigma}$ between the *ABCG2 c.421C>A* variant and wt subjects is attributable to the fact of variant allele carriage.

Present study is limited by a modestly sized single-center sample, the fact that MPAG was not measured (as not a part of routine TDM), and, relatedly, by no insight into possible mechanisms of the observed effect. A study in Japanese patients [11] reported higher steady-state MPAG concentrations in 44 *c.421C>A*variant carriers than in 36 wt controls (median 1540 vs. 1195 mg × h/L; P=0.029; corresponds to ROM=1.29), and suggested involvement of ABCG2 in MPAG-MPA recirculation. Current observations (Figure 1, Table 5) of closely similar C_{max} (at around 2 hours post-dose), but clearly larger $AUC_{\tau,\sigma\varsigma}$ in variant carriers vs. wt controls indirectly support such a possibility: the difference in AUC is primarily due to differences that occurred between 3 and 12 hours post-dose, which is in agreement with hypothetical differences in MPAG recirculation.

Conclusions

Present data strongly suggest that the variant *ABCG2 c.421C>A* allele increases steady-state exposure to MPA in stable renal transplant patients. Further studies are needed to validated this observation and elucidate underlying mechanism(s).

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Compliance with Ethics Guidelines. Study was approved by the Ethics Committee of the University Hospital Center Zagreb (approval No. 8.1-17/242-2 02/21, January 30, 2018). All procedures performed in the study were in accordance with the 1964 Declaration of Helsinki and its later amendments. All patients included in the present analysis underwent standard routine therapeutic drug monitoring in their post-transplant period. Those meeting inclusion criteria were included only if they signed an informed consent for genotyping of pharmacogenes for research purposes.

Data availability . The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Table 1 Single nucleotide polymorphisms genotyped in the 68 included renal transplant recipients and donors.

Recipient		*ABCB1 1236C>T* (rs1128503)	
ABCG2 421C>A (rs2231142)		CC	19 (28.0)
CC	56 (82.3)	CT	37 (54.4)
CA	12 (17.7)	TT	12 (17.6)
AA	0	*UGT2B7 -161C>T* (rs7668258)	
*CYP3A4*22* (rs35599367)		CC	13 (17.6)
*1/*1	67 (98.5)	CT	40 (58.8)
*1/*22	1 (1.5)	TT	15 (22.1)
*CYP3A5*3* (rs776746)		*SLCO1B1 521T>C* (rs4149065)	
*3/*3	59 (86.8)	TT	43 (63.2)
*1/*3	9 (13.2)	TC	24 (35.3)
UGT1A9 -275T>A (rs6714486)		CC	1 (1.5)
TT	65 (95.6)	*ABCC2 -24C>T* (rs717620)	
TA	3 (4.4)	CC	47 (69.1)
AA	0	CT	19 (27.9)
UGT1A9 -2152C>T (rs17868320)		TT	2 (2.9)

Recipient		*ABCB1 1236C>T* (rs1128503)	
CC	65 (95.6)	*ABCC2 1249G>A* (rs2273697)	
CT	3 (4.4)	GG	41 (60.3)
TT	0	GA	22 (32.3)
ABCB1 2677 G>T/A (rs2032582)		AA	5 (7.4)
GG	18 (26.5)	*Donor*	
GA	1 (1.5)	*ABCC2 1249G>A* (rs2273697)	
GT	35 (51.5)	GG	41 (60.3)
TA	2 (2.9)	GA	21 (30.9)
TT	12 (17.6)	AA	6 (8.8)
ABCB1 3435C>T (rs1045642)			
CC	13 (19.1)		
CT	37 (54.4)		
TT	18 (26.5)		

Table 2 Characteristics of patients with variant and wild type*ABCG2 c.421C>A* genotypes.

	Variant carriers	Wild type
N	12	56
Men	6 (50.0)	30 (53.6)
Age (years)	46.5±13.3 (26-68)	50.5±12.8 (16-71)
Body mass index (kg/m^2)	24.9±6.3 (17.7-39.5)	24.0±3.3 (14.5-30.4)
Mycophenolate mofetil	2 (16.7)	21 (37.5)
1000 mg bid	1	17
750 mg bid	0	2
500 mg bid	1	2
Enteric coated mycophenolate	10 (83.3)	35 (62.5)
Daily dose (mg)	All 720 mg bid	All 720 mg bid
Cyclosporine A	6 (50.0)	37 (66.1)
Morning dose (mg/kg)	160 (144-175; 125-175)	150 (125-188; 75-250)
Evening dose (mg/kg)	162 (125-181; 125-200)	150 (125-200; 75-250)
Morning trough (µg/L)	212±53 (169-311)	204±64 (70-341)
Tacrolimus	6 (50.0)	19 (33.9)
Morning dose (mg)	2.0 (1.7-3.9; 1.0-5.0)	3.0 (2.5-3.0; 1.5-4.0)

	Variant carriers	Wild type
Evening dose (mg)	3.3 (2.2-4.3; 1.5-5.0)	3.0 (3.0-4.0; 2.0-4.0)
Morning trough (µg/L)	11.0±3.2 (7.8-16.9)	10.8±5.8 (3.4-27.4)
Prednisone equivalent (mg/day)	40 (30-40; 30-60)	35 (30-47; 20-75)
Urine output (L/day)	2.4 (2.1-2.6; 1.8-4.3)	2.6 (2.1-3.2; 1.1-5.7)
Estimated creatinine clearance (mL/min)	45±16 (29-84)	41±11 (23-74)
*CYP3A4*22* genotype		
*1/*1	12 (100)	55 (98.2)
*1/*22	0	1 (1.8)
*CYP3A5*3* genotype		
*3/*3	11 (91.7)	48 (85.7)
*1/*3	1 (8.3)	8 (14.3)
UGT2B7 -161C>T genotype		
Variant allele	9 (75.0)	47 (83.9)
Wild type	3 (25.0)	9 (16.1)
UGT1A9 -275 / -2152 diplotype		
Variant allele	0	3 (5.4)
Wild type	12 (100)	53 (94.6)
ABCB1 2677 / 3435 / 1236 diplotype		
Wild type or 1 variant allele	3 (25.0)	15 (26.8)
2-3 variant alleles	6 (50.0)	25 (44.6)
4-6 variant alleles	3 (25.0)	16 (28.6)
SLC01B1 521T>C genotype		
Variant allele	4 (33.3)	21 (37.5)
Wild type	8 (66.7)	35 (62.5)
ABCC2 -24 C>T genotype		
Variant allele	2 (16.7)	19 (33.9)
Wild type	10 (83.3)	37 (66.1)
ABCC2 1249 G>A genotype		
Variant allele	4 (33.3)	23 (41.1)
Wild type	8 (66.7)	33 (58.9)

	Variant carriers	Wild type
Donor's *ABCC2* *1249 G>A* genotype		
Variant allele	6 (50.0)	21 (37.5)
Wild type	6 (50.0)	35 (62.5)

Data are counts (percent), mean±SD (range) or median (quartiles, range)

Table 3 Factors known or suggested to affect pharmacokinetics of mycophenolic acid (MPA PK) in renal transplant recipients controlled for by different means in the present analysis (see also Patients and Methods: Study outline, and Electronic supplementary material – Supplemental Methods A [Figure S1B] for details).

Factors controlled for by inclusion-exclusion criteria
Drugs affecting MPA pharmacokinetics
Serum creatinine dynamics and diuresis over 5-7 postoperative days (before MPA PK assessment)
Hypoalbuminemia at baseline of MPA PK assessment
Postoperative complications, acute rejection, infectious, cardiovascular, metabolic or hepatic co-morbidity that may affect M
Drugs that interfere (apart from CNI) with ABCG2 – by inclusion-exclusion criteria pertinent to drugs affecting MPA PK (
Drugs affecting CNI pharmacokinetics – by inclusion-exclusion criteria pertinent to drugs affecting MPA PK (some are com
Factors controlled for by matching/statistical adjustment
Type of MPA formulation
Estimated creatinine clearance at baseline of MPA PK assessment
Type of CNI
CNI trough concentrations at baseline of MPA PK assessment
Age and body mass index
Polymorphisms: *UGT1A9 -2152/-275* diplotypes; *UGT2B7 -161* genotype; *ABCB1 2677 / 3435 / 1236* diplotypes; *SLC01*
Measured factors, but not included in matching/adjustment
*CYP3A4*22* and *CYP3A5*3* polymorphisms: a) only a few subjects overall had variant alleles. Considering that CYP3A4/
Prednisone-equivalent doses: a) doses were closely similar between *ABCG2 421C>A* variant carriers and wild-type controls

Table 4 Characteristics of *ABCG2* variant allele carriers and wild type (wt) homozygotes before (full dataset) and after matching. Data are counts (%), mean±SD or geometric mean (geometric coefficient of variation, %) for pharmacokinetic variables. Differences are expressed as standardized mean difference (d). For pharmacokinetic parameters, *d* was calculated based on mean (SD) of ln-transformed data. Values <0.1 indicate irrelevant differences. Variables included in the matching procedure are shaded.

