Precision Medicine Approaches to Immunosuppression Using Pharmacogenomics and the Microbiome

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Introduction:

Twenty years into the 21st century, the concept of personalized medicine is well established and implemented into medical care. The emergence of advanced technology of DNA sequencing and analysis of big data has led to publications that demonstrate the benefits of applying precision medicine concepts[1,2]. Conventional treatment modalities are based on the concept of "*one size fits all*" and ignores inter-individual variations in physiological and biochemical processes. In contrast, precision medicine refers to tailoring the therapeutic approach to nuanced attributes including genetics of individuals. Precision medicine is a general concept comprising several scientific areas known as -*omics*: genomics, transcriptomics, proteomics, metabolomics, epigenomics and microbiomics.

Genomics is the study of genes and their variations. Pharmacogenomics is when these variations are associated with pharmacokinetics and/or pharmacodynamics of a drug and one form of precision medicine. A classic example of pharmacogenomics is the influence of variation in the genes *VKORC1, CYP4F2* and *CYP2C9* which profoundly influence warfarin dosing[3,4].

Microbiomics is the study of microbial communities (microbiota) and their collective genes (microbiome). Pharmacomicrobiomics is another form of precision medicine and is the study of the interaction between the microbiota/microbiome and drug disposition and/or response. This field is less well developed than pharmacogenomics and there are only a few examples where microbiome is used in clinical care (i.e. *Clostridium Difficile* treatment)[5–7].

In this thesis, I provide two examples of precision medicine applications to currently unsolved drug related problems. In chapter 1, the influence of the microbiome on the enterohepatic recirculation of mycophenolate mofetil (MMF) in hematopoietic stem cell transplant recipients (HCT) is described. There is substantial unexplained interindividual variability in MMF pharmacokinetics. This work illustrates that variability in the gut microbiome composition is associated with enterohepatic recirculation of the mycophenolic acid (MPA), the active metabolite of MMF, and consequently differences

in drug exposure. In chapter 2, the influence of *CYP3A4 and CYP3A5* genotypes on the magnitude of the drug-drug interaction between tacrolimus and steroids in kidney transplant recipients is described. This drug-drug interaction, while well-known, is unpredictable. Some individuals have an induction of tacrolimus clearance in the presence of steroids and others have little to no changes in clearance. This analysis shows that individuals who carry a loss of function allele such as *CYP3A5*3* have a minor and clinically insignificant drug-drug interaction whereas individuals who express CYP3A5 and carry at least one CYP3A5*1 allele have a significant tacrolimus-steroid interaction which results in higher tacrolimus dose requirements during steroid use.

Chapter 1 :
Reduced Enterohepatic Recirculation of Mycophenolate and Lower Blood Concentrations are Associated with the Stool Bacterial Microbiome After Hematopoietic Cell Transplantation

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Overview

Background: Mycophenolate mofetil (MMF) is an important immunosuppressant used after allogeneic hematopoietic cell transplant (HCT). MMF has a narrow therapeutic index and blood concentrations of mycophenolic acid (MPA), the active component of MMF, are highly variable. Low MPA concentrations are associated with risk of graft vs host disease (GvHD) while high concentrations are associated with toxicity. Reasons for variability are not well known and may be due, at least in part, to the presence of β-glucuronidase producing bacteria in the gastrointestinal tract which enhance MPA enterohepatic recirculation (EHR) by transforming MPA metabolites formed in the liver back to MPA.

Objective: To determine if individuals with high MPA EHR have a greater abundance of β-glucuronidase producing bacteria in their stool and higher MPA concentrations relative to those with low EHR.

Study Design: We conducted a pharmacomicrobiomics study in 20 adult HCT recipients receiving a myeloablative or reduced intensity preparative regimen. Participants received MMF 1g IV every 8 hours with tacrolimus. Intensive pharmacokinetic sampling of mycophenolate was conducted before hospital discharge. Total MPA, MPA glucuronide (MPAG) and acylMPAG were measured. EHR was defined as a ratio of MPA area under the concentration-versus-time curve $(AUC)_{4\text{-}8}$ to MPA $AUC_{0\text{-}8}$. Differences in stool microbiome diversity and composition, determined by shotgun metagenomic sequencing, were compared above and below the median EHR (22%, range 5-44%).