	Before matching	Before matching	Before matching	Before matching	Before matc
	Variant		Wt		d
N	12		56		—
MMF	2 (16.7)		21 (37.5)		-0.715
EC-MPS	10 (83.3)		35 (62.5)		0.715
Cyclosporine	6 (50.0)		37 (66.1)		-0.715
Tacrolimus	6 (50.0)		19 (33.9)		0.715
SLCO1B1 variant	4 (33.3)		21 (37.5)		-0.087
SLCO1B1 wild type	8 (66.7)		35 (62.5)		0.087
ABCC2 -24 variant	2 (16.7)		19 (33.9)		-0.405
ABCC2 -24 wild type	10 (83.3)		37 (66.1)		0.405
ABCC2 1249 variant	4 (33.3)		23 (41.1)		-0.161

	Before matching	Before matching	Before matching	Before matching	Before matc
ABCC2 1249 wild type	8 (66.7)		33 (58.9)		0.161
UGT2B7 variant	9 (75.0)		47 (83.9)		-0.222
UGT2B7 wild type	3 (25.0)		9 (16.1)		0.222
UGT1A9 variant diplotype	0		3 (5.4)		-0.336
UGT1A9 wild type diplotype	12 (100)		53 (94.6)		0.336
ABCB1 wild type/ 1 var allele	3 (25.0)		15 (26.8)		-0.041
ABCB1 2 -3 variant alleles	6 (50.0)		25 (44.6)		0.108
ABCB1 4 -6 variant alleles	3 (25.0)		16 (28.6)		-0.081
Donors' *ABCC2* 1249 variant	6 (50.0)		21 (37.5)		0.282
Donors' *ABCC2* 1249 wild type	6 (50.0)		35 (62.5)		-0.282
Age (years)	46.5±13.3		50.0±12.8		-0.301
BMI (kg/m^2)	24.9±6.3		24.0±3.3		0.182
eCrCl (mL/min)	45±16		41±11		0.315
Rescaled ln(CNI trough) (µg/L)[1]	5.34±0.21		5.26±0.37		0.230
PK outcomes					
$AUC_{\tau,\sigma\varsigma}$ (mg*hr/L)	73.2 (57.3)		50.3 (44.8)		0.775
$C_{max,ss}$ (mg/L)	16.1 (64.9)		15.9 (60.5)		0.023
C_0 (mg/L)	4.6 (91.0)		2.8 (101)		0.625
C_{12} (mg/L)	2.8 (149)		1.8 (101)		0.456
$CL_{T/F,ss}$ (mL/min/kg)	2.2 (77.0)		3.3 (48.0)		-0.715
C_{max}/AUC_τ (1/hr)	0.22 (51.0)		0.32 (56.0)		-0.715

$AUC_{t,ss}$ – area under the concentration-time curve during dosing interval at steady-state; BMI – body mass index; $C_{max,ss}$ – peak plasma concentration at steady-state; C_0 – morning pre-dose trough; C_{12} – evening trough; $CL_{T/Fss}$ – apparent total body clearance at steady-state; CNI – calcineurin inhibitor; EC-MPS – enteric-coated mycophenolate sodium; eCrCl – estimated creatinine clearance; MMF – mycophenolate mofetil

[1]Linear transformation to ln(cyclosporine trough) scale

Table 5 Comparison of pharmacokinetic parameters between*ABCG2 c.421C>A* variant allele carriers and wild type homozygotes (as geometric means ratios, GMR for variant/wild type) in the entire data set (unmatched and unadjusted) and in matched data set (with additional adjustment).

	Unmatched/unadjusted	Unmatched/unadjusted	Unmatched/unadjusted	Unmatched/unadju
	Frequentist	Frequentist	Frequentist	
	GMR (95%CI)		P	
$AUC_{\tau,\sigma\varsigma}$ (mg*hr/L)	1.45 (1.10-1.92)		0.009	
$C_{max,ss}$ (mg/L)	1.01 (0.71-1.44)		0.940	
C_0 (mg/L)	1.66 (0.99-2.79)		0.056	
C_{12} (mg/L)	1.57 (0.88-2.81)		0.123	
$CL_{T/F,ss}$ (mL/min/kg)	0.66 (0.48-0.90)		0.010	
C_{max}/AUC_τ (1/h)	0.70 (0.50-0.96)		0.029	

[1]Additionally adjusted for body mass index, estimated creatinine clearance and donors' *ABCC2 1249 G>A*genotype.

$AUC_{t,ss}$ – area under the concentration-time curve during dosing interval at steady-state; $C_{max,ss}$ – peak plasma concentration at steady-state; C_0 – morning pre-dose trough; C_{12} – evening trough; $CL_{T/Fss}$ –

apparent total body clearance at steady-state

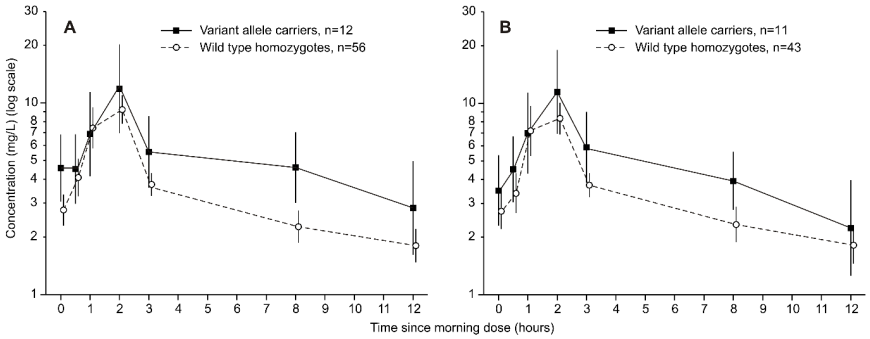

Figure 1 Average (geometric mean, 90%CI) mycophenolic acid (MPA) dose-adjusted concentrations over-dosing interval at steady state in*ABCG2 c.421C>A* variant allele carriers and wild type controls: raw (measured) data (**A**), data in matched sets (**B**).