Results: Median EHR was 12% and 29% in the low and high EHR groups, respectively. MPA troughs, MPA $AUC_{4\text{-}8}$ and acylMPAG $AUC_{4\text{-}8}/AUC_{0\text{-}8}$, were greater in the high EHR group vs low EHR group [1.53 vs 0.28 mcg/mL, *p = 0.0001*], [7.33 vs 1.79 hr*mcg/mL, *p = 0.0003*] and [0.33 vs 0.24 hr*mcg/mL, *p = 0.0007*], respectively. MPA $AUC_{0\text{-}8}$ was greater in the high EHR than the low EHR group and trended towards significance [22.8 vs. 15.3 hr*mcg/mL, *p=0.06*]. *Bacteroides vulgatus*, *stercoris* and *thetaiotaomicron* were 1.2-2.4 times more abundant (*p=0.039, 0.024, 0.046*, respectively) in the high EHR group. MPA EHR was positively correlated with *B. vulgatus* (ρ=0.58,

p≤0.01) and *B. thetaiotaomicron* (ρ=0.46, *p<0.05*) and negatively correlated with *Blautia hydrogenotrophica (*ρ=-0.53, *p<0.05)*. Therapeutic MPA troughs were achieved in 80% of patients in the high EHR group and 0% in the low EHR. There was a trend towards differences in MPA AUC_{0-8} and MPA C_{ss} mcg/mL in high vs. low EHR groups (*p=0.06*).

Conclusion: MPA EHR was variable. Patients with high MPA EHR had greater abundance of Bacteroides species in stool and higher MPA exposure than patients with low MPA EHR. Bacteroides may therefore be protective from poor outcomes such as graft vs host disease but in others it may increase the risk of MPA adverse effects. These data need to be confirmed and studied after oral MMF.

Introduction

Numerous diseases have been linked to the gut microbiota mainly through interactions with the host immune system. In hematopoietic cell transplant (HCT) there is an association between the stool microbiome, especially bacterial diversity, and graft vs host disease (GVHD) risk[8–10]. Holler et al found that an increased abundance of *Enterococci* was associated with GVHD[11]. Jenq et al[12] showed that a highly diverse intestinal microbiota was associated with lower incidence of GVHD mortality. In particular, the genus *Blautia* was associated with reduced mortality due to GVHD. Furthermore, GVHD was found to be significantly associated with decreasing nonpathogenic Clostridia abundance, possibly due to clindamycin exposure[13].

It is well-established that certain diseases and drugs profoundly alter the composition of the gut microbiome leading to dysbiosis. HCT patients receive antibiotics and non-antibiotic medications during the course of transplantation that cause dysbiosis that may adversely influence effectiveness of other drugs and specifically mycophenolate[14,15]. Mycophenolate mofetil (MMF) is a common immunosuppressant and is critical for donor engraftment and prevention of acute GVHD (aGVHD). It reversibly inhibits inosine monophosphate dehydrogenase, reducing T and B lymphocyte proliferation[16]. Despite the use of mycophenolate and other potent immunosuppressants, acute GVHD still occurs in up to 50% of patients and is associated with non-relapse mortality[17]. Variability in immunosuppressant exposure is an important contributor to failure of immunosuppressants leading to under exposure and loss of immune control, or over exposure and toxicity necessitating dose reduction or discontinuation. Pharmacokinetic variability of mycophenolic acid (MPA), the active component of MMF, is high in HCT[18]. We and others have shown that low MPA concentrations are associated with poorer engraftment and higher incidence of acute GVHD whereas high concentrations are associated with risk of toxicity[19–23].

Mycophenolate disposition and metabolism is complex. MPA undergoes enterohepatic recirculation (EHR) that is mediated by intestinal β-glucuronidases produced primarily by Gram-negative anaerobes[24]. EHR is important because it contributes to maintaining

MPA blood concentrations. Interpatient and intrapatient variability in EHR may lead to unpredictable immunosuppression which may adversely affect engraftment, GVHD protection and MMF toxicity. Ex vivo culturing systems of microbial communities have identified microbiome-derived metabolism in 28 pharmacological classes including mycophenolate resulting in parent drug depletion or appearance of new metabolites[25]. In an in vitro microbiome model, MMF was one of the most highly metabolized drugs with ~70 bacteria showing metabolic activity[26].

Zimmerman-Kogadeeva et al simulated host-microbiome deglucuronidation and glucuronyltransferase enzyme activity using experimental data and tested under which conditions EHR affects drug exposure. They were able to predict the concentrations of drugs using these models establishing the potential role of microbiome on the pharmacokinetics of [27].

There are little data on EHR in transplant recipients. Disease and xenobiotic compounds used in patient populations change the composition and/or abundance of β-glucuronidase forming bacteria in the gut microbiota. β-glucuronidase enzymes are primarily produced by gut microbes from the Bacteroidetes (produces the largest number of enzymes), Firmicutes, Verrucomicrobia, and Proteobacteria phylum[28]. Any disruption in these organisms may reduce the amount of β-glucuronidase in the gut, reduce EHR, lower systemic MPA exposure and increase the risk of mycophenolate failure. In addition, MMF is associated with diarrhea and in kidney transplant recipients higher fecal β-glucuronidase activity is associated with a longer course of diarrhea compared to lower β-glucuronidase activity[29]. No studies have investigated the effect of gut microbiota on MPA exposure in HCT recipients.

The primary objective of this study was to evaluate the association of the stool microbiome composition and abundance with MPA EHR, and the pharmacokinetics of MPA and its metabolites. As a secondary objective, the association between microbiome β-glucuronidase gene abundance in the stool with MPA EHR was determined.

Materials and Methods

Study Design and Participant Enrollment

We conducted a prospective, observational, pharmacomicrobiomics study. Eligible participants were adults 18-75 years of age with hematologic malignancies undergoing myeloablative or reduced intensity allogeneic HCT. Participants received an HLA-haploidentical related HCT using a reduced intensity conditioning or a myeloablative preparative regimen followed by an HLA-matched, haploidentical or unrelated donor transplant. Participants received post-transplant cyclophosphamide for GVHD prophylaxis on days 3 and 4. Immunosuppression with mycophenolate mofetil and tacrolimus started on day +5 post-transplant. Participants were excluded if they had a previous HCT or organ transplant, liver dysfunction defined as total bilirubin 2-fold greater than the upper limit normal range, or kidney dysfunction defined as a CrCl below 30mL/min. Standard antibiotic prophylaxis consisted of levofloxacin beginning on day −1 until neutrophil recovery. Cefepime was the recommended initial empiric antibiotic for neutropenic fever. All antibiotic use and changes were at the discretion of the treating physician. No patients received probiotics. All study participants signed a voluntary informed consent. The protocol was approved by the IRB (Study# 00005621).

Pharmacokinetic Sampling of Mycophenolate

Participants received MMF 1g IV every 8 hours over 2 hours and tacrolimus 0.03 mg/kg IV over 24 hours beginning on day +5. Pharmacokinetic sampling was conducted on day +7 in the inpatient unit over one IV MMF dosing interval at steady state (a minimum of 3 unchanged doses). At the time of pharmacokinetic sampling, the CrCl was required to be > 30 ml/min and total bilirubin < 3 times the upper limit of normal. If CrCl in renal or hepatic function was not within these parameters, the patient was excluded from the study and replaced. No patients were excluded due to these criteria. Whole blood samples (4mL in purple top tube) for pharmacokinetics were obtained at hours 0, 2, 3, 4, 5, 6, 7, and 8 following the start of the 2-hour infusion.