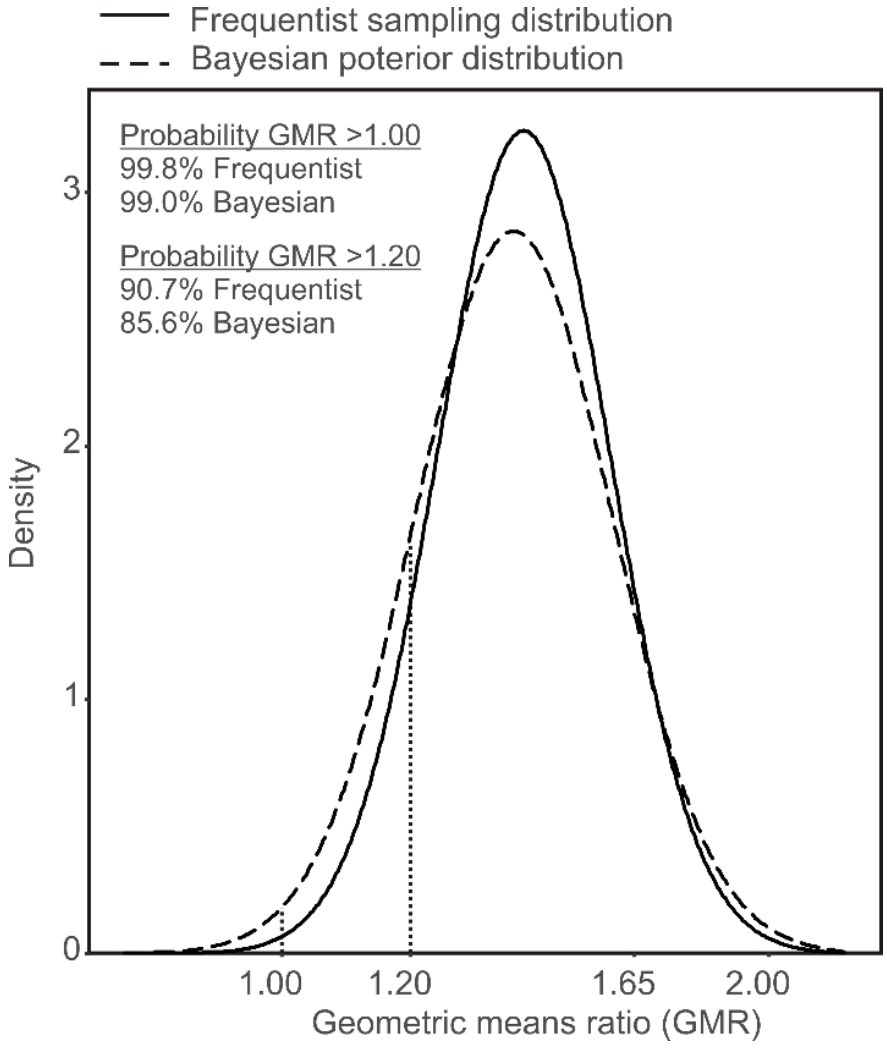

Figure 2 Frequentist sampling distribution and Bayesian posterior distribution of the geometric means ratio (GMR) generated in the matched/adjusted comparison of total exposure ($AUC_{\tau,\sigma\varsigma}$) in variant allele carriers and wild type controls. Dotted vertical lines indicate GMR=1.00 ("no effect") and GMR=1.20 – depicted are probabilities of GMRs >1.0 and >1.20.

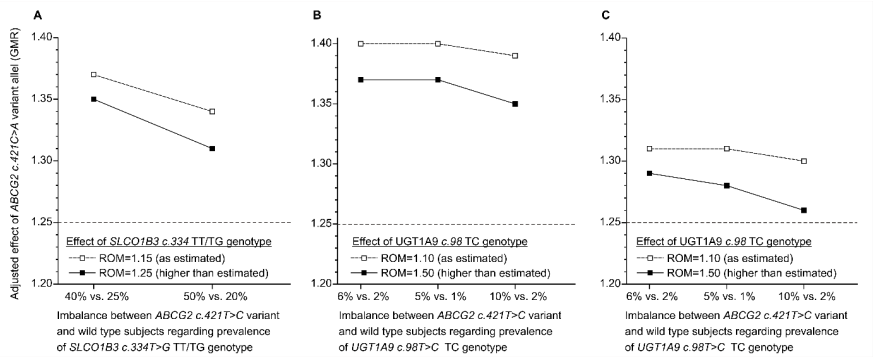

Figure 3 Sensitivity analysis of the effect of *ABCG2 c.421C>A* variant allele on MPA $AUC_{\tau,\sigma\varsigma}$(GMR=1.40) to account for hypothetical bias arising from not accounting for the *SLCO1B3 c.334T>G* and *UGT1A9 c.98T>C* SNPs. Effect of the TT/TG (vs. GG) *SLCO1B3 c.334* genotype is estimated at ROM=1.136 (95%CI 0.949-1.361) (Figure S2); effect of the TC (vs. TT) *UGT1A9 c.98* genotype is estimated at ROM=1.098 (0.548-2.198) (Figure S3); and prevalence of the TT/TG and TC genotypes is estimated at 31% and 3.9%, respectively (Figure S4). Shown are bias-adjusted effects (GMRs) of the *ABCG2 c.421C>A* variant allele assuming considerable imbalance between variant carriers and wild type controls in prevalence of confounders (i.e., *SLCO1B3 c.334* TT/TG genotype and *UGT1A9 c.98* TC genotype) and different effects of confounders (expressed as ratio of means, ROM) – those estimated based on published studies (Figure S2, Figure S3), and larger effects. **A** . Confounder to adjust for is *SLCO1B3 c.334T>G* genotype TT/TG (vs. GG). Effect to adjust is GMR=1.40. The confounder effect is estimated at ROM=1.136, rounded-up to 1.15. The effect "higher than estimated" (ROM=1.25) is effect somewhat higher than that reported in the largest individual study with 110 TT/TG and 218 GG subjects [31].**B.** Confounder to adjust for is *UGT1A9 c.98T>C* genotype TC (vs. TT). Effect to adjust is 1.40. The confounder effect is estimated at 1.098, rounded-up to 1.10. The effect "higher than estimated" (ROM=1.50) is effect somewhat higher than that reported in the largest individual study with 10 TC and 328 TT subjects [32]. **C** . Assumed is simultaneous and maximum imbalance between *ABCG2 c.421* variant carriers and wild type controls regarding *SLCO1B3 c.334T>G TT/TG* genotype (50% vs. 20%) and regarding *UGT1A9 c.98T>C* TC genotype (10% vs. 2%) with consecutive adjustment for their larger effects (ROM=1.25, ROM=1.50): initial estimate (GMR=1.40) is first adjusted for TT/TG to GMR=1.31, and GMR=1.31 is further adjusted for *UGT1A9* SNP.

Dashed horizontal line depicts the conventional upper limit of equivalent exposure (GMR=1.25).

Electronic Supplementary Material

Loss of function *ABCG2 c.421C>A* (rs2231142) polymorphism increases steady-state exposure to mycophenolic acid in stable renal transplant recipients: exploratory matched cohort study

Borić Bilušić A[1], Božina N[2], Lalić Z[3], Lovrić M[3], Nađ-Škegro S[4], Penezić L[4], Barišić K[5], Trkulja V[2]

[1]Agency for Medicinal Products and Medical Devices of Croatia,Zagreb, Croatia

[2] Department of Pharmacology, Zagreb University School of

Medicine, Zagreb, Croatia

[3] Department of Laboratory Diagnostics, Analytical Toxicology and

Pharmacology Division, University Hospital Center Zagreb, Zagreb, Croatia

[4] Department of Urology, University Hospital Center Zagreb, Zagreb, Croatia

[5] Department of Medical Biochemistry and Hematology, Faculty of Pharmacy and Biochemistry, Zagreb University, Zagreb, Croatia.

Corresponding author: Vladimir Trkulja

Department of Pharmacology

Zagreb University School of Medicine

Šalata 11, 10000 Zagreb, Croatia

e-mail: vladimir.trkulja@mef.hr

Content

Supplemental Methods A – Methods to achieve conditional exchangeability

Currently, ABCG2 is not considered in the context of MPA (or MPAG) pharmacokinetics [1] and there is no explicit evidence that either MPA or MPAG are ABCG2 substrates – but there is also no explicit evidence that they are not [2]. We aimed to estimate effect of the*ABCG2 c.421C>A* (rs2231142) SNP (results in reduced transporter numbers and function), i.e., of carrying a variant allele, on steady-state exposure to MPA. As reviewed, studies (so far) have failed to detect association between this SNP and exposure to MPA (as AUC_{τ} or as trough concentrations) [3]. However, in one study in Japanese renal transplant recipients, variant carriers (n=44) had higher (adjusted for MMF dose; expressed per 1000 mg) MPAG $AUC_{0\text{-}12}$ than 36 wt controls (median 1540 vs. 1195 mg × h/L; P=0.029) [4]. Authors suggested that ABCG2 might be included in biliary excretion of MPAG [4]. By analogy with reports of association between OATP1B1 and OATP1B3 polymorphisms (MPAG is a substrate, MPA is not) [1] and systemic bioavailability of MPA (reviewed in [3]), the *ABCG2 c.421* SNP might (as well) reflect on the systemic exposure to MPA. Current "failures" in this respect might be due to methodological study characteristics, and/or could indicate that even if it existed, the effect was mild-moderate, i.e., not robust enough as to be spotted under certain methodological circumstances.