Processing and Bioanalysis of Mycophenolate

Samples were transferred within one hour of collection on ice in an insulated container to the lab and processed. Mycophenolate blood samples were centrifuged at 2200 RPM for 10 minutes at 4 ºC. After centrifugation, plasma (~0.5-1 mL) was transferred into a screw top containing 85% H_3PO_4 at a ratio of 1:50 of 85% H_3PO_4 to plasma for total MPA, MPAG and acylMPAG. Acidification is required to prevent the breakdown of the acylMPAG metabolite. Tubes were stored at -80 ºC until time of assay. Samples were assayed using HPLC-MS assays for total MPA, MPAG and acylMPAG [30,31]. The lower limits of quantification are 25 ng/mL for total MPA, 1 mcg/mL for MPAG, 25 ng/mL for acylMPAG.

Pharmacokinetic Analysis and Determination of MPA EHR

MPA, MPAG and acylMPAG concentration-time data were analyzed by non-compartmental analysis. WinNonLin Phoenix (Certara, L.P., Princeton, New Jersey) was used to calculate MPA, MPAG and acylMPAG area under the curve (AUC) using the linear trapezoidal rule. Concentration at steady state (C_{ss}) was determined by dividing AUC_{0-8} by the dosing interval in hours. The concentration-time curves of MPA were visually inspected in all patients. A second MPA peak, when present, was observed 4 or more hours after the dose. Therefore, MPA EHR was defined as a ratio of MPA AUC_{4-8} to MPA AUC_{0-8} and was computed for each individual. Individuals with MPA EHR above the median were considered to have high EHR and those below the median had low EHR.

Stool Collection and Microbiome Analysis

Stool samples for DNA analysis were collected on the day of MMF pharmacokinetic sampling (±48 hours) and stored at -80 ºC after collection. The stool sample for microbiome characterization was collected by swabbing the stool sample obtained from a fresh collection in the patient's room. The stool swab was immediately placed in a tube containing 95% ethanol. The characterization and abundance of the microbiome and β-glucuronidase gene abundance was determined in each sample.

DNA was extracted from the swabbed stool samples using the DNeasy PowerSoil DNA isolation kit. The DNA was then used for shotgun sequencing using 1/4NexteraXT libraries[32] on the Illumina NextSeq instrument. A mean read depth of 2.9M raw reads (range: 0.4 - 5.7M reads) per sample was achieved. Bioinformatic processing and annotations were done using MetaPhlAn3 and HUMAnN3[33]. Functional genes were annotated against the MetaCyc [34] and Uniref[35] databases and classified using level 4 EC categories. Taxonomic data were reported as percent of total reads and functional gene data were normalized as copies per million (cpm). Sequencing data are deposited in the SRA archive under BioProject accession number SRP303698.

Statistical analysis

Wilcoxon rank sum test was used to test the difference between pharmacokinetic measures by EHR group at 0.05 significance level. Species composition data were reported as relative abundances and abundances of β-glucuronidase genes were normalized as copies per million (cpm). Differences among microbial communities were determined based on Bray-Curtis dissimilarities calculated from species abundance tables and evaluated by analysis of similarity (ANOSIM) using vegan[36]. Communities were visualized by ordination using principal coordinate analysis, and species that were significantly correlated with axis position (Spearman correlation) were overlaid using the corr.axes command in mothur[37]. Differences in relative abundances of species, β-glucuronidase gene abundances, and alpha diversity measured by the Shannon index were determined using Kruskal-Wallis test. All statistics were evaluated at $\alpha = 0.05$.

Results

Twenty HCT recipients (12 males and 8 females) were studied. Demographics are shown in Table 1.1. The median (interquartile range [IQR]) serum creatinine and total bilirubin on the day of pharmacokinetics was 0.58 (0.16) mg/dL and 0.55 (0.32) mg/dL, respectively. Pharmacokinetic sampling was performed at a median (range) of day 7 (6-8) posttransplant. Plasma MPA, MPAG and acylMPAG AUC_{0-8} in all patients were 19.36

hr*mcg/mL (IQR: 10.5), 207.69 hr*mcg/mL (IQR: 109.95) and 2.06 hr*mcg/mL (IQR: 1.52), respectively.

Pharmacokinetics by EHR Classification

MPA, MPAG and acylMPAG $AUC_{0\text{-}4}$, $AUC_{4\text{-}8}$, and $AUC_{0\text{-}8}$ were calculated and reported in (Figure 1.1 and supplementary Table 1.1). EHR was the ratio of MPA $AUC_{4\text{-}8}$/MPA $AUC_{0\text{-}8}$ and the overall median was 0.22 (range 0.05-0.44). The median ratio in those classified as high EHR and low EHR was 0.29 and 0.12 respectively (Figure 1.1, supplementary Table 1.1). MPA trough at 8 hours post dose was greater in patients with high EHR relative to low EHR (1.53 vs 0.28 mcg/mL, *p=0.0001*). MPA $AUC_{4\text{-}8}$ hr*mcg/mL was higher in the high EHR group (7.33 vs 1.79 hr*mcg/mL, *p=0.0003)*. The median ratio of acylMPAG $AUC_{4\text{-}8}/AUC_{0\text{-}8}$ was higher in those classified as high EHR compared to those classified as low EHR (0.33 vs. 0.24 hr*mcg/mL, *p=0.0007*). There was a trend towards differences in both MPA $AUC_{0\text{-}8}$ and MPA C_{ss} mcg/mL in high vs. low EHR groups (*p=0.06*). There are differences in the MPA concentration-time profiles where beginning around 4 hours concentrations are greater in the high EHR group (Figure 1.2). In the high EHR group, 80% of the participants had MPA trough concentrations within the therapeutic range and 0% in the low EHR group (Table 1.2).

Microbiome association with EHR

Community composition of stool samples varied significantly between patients with high *vs.* low EHR (ANOSIM R = 0.15, *p = 0.012*; Figure 1.3. Panels A-C). *B. vulgatus, B. stercoris* and *B. thetaiotaomicron* had significantly greater relative abundances in individuals with high MPA EHR *(p=0.039*, *0.024*, *0.046* respectively; Figure 1.3. Panels D-F). MPA EHR was also positively correlated with the presence of *B. vulgatus* (ρ=0.58, *p=0.01*) and *B. thetaiotaomicron* (ρ=0.46, *p=0.04*) (Table 1.3). *Blautia hydrogenotrophica* abundance was inversely correlated with MPA EHR (ρ=-0.53, *p=0.02*), MPAG $AUC_{4\text{-}8}/AUC_{0\text{-}8}$ ratio (ρ=-0.57, *p=0.01),* AcylMPAG $AUC_{4\text{-}8}/AUC_{0\text{-}8}$ ratio (ρ=-0.50, *p=0.03*) and MPA steady state (C_{ss}) (ρ=-0.50, *p=0.03*). *Parabacteroides distasonis* abundance was significantly and positively correlated with the MPAG $AUC_{4\text{-}8}/AUC_{0\text{-}8}$ ratio (ρ=0.69, *p=0.001*) and MPA C_{ss} (ρ=0.53, *p=0.02*). *B. uniformis*

abundance was positively correlated with MPAG AUC_{0-8} (ρ=0.48, *p=0.04*). Abundances of genes annotated as β-glucuronidase did not differ significantly among samples from patients based on EHR status (Kruskal-Wallis *p = 0.496*), although greater variability in gene abundances was observed among patients with low EHR (Figure 1.3G). There was no significant difference in alpha diversity (Shannon index) between the high and low EHR groups (Figure 1.3H), which is surprising given the higher burden of antibiotics in the low EHR group. This may be due to the use of a relatively small shotgun sequencing depth and the need for extensively deeper sequencing for comprehensive community characterization[38].