Figures S1A and S1B schematically represent the setting in which the effect of variant *c.421C>A* allele was to be assessed and measures undertaken to control for confounding. We followed the concepts developed and presented by Pearl [5] and further elaborated by VanderWeele [6, 7], and implemented in R package *dagitty*[8]. Major elements are depicted in Figure S1A (some are based on explicit *in vitro* and/or *in vivo* evidence, some are implied based on circumstantial evidence and are partly hypothetical): 1. For simplicity, in the main text *ABCG2 c.421C>A*SNP is designated as a *binary treatment* (variant allele=1, treated; wt homozygous=0, control). Its immediate consequence is reduced number (and activity) of ABCG2 (increased degradation). Hence, the actual treatment (or exposure) is *ABCG2 activity* (may be dichotomized as "reduced" [variant allele]=treatment; and "preserved" [wt homozygous]= control). However, *in vivo*ABCG2 activity cannot be measured, hence the true exposure remains unobserved, and we use *ABCG2 c.421* genotype as an instrumental variable – it has no other effect on the outcome or on any other variable (*in vitro* , both CsA and tacrolimus are potent ABCG2 inhibitors [9], but neither is an ABCG2 substrate [10, 11]) beyond that conveyed through reduced transporter function; 2. Steady-state MPA pharmacokinetic indicators (MPA PK) are continuous outcomes; 3. The connection between the exposure (ABCG2 activity,*c.421*

SNP) and the outcome might be a direct one (assumes that MPA is an ABCG2 substrate), and/or an indirect one (as suggested in the Japanese study [4]) with MPAG as a hypothetical mediator (unmeasured in the present study). The setting is such that it can estimate the total effect of exposure (instrument) on the outcome.

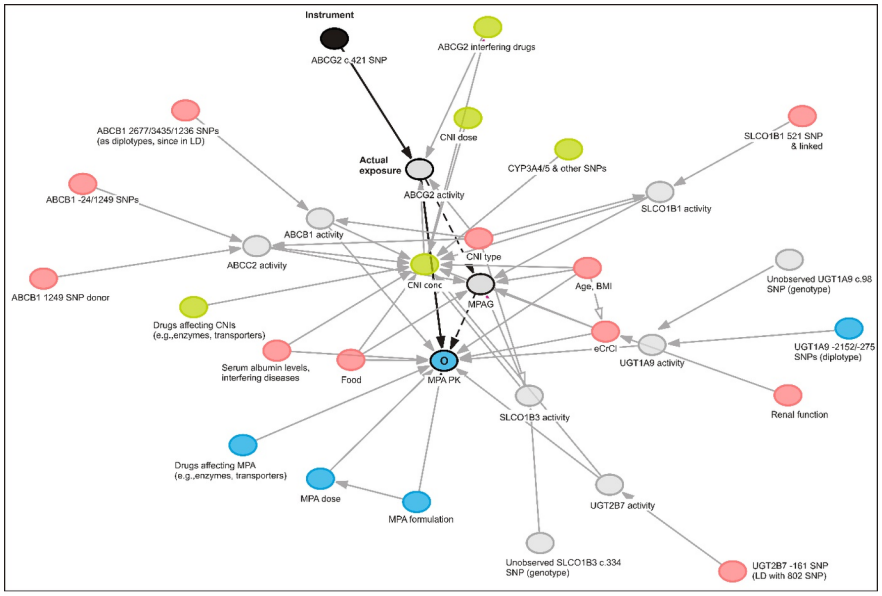

Figure S1A . Directed acyclic graph (DAG) representing the setting to estimate the effect of *ABCG2 c.421* SNP, i.e., reduced (vs. preserved) ABCG2 function resulting from variant allele carriage (vs. wt homozygosity) on steady-state pharmacokinetics of MPA (MPA PK – outcome, O). The measured "treatment" – *ABCG2 c.421C>A* genotype - is an instrument (black circle), since ABCG2 activity (actual exposure) is not measured. The causal path (thick black arrow) might be a direct one and/or mediated (dashed black arrow) through an unmeasured (hypothetical) mediator, i.e., MPAG levels. Pale red circles represent confounders (ancestors of both the treatment i.e., actual exposure, and the outcome), blue circles represent ancestor of the outcome and green circles represent ancestors of (actual, but unmeasured) exposure (ABCG2 activity). Gray arrows depict biasing paths. Gray filled/outlined circles represent unmeasured variables - one is a suggested but unmeasured confounder – *SLCO1B3 c.334* SNP, and one is unmeasured ancestor of the outcome- *UGT1A9 c.98* SNP; the others indicate transporter/enzyme activities (presumably) affected by exposure/outcome ancestors (see text for details).

4. Number of baseline covariates that can interfere with the (tested)*ABCG2 c.421* (ABCG2 activity) effect is high (although Figure S1A is somewhat simplified). In a scenario in which the *ABCG2 c.421* variant would be "treatment", none of them would meet the "classical" definition of a confounder, since "treatment" is defined at conception, and the current knowledge about possible epigenetic regulation of *ABCG2* is virtually non-existing. In such a case, they would qualify as "ancestors of the outcome" (i.e., factors known or suspected to affect MPA PK, thorough different mechanisms [paths]). As illustrated in Figure S1A, when ABCG2 activity is considered as an actual but unobserved "treatment" (but adequately represented by an instrument), then some of these variables should justifiably be considered ancestors of both the "treatment" (may affect ABCG2 activity) and the outcome (may affect MPA PK, by different mechanisms); 5. Variables that may be considered ancestors of both the "treatment" and the "outcome" (depicted in pale red in Figure S1A) include: i) type of CNI (CsA or tacrolimus). They are both (*in vitro*) potent ABCG2 inhibitors, but it is possible that *in vivo*(at therapeutic doses) they differ in their inhibitory effect –*in vitro* , CsA is particularly (and more) potent when the number of transporter is reduced [12] (as in the case of the *ABCG2c.421* SNP). Next, CsA inhibits ABCC2 (ABCC2 activity is another unmeasured variable in this

setting) and affects MPAG/MPA recirculation and exposure, while tacrolimus does not [13]. Also, both CsA and tacrolimus may both inhibit and induce ABCB1 activity (a further unmeasured variable) [14], and may differ in this respect, and MPA is a substrate of ABCB1 [15]. Also, CsA, but not tacrolimus, is listed among SLCO inhibitors [15] – thus, it can affect SLCO1B1 and/or 1B3 activity (further unmeasured variables), and MPAG is a substrate of both [1]; ii) *ABCB1 2677/345/1236* SNPs (as diplotypes, since in LD) reflect on ABCB1 activity (not measured), hence they affect MPA (outcome), and also the exposure: both CsA and tacrolimus are also ABCB1 substrates [14], hence altered ABCB1 activity may reflect on their trough concentrations, and this may result in a variable effect on ABCG2 activity (exposure); iii) *ABCC2 -24 or/and 1249* SNPs may reflect on ABCC2 activity and MPAG is an ABCC2 substrate. Also, although ABCC2 is not considered relevant in CsA and tacrolimus pharmacokinetic pathways [10], ABCC2 (and -24/1249 SNPs) may affect tacrolimus [16] – hence, affect its concentrations which might reflect on its effect on ABCG2 activity; iv) donor's *ABCC2 1249* SNP might reflect on MPA (presumably, by affecting MPAG in the kidney) [17], and, at least theoretically, on tacrolimus [16] (although renal excretion is of minor relevance for tacrolimus [10]), and thus contribute to the variability of tacrolimus effect on ABCG2 activity; v) *UGT2B7 -161* SNP is in a complete LD with the *UGT1B7 802* SNP [18], hence it "represents" the 802 SNP. By affecting UGT2B7 activity (not measured), it would affect MPA glucuronidation. On the other hand, one study demonstrated direct glucuronidation of CsA and tacrolimus in human gut and liver by UGT2B7 [19] – hence, it might affect CNI concentrations, and, consequently, their effect on ABCG2 activity; vi) *SCLO1B1 521* SNP (and linked SNPs) – may affect SLCO1B1 activity (not measured) and MPAG is a substrate to SLCO1B1. However, SLCO1B1 may also transport tacrolimus [20], hence affect its concentrations and the effect on ABCG2; vii) serum albumin levels and diseases that might interfere with pharmacokinetics of MPA and of CNI – Figure S1A is simplified in that these factors were considered jointly (since also possibly inter-related): hypoalbuminemia is known factor affecting exposure to MPA, and various systemic conditions in the early post-transplant stage can be reasonably considered as factors that could affect both exposure to MPA and CNI levels (and, thus, CNI effects on ABCG2 activity); viii) Food (concomitant) may interfere with absorption of both MPA and CNIs (and their concentrations); ix) Renal function and its commonly measurable "proxy" – estimated creatinine clearance (eCrCl) – may reflect on bioavailability of MPA and of CNI (although, this is a minor pathway for CNIs [10]), hence on both "exposure" and the "outcome"; x) age, body mass index (Figure S1B is simplified in that it combines these two demographic factors and omits all possible interconnections between demographics, concomitant morbidity, liver and renal function) – have been found related to bioavailability of all immunosuppressants (at least to some extent; e.g., by reflecting on renal function, liver function, or in any other way), hence they affect both the "exposure" and the "outcome". 6. **Unobserved (known) confounder** – *SLCO1B3 c.334* SNP (and linked SNPs). In complex pharmacogenetic settings, a number of unmeasured/unknown confounders are possible. *SLCO1B3 c.334* SNP would qualify as a known (although not unambiguously) possible confounder which however remained unobserved (patients were not genotyped for this SNP). By (presumably) affecting SLCO1B3 activity it would reflect on MPAG (MPAG is a substrate), but it may also reflect on tacrolimus concentrations [20], and thus on its effect on ABCG2 activity. 7. Ancestors of the outcome (depicted in blue in Figure S1A). A number of covariates qualified as ancestors of the outcome (they can be plausibly related to MPA PK, but not to "exposure", i.e., ABCG2 activity): i) drugs affecting MPA PK (by effects on UGTs, transporters or by any other mechanism) – Figure S1A is simplified in that it considers all such drugs jointly and omits their relationship to "effector molecules", e.g., UGT enzymes, ABCB1, ABCC2, SLCO1B1/B3 or others; ii) MPA formulation and MPA dose – IR MMF and EC-MPA formulations are not bioequivalent; they deliver different molar doses and MPA concentration-time profiles are not equivalent [21]. Clearly, choice of formulation (specific molar dose and release particulars) affects bioavailability of MPA; iii) *UGT1A9 -2152/-275* SNPs (as diplotypes, since in complete LD) reflect on the enzyme activity (unmeasured), and thus on MPA PK; 8. **Unobserved (known) ancestor of the outcome** – *UGT1A9 c.98T>C* : variant allele carriage has been suggested (although with high uncertainty) associated with higher exposure to MPA, but its prevalence is very low. 9. Ancestors of exposure (depicted in Figure S1A in green). Variables that affect exposure and have no effect on the outcome (apart that executed through their effect on exposure) are instrumental variables. When actual exposure is quantified, adjustment

for instruments worsens or introduces bias, does not remove it [5-7]. In the present study, actual exposure is unobserved, and we use an instrument (*ABCG2 c.421C>A* genotype). In such a setting, accounting for other instrumental or near-instrumental variables (affect "exposure", while effect on the outcome is minor) is needed in order for the instrument to adequately represent the (actual) exposure: i) drugs that interfere with ABCG2 activity (beyond CNIs). Figure S1A is simplified in that it considers all such drugs jointly. It also allows for a possibility that these drugs could (by any mechanism) affect CNI concentrations (which could also reflect on ABCG2 activity); ii) CNI concentration (morning trough at the beginning of the 12-hour MPA sampling period) regardless of the CNI type [ln(tacrolimus) troughs rescaled to ln(CsA troughs) scale by linear transformation]. CNI concentration is a descendant of several variables that may affect it. Putting both "CNI type" and "CNI concentration" into the network is reasonable: in some aspects, tacrolimus and CsA differ qualitatively (e.g., CsA inhibits ABCC2 and SLCO1B1, tacrolimus does not), while the effect on "exposure" (ABCG2 activity) might be concentration-dependent (along with a possibility of a qualitative difference between the two); iii) CNI dose directly affects CNI concentrations; iv) *CYP3A4/5*SNPs (those genotyped in the present sample and other potentially relevant [10]) may affect CNI concentrations; v) drugs affecting pharmacokinetics of CNIs – Figure S1A is simplified in that it considers all such drugs jointly and omits their mechanisms (e.g., effects on CYPs, transporters or any other "effector").

Figure S1B contains all the same elements as Figure S1A, but depicts the minimal adjustment set required (and sufficient) to block biasing paths, as to (unbiasedly) estimate the causal effect of treatment (*ABCG2 c.421C>A* variant allele – reduced transporter function) on the outcome (MPA PK): adjustment (by different means) for outcome/exposure ancestors (but not for colliders) [5-7]. The minimal adjustment set includes (variables depicted in Figure S1B as open, black-outlined circles): i) *ABCB1 2677/3435/1236* SNPs considered as diplotypes (since in strong LD; 3 levels based on the number of variant alleles) – by matching; ii) *ABCC2 -24* and*1249* SNPs and also donor's *ABCC2 1249* SNP, dichotomized as variant carriers and wt subjects – by matching (the latter also by statistical adjustment); iii) *UGT2B7 -161* SNP (represents the 802 SNP since in a complete LD), dichotomized as variant allele or wt – by matching; iv) *UGT1A9 -2152* and *-275* SNPs considered as diplotypes (since in complete LD), dichotomized as variant or wt diplotype – by matching; v) *SLCO1B1 521* SNP (may include other SNPs in a complete LD), dichotomized as variant or wt – by matching; vi) Food-drug interaction – by clinical procedure: blood sampling after an overnight fast; vii) MPA formulation (IR MMF or EC-MPA) – by matching; viii) MPA dose – all PK parameters calculated using dose-adjusted MPA concentrations; ix) CNI type (CsA or tacrolimus) – by matching; x) CNI concentration (trough) – by matching; xi) Age, BMI – by matching (latter also by statistical adjustment); xii) Renal function – by inclusion criteria (patients had to have by at least 33% improved creatinine vs. post-operative Day 1 with absolute value <300 μmol/L and stable diuresis);

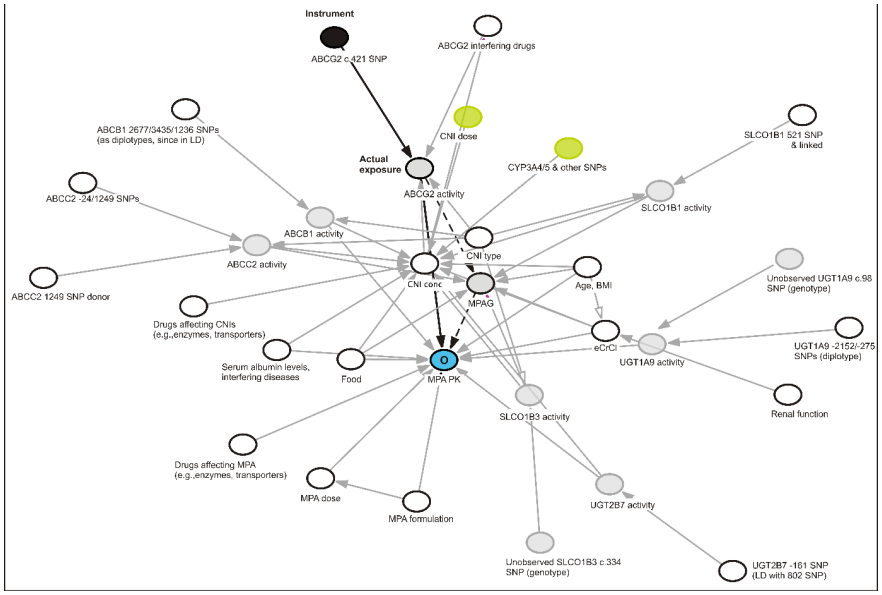

Figure S1B . The same DAG as in figure S1A, but with depicted outcome/exposure ancestors controlled for by different means depicted as open, black-outlined circles - minimal sufficient adjustment set. Black circle is the instrument, gray/black-outlined circles depict unmeasured exposure defined by the instrument (ABCG2 activity) and a hypothetical mediator (MPAG levels) on a causal path - direct (thick full black arrow) and/or indirect (dashed black arrow) – between the instrument (unmeasured exposure) and the outcome. Completely gray circles are unmeasured variables – enzyme/transporter activities that are (presumable) mediators on some of the biasing paths (descendants of outcome/exposure ancestors). They did not need to be adjusted for, since their ancestors were included in the adjustment set. One suggested confounder and one ancestor of the outcome (based on some of the literature data) remained unmeasured – the *SLCO1B3 c.334* SNP and *UGT1A9 c.98* SNP. Two green circles depict ancestors of exposure that did not need to be in the adjustment set, since their descendant – CNI concentration – is adjusted for (see text for details).

xiii) Additionally – estimated creatinine clearance – by matching and statistical adjustment; xiv) Hypoalbuminemia – by inclusion criteria: patients had to have serum albumin >31 g/L; xv) Medical conditions that may interfere with bioavailability of both MPA and CNIs – by inclusion criteria: patients had to be generally "well-doing" (free of surgical complications, infections, relevant metabolic, gastrointestional, hepatic or cardiovascular conditions); xvi) Drugs interfering with MPA pharmacokinetics – by inclusion criteria: patients had to be free of treatment known to affect MPA during the pre-study days and on the study day (antacids, phosphate binders, proton pump inhibitors, antibiotics, rifampicin, oral iron, calcium or magnesium; also, co-medication was restricted by exclusion based on comorbidities); xvii) Drugs interfering with CNIs – by inclusion/exclusion criteria: no specific list was formed, but co-medication was restricted already regarding MPA, and comorbidities; also, CNI concentrations were accounted for in matching; xviii) Drugs interfering with ABCG2 activity (apart from CNIs) – by inclusion/exclusion criteria: no specific list was formed, but considering the known ABCG2 substrates and inhibitors [11], relevant co-medication was practically completely restricted already regarding MPA and comorbidities.

Variables that did not need to be included in the adjustment set (depicted as green circles in Figure S1B): i) CNI dose – as it is represented by its descendant, CNI concentrations; ii) The same applies to the two genotyped CYP3A4/5 SNPs. CYPs are of marginal relevance for MPA clearance, hence the two genotyped CYPs and other possible SNPs affecting CYP3A4/5 or other CYPs and/or transporters are of relevance primarily for their effect on CNI concentrations [1, 10] – and are thus controlled for by adjustment for CNI concentrations. By the same logic, drugs affecting CNI concentrations (by any means) did not necessarily need to be included in the adjustment set, but by comorbidity and co-medication restrictions pertinent to

MPA, they were, to a great extent. As reviewed [3], *in vitro* data and a few studies in humans (vs. several "negative" studies) suggest that*SLCO1B3 c.344* SNP and *UGT1A9 c.98* SNP may be relevant for exposure to MPA – in the present study, these SNP was not genotyped, i.e., remained "unobserved" (indicated as gray circles [for unobserved/unmeasured variables] in Figure S1B.

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Supplemental Methods B – Matching

In respect to variables that needed to be controlled for (in addition to inclusion-exclusion criteria), the present setting was extremely complex. For example, based on categorical covariates depicted in Figure S1, 768 strata of variable combinations could be formed. We used exact matching combined with optimal full matching based on Mahalanobis distance with age, BMI, eCrCl and CNI concentrations as (further) continuous matching variables. Exact matching achieves balance that corresponds to a fully blocked randomization. Optimal full matching also allows one-to-many matching, and when Mahalanobis is a distance measure, it approximates fully blocked randomization. The process first completes exact matching on specified covariates (forms subclasses of "treated" and "controls" exactly matched on a set of covariates) and then uses Mahalanobis distance (calculated using the entire data set) to further minimize within-subclass distances regarding covariates not included in exact matching [1-3]. Choice of (categorical) covariates for exact matching was guided by the intention to retain as many as possible "treated" subjects, but also on their practical importance: hence, exact matching was in respect to the type of CNI (CsA or tacrolimus), *UGT2B7* and *UGT1A9* SNPs.

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Supplemental Methods C – Sensitivity to unmeasured confounding

A number of factors, known or suggested (but not unambiguously) to affect (to some extent) pharmacokinetics of MPA are depicted in Figure S1. However, "pharmacogenetic settings" are complex, and one should *a priori* consider that there are inevitably unmeasured, although reasonably possible (e.g., *SLCO1B3 c.334* and *UGT1A9 c.98*SNPs), but also unknown confounders, i.e., those not indicated in Figure S1.

E-value [1] represents the minimum strength of association, on the risk ratio scale, that unmeasured confounder(s) need(s) to have with the outcome and the treatment to explain away a specific treatment-outcome association, conditional on the measured covariates. A large E-value implies that a considerable unmeasured confounding effect is needed for the purpose. E-value can be determined for different effect measures, i.e., relative risk, odds ratio, hazard ratio, rate ratio, mean difference or a regression coefficient, but is expressed on the risk ratio scale [1]. The effect measure in the present study was geometric means ratio (GMR), i.e., exponentiated difference between two (adjusted) means of ln-transformed values of pharmacokinetic indicators. Calculation of E-value for difference in means is based on standardized difference (d), requires several effect measure conversions, several assumptions, and its interpretation is not very intuitive [1]. However, GMR and RR share some common features: (i) both are exponents of difference in means of ln-transformed quantities (risk or a continuous variable); (ii) both ln(risk) and ln(right-tailed continuous variable) have a normal distribution and their interpretation is similar as they provide information about a relative difference between a treatment and a control. If for a treatment vs. control RR >1.0, e.g., 1.5, it means relatively by 50% higher risk with treatment, just as is the case with GMR: if 1.5, it means relatively by 50% higher value of the measured quantity with treatment. Similar is the relationship between a relative risk (risk ratio)

and relative rate (rate ratio), where E-value calculation for a relative rate is identical as for the relative risk [1]. Therefore, in calculation of E-values we "treated" GMRs as relative risks. One can calculate E-value that indicates size of the effect of unmeasured confounding needed to completely explain away the observed effect, i.e., to "push" the estimate of a ratio to 1.0 or a difference to 0.0; but E-value can be determined in respect to any desired level [1]. We determined E-values needed to "push" the GMR point-estimates to 1.20. We considered that GMR 1.20 indicated a cut-off value at which difference (1.20 and higher) becomes practically relevant: in the classical context of equivalent relative exposure, GMR point-estimates of 1.20 are at the upper limit of the conventional acceptance range. E-value may be considered as a total effect of all unmeasured confounding.

Since current literature data [2,3] suggest *SLCO1B3 c.334T>G* and *UGT1A9 c.98T>C* SNPs as potentially relevant factors for MPA bioavailability – which remained unmeasured in the present study (Figure S1) – the estimated GMRs (for variant *ABCG2 c.421C>A* vs. wt genotype) were submitted to a sensitivity analysis in which they were corrected for bias arising from not adjusting for the these two SNPs [4]. For this purpose, using two recent systematic reviews [2,3], we identified studies reporting on associations between *SLCO1B3 c.334T>G* (as a contrast between TT/TG patients vs. GG patients) and *UGT1A9 c.98T>C* (as a contrast between variant allele carriers and wt subjects) SNPs, and MPA $AUC_{\tau,\sigma\varsigma}$, and we generated meta-analytical pooled estimates (random-effects, REML variance estimator and Hartung-Knapp-Sidik-Jonkman correction using package *meta* in R [5]) expressed as ratios of means (ROM) [6]. We also identified studies conducted in samples from European populations that provided information about prevalence of *SLCO1B3 c.334* TT/TG patients and of *UGT1A9 c.98T>C* variant carriers to generate prevalence estimates (random-effects, generalized linear mixed model fitted to logit-transformed proportions with ML variance estimator, package *meta* in R [5]) needed for the sensitivity analysis. We "treated" GMRs and ROMs as relative risks, and calculated bias-adjusted GMRs at different levels of confounder effects using package *episensr* in R [7].

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Supplemental Results – Sensitivity analysis (Figures S2-S4, Table S1 and S2)

Identification of unmeasured confounder effects and prevalence

We identified 4 studies reporting total exposure to MPA over dosing interval at steady-state [$AUC_{0\text{-}12}$ (mg ×h/L) calculated based on dose-adjusted MPA concentrations] in renal transplant patients classified in respect to the *SCLO1B3 c.334T>G* SNP as combined TT/TG subjects vs. GG subjects: one French (Picard 2010 [1]) and one Dutch (Bouamar 2012 [2]) study, each reporting (separately) values in patients on IR MMF co-treated with either CsA or macrolactams (mostly tacrolimus, less commonly sirolimus); one

Chinese study (Geng 2012 [3]) in which IR MMF was combined with CsA; and one Japanese study (Miura 2007 [4]) in which IR MMF was combined with tacrolimus. All values were crude, unadjusted mean±SD $AUC_{0\text{-}12}$ quantified at different post-transplantation times (Figure S2).

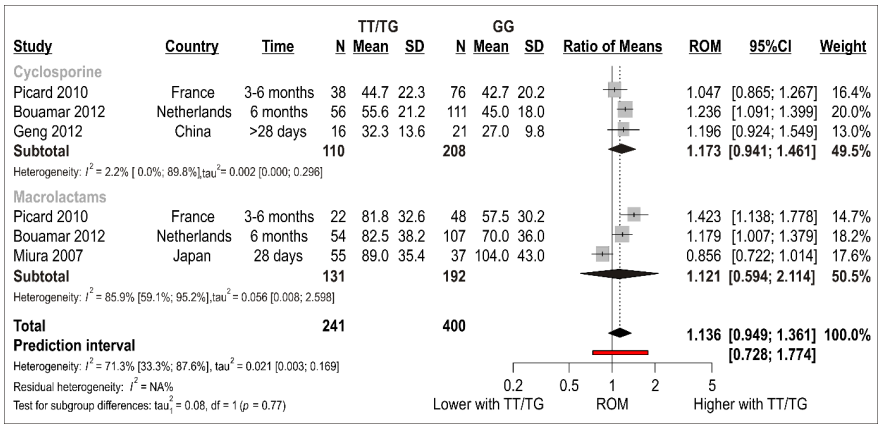

Figure S2 . Meta-analysis of studies reporting mean±SD MPA $AUC_{0\text{-}12}$ at steady-state in renal transplant recipients treated with IR MMF and co-treated with CsA or macrolactams (tacrolimus or sirolimus) and genotyped for *SLCO1B3 c.334T>G*SNP, classified as TT/TG vs. GG patients. The effect measure is ratio of means (ROM).

In CsA co-treated subjects, one study indicated no relevant difference between TT/TG and GG patients, while two suggested somewhat higher AUC in TT/TG patients: the pooled estimate was ROM=1.173 (0.941-1.461) (Figure S2) indicating a tendency of slightly higher exposure in TT/TG patients (total N=110) than in GG patients (total N=208).

In macrolactam co-treated patients, two studies suggested somewhat higher AUC in TT/TG vs. GG patients, and one suggested somewhat lower values: the pooled estimate was ROM=1.121 (0.594-2.114) (Figure S2) not indicating any relevant difference in exposure between TT/TG (total N=131) and GG patients (total N=192).

The overall estimate (ROM=1.136, 0.949-1.361) (Figure S2) indicated a possibility of slightly higher exposure in TT/TG (total N=241) than in GG patients (total N=400), but uncertainty (due to heterogeneity best illustrated by the wide prediction intervals extending from 27% lower to 77% higher AUC) about the size of the effect (difference) was considerable.

We identified 3 studies reporting total exposure to MPA over dosing interval at steady-state [$AUC_{0\text{-}12}$ (mg ×h/L) calculated based on dose-adjusted MPA concentrations] in renal transplant patients classified in respect to the *UGT1A9 c.98T>C*SNP as variant carriers (TC) vs. wild type subjects (TT): one French (Picard 2010 [1]) and one Dutch (van Schaik 2009 [5]), each reporting values in patients on IR MMF co-treated with either CsA or macrolactams; one Belgian (Kuypers 2005 [6]) in which MMF was combined with tacrolimus. Two studies (Picard 2010, Kuypers 2005 [1, 6]] reported crude, unadjusted mean±SD $AUC_{0\text{-}12}$quantified at different post-transplantation times (Figure S3).

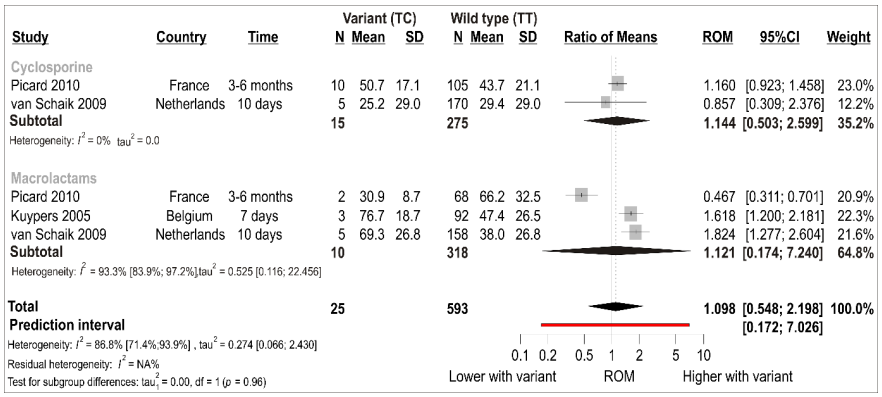

Figure S3 . Meta-analysis of studies reporting mean±SD MPA $AUC_{0\text{-}12}$ at steady-state in renal transplant recipients treated with IR MMF and co-treated with CsA or macrolactams (tacrolimus or sirolimus) and genotyped for *UGT1A9*3 (c.98T>C)*SNP, classified as variant carriers (heterozygous, TC) vs. wild type homozygous subjects. The effect measure is ratio of means (ROM).

van Schaik 2009 [5] used reduced sampling schedule (at pre-dose, 0.5 and 2 hours post-dose) to project $AUC_{0\text{-}12}$ at 3 and 10 days, and at 1, 3, 6 and 12 months postoperatively. They graphically reported geometric mean AUCs (no measures of spread or precision) at these time-points for 5 variant vs. 170 wt patients co-treated with CsA and, separately, for 5 variant vs. 158 wt patients co-treated with tacrolimus (Figure 3 in [5]). We graph-read (using digitizing software) the geometric mean values at Day 10 (considering the timing of the present measurements) and used p-values to recover common SD – this is shown in Figure S3.

The number of variant allele carriers was very low in each individual study and across studies by co-treatment and overall (Figure S3). van Schaik [5] reported that based on mixed-modelling of repeatedly assessed AUCs over time (at 6 time-points), among tacrolimus co-treated subjects, 5 TC patients vs. 158 wt subjects had by around 50% higher time-averaged AUCs (adjusted for age, sex, creatinine clearance, delayed-graft function and MPA measurement technique); and the same was reported for 5 TC subjects vs. 170 wt subjects co-treated with CsA [5]. However, considering the AUCs on postoperative day 10, the TC-wt difference in macrolactam co-treated subjects appeared even greater, but there was apparently no TC-wt difference among CsA co-treated patients (Figure S3). Kuypers [6] reported higher AUC in 3 TC vs. 92 wt subjects (Figure S3), while Picard [1] reported comparable AUCs in 10 TC vs. 105 wt subjects co-treated with CsA (Figure S3), and lower AUCs in 2 TC vs. 68 wt subjects co-treated with tacrolimus (Figure S3). When pooled, this scarce data rather consistently indicate lack of a relevant difference between TC (N=15) and wt subjects (N=275) when CsA is co-treatment (Figure S3). When marcolactams are co-treatment (Figure S3), individual study results are heterogeneous indicating also no major difference between TC (N=10) and wt subjects (N=318). In agreement, the overall pooled estimate (Figure S3) does not signal a major difference between TC (N=25) and wt subjects (N=593), but heterogeneity is huge, with 95% prediction intervals indicating from 7 times lower to 7 times higher AUCs in TC vs. wild-type subjects, i.e., approximately equal probability of TC being associated with a considerably lower or considerably higher exposure to MPA.

Based on studies including exclusively or predominantly Caucasians of European descent [1,2, 5-12] (pertinent for the present sample), prevalence of the TT/TG *SLCO1B3 c.334T>G* genotype is rather consistently estimated at 31.1% (95%CI 28.2-34.2; prediction interval 24.9-38.1) (Figure S4), and prevalence of variant *UGT1A9*3 (c.98T>C)* allele carriage is consistently estimated at 3.9% (95%CI 3.1-4.9, prediction interval 3.0-5.2) (Figure S4).

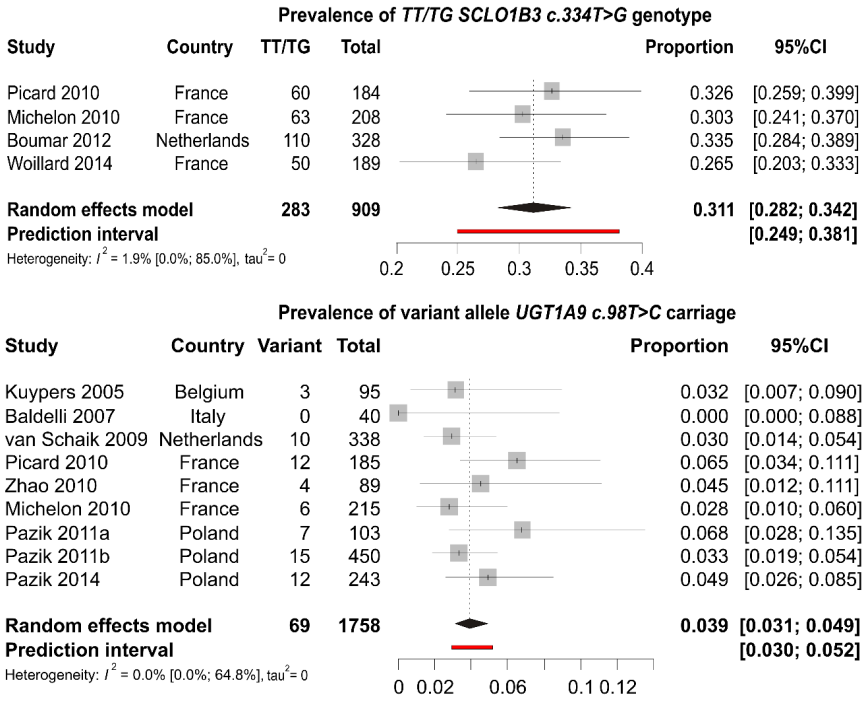

Figure S4 . Meta-analysis of prevalence of TT/TG *SCLO1B3 c.334T>G* genotype and of variant *UGT1A9 c.98T>C* allele carriage in studies including exclusively or predominantly Caucasian Europeans.

Sensitivity to bias due to SLCO1B3 c.334T>G SNP

There is no biologically plausible reason to expect prevalence of TT/TG*SLCO1B3 c.334T>G* genotype (estimated in Figure S4 at 31% in Caucasian Europeans) to differ between *ABCG2 c.421C>A* variant carriers ("treated") and wt subjects ("controls"). However, one could assume a range of different imbalances in prevalence of TT/TG subjects occurring by chance. Table S1 illustrates scenarios in which a considerable imbalance occurred by chance - prevalence of TT/TG (associated with higher MPA $AUC_{0\text{-}12}$) among *ABCG2 c.421C>A*variant carriers (treated) is at the upper limit of the 95% prediction interval (Figure S4) and is 40%, while in wt subjects (controls) it is at the lower limit of the prediction interval and is 25%. It also illustrates scenarios with an even greater imbalance with 2.5-fold difference in prevalence of TT/TG in *ABCG2 c.421* variant carriers (50%) and wt controls (20%). Even if the effect of TT/TG is much higher than estimated in Figure S2 (i.e., it is ROM=1.25, which should be considered markedly higher than the pooled estimate, since it was based on raw, unadjusted [reported] values) – the effect of the variant *ABCG2 c.421* allele would still be at least GMR=1.31 (vs. the estimated 1.40).

Table S1 . Sensitivity analysis of the effect of *ABCG2 c.421C>A* variant allele on MPA $AUC_{\tau,\sigma\varsigma}$(estimated in the main analysis as GMR=1.40) to account for hypothetical bias arising from not accounting for the *SLCO1B3 c.334T>G* SNP. The effect of the TT/TG (vs. GG)*SLCO1B3 c.334* genotype is estimated at ROM=1.135 (95%CI 0.949-1.361) (Figure S2) and prevalence of the TT/TG genotype is estimated at 31% (Figure S4). Shown are bias-corrected effects (GMRs) of the *ABCG2 c.421C>A* variant (vs. wt) assuming considerable imbalance between variant carriers and wt controls in prevalence of the *SLCO1B3 c.334 TT/TG* genotype, and assuming TT/TG effect as estimated (i.e., 1.15) and higher, up to 1.25.

Prevalence of TT/TG in *ABCG2* variant carriers	Prevalence of TT/TG in *ABCG2* wt controls	Effect of *TT/TG SLCO*
40%	25%	1.15
40%	25%	1.20

Prevalence of TT/TG in *ABCG2* variant carriers	Prevalence of TT/TG in *ABCG2* wt controls	Effect of *TT/TG SLCO*
40%	25%	1.25
50%	20%	1.15
50%	20%	1.20
50%	20%	1.25

Sensitivity to bias due to UGT1A9 c.98T>C SNP

Prevalence of the variant *UGT1A9 C.98T>C* allele is very low. None of the studies in Figures S3 and S4 identified a single variant homozygous subject – only TC heterozygotes were found. Their prevalence (estimated at 3.9% in Figure S4) suggests that one could expect 2 TC subjects in the present sample of 54 matched patients, but probability of observing 2 variant carriers in the present sample is only 27.4%, probability of observing none is 11%, probability of observing not more than one is 35.8% and probability of observing 2 or more is 36.7%. Overall, TC patients were most likely only a few – if any – in the present sample. There is no biologically plausible reason to expect prevalence of *UGT1A9 c.98T>C* variant carriers to differ between *ABCG2 c.421C>A* variant carriers ("treated") and wt subjects ("controls"). However, one could assume a range of different imbalances in prevalence occurring by chance. Table S2 illustrates two types of scenarios: in one, the "starting" effect of the *ABCG2 c.421C>A* variant allele that needs to be adjusted for bias is GMR=1.40 (as estimated in the main analysis); in the other one, the "starting" effect of the *ABCG2 c.421* variant allele is GMR previously adjusted for bias arising from not accounting for *SLCO1A9 c.334T>G* SNP with a high imbalance in TT/TG prevalence between *ABCG2 c.421* variant carriers and wt controls (50% vs. 20%) and a marked effect of the TT/TG genotype (ROM=1.25), i.e., GMR=1.31. The effect of the TC *UGT1A9 c.98* genotype (associated with higher MPA $AUC_{0\text{-}12}$) is ROM=1.098, as estimated in Figure S3, or GMR=1.50, as suggested by van Schaik [5] based on time-averaged AUCs over a 12-month period in CsA or tacrolimus co-treated patients; and there is a considerable imbalance between *ABCG2 c.421* variant carriers and wt controls in prevalence of *UGT1A9 c.98T>C* heterozygotes: a 3-fold difference as 6% vs. 2%, or a 5-fold difference as 5% vs. 1% or 10% vs. 2%. As demonstrated in Table S2, even in the "worst case scenario", with the TC effect of GMR=1.50 and a huge imbalance between treated and controls (10% vs. 2%), already adjusted *ABCG2 c.421* variant allele effect would still be 1.26, i.e., beyond the conventional limit of equivalent total exposure.

Table S2 . Sensitivity analysis of the effect of *ABCG2 c.421C>A* variant allele on MPA $AUC_{\tau,\sigma\varsigma}$ to account for hypothetical bias arising from not accounting for the *UGT1A9 c.98T>C* SNP. The effect of the TC (vs. TT) *UGT1A9 c.98* genotype is estimated at ROM=1.098 (95%CI 0.548-2.198) (Figure S3) or as GMR=1.50 [5], and prevalence of the TC genotype is estimated at 3.9% (Figure S4). Shown are bias-corrected effects (GMRs) of the *ABCG2 c.421C>A* variant (vs. wt) assuming considerable imbalance between variant carriers and wt controls in prevalence of the *UGT1A9 c.98 TC* genotype, and assuming TC effect as estimated (i.e., 1.10 or 1.50). The *ABCG2 c.421* effect that is being bias-corrected is either GMR=1.40 (as estimated in the main analysis) or GMR=1.31 – if adjusted for hypothetical bias arising from not adjusting for *SLCO1B3 c.334T>G* genotype, under a large TT/TG imbalance between *ABCG2* variant carriers and wt controls, and the TT/TG effect ROM=1.25 (see Table S1).

Prevalence of TC in *ABCG2* variant carriers		Prevalence
GMR that needs to be adjusted for bias =1.40	**GMR that needs to be adjusted for bias =1.40**	**GMR tha**
6%		2%
5%		1%
10%		2%
6%		2%
5%		1%
10%		2%
GMR that needs to be adjusted for bias =1.31	**GMR that needs to be adjusted for bias =1.31**	**GMR tha**

Prevalence of TC in *ABCG2* variant carriers	Prevalence
6%	2%
5%	1%
10%	2%
6%	2%
5%	1%
10%	2%

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