More individuals in the high EHR group (8/10) received levofloxacin prophylaxis in the 72 hours prior to pharmacokinetic sampling than the low EHR group (3/10) (Table 1.4). In the low EHR group, 8 of 10 (80%) of individuals received IV cefepime, cefpodoxime, cefdinir, azithromycin, and/or vancomycin prior to pharmacokinetics compared to only 2 of 10 (20%) in the high EHR group.

Discussion

Enterohepatic recirculation is an important pharmacologic characteristic where biliary excreted drugs are reabsorbed in the intestine instead of being eliminated. Mycophenolate is a classic example of a recirculated drug. Multiple gut bacteria produce β-glucuronidase and changes in abundance or composition of these organisms may affect mycophenolate recirculation and systemic exposure. In this study we demonstrated that β-glucuronidase producing bacteria and others may be associated with changes in MPA recirculation. When given orally, MPA is absorbed primarily in the stomach and proximal small intestine[16] whereas when given intravenously (IV), it bypasses first pass metabolism. MPA is biotransformed in the liver primarily by uridine 5'-diphospho-glucuronosyltransferases (UGTs) 1A9 and 1A8 to the inactive metabolite MPA glucuronide (MPAG), and to a lesser extent to the active metabolite acylMPAG by UGT2B7[24]. MPAG and possibly acylMPAG are excreted renally and into the bile where they are subject to EHR. From the bile, MPAG is excreted into the small intestine where deconjugation occurs via β-glucuronidases and active MPA is reformed. After

deconjugation, MPA is reabsorbed back into the circulation from the distal regions of the small intestine[24,39]. In normal volunteers, EHR is a substantial portion of overall exposure and is estimated to be 37% of the MPA AUC (range 10-61%)[40].

Therapeutic concentrations of MPA have been shown to be important towards engraftment and aGVHD and were consistently lower in the low EHR group. No patients with low MPA EHR had a therapeutic MPA trough (>1mcg/mL) whereas 80% were therapeutic in the high EHR group. Median MPA AUC_{0-8} was lower in the low EHR group (15.35 hr*mcg/mL) vs (22.82 hr*mcg/mL) in the high EHR (*p=0.06*). MPA C_{ss} was 2.85 mcg/mL in the high EHR group and 1.92 mcg/mL in the low EHR group (*p=0.06*). In our previous studies we have found that MPA trough, AUC and C_{ss} targets are hard to attain even on doses of 3gm/day and it is likely that altered EHR contributes to this difficulty[41].

In our study, we found that greater abundances of the species *B. vulgatus, B. stercoris* and *B. thetaiotaomicron* were associated with higher MPA EHR. These anaerobic, Gram-negative rods likely produce β-glucuronidase, which deconjugates MPAG in the intestine to MPA and is then readily enterohepatically recirculated[42]. In a HCT microbiome study, the Bacteroidetes phylum was more abundant in children without aGVHD relative to those who developed aGVHD[43]. This is consistent with presence of more β-glucuronidase enzyme, higher MPA EHR and greater GVHD protection. The genus *Bacteroides* are important in humans and comprise a large proportion of bacteria in the human colon. They produce short chain fatty acids which are utilized by the host as an important energy source and other functions such as providing protection from pathogens and nutrients to other microbial residents. The three individuals with the lowest MPA EHR had no detectable *Bacteroides* (Figure 1.3A).

We also found that relative abundances of *Parabacteroides distasonis* were positively correlated with MPAG ratio and MPA average concentration over a dosing interval (C_{ss}). *P. distasonis* grows in bile and in mice it alters the production of secondary bile acids and increases levels of lithocholic acid and ursodeoxycholic acid[44]. MPAG is excreted into the gallbladder from the liver and efficient deconjugation of MPAG and formation of MPA may be dependent on sufficient primary bile acid production. Thus, the presence of

P. distasonis may represent good hepatobiliary system functioning. *Bacteroides uniformis* is positively correlated with MPAG AUC_{0-8}. This organism and other β-glucuronidase producing bacteria are associated with the deconjugation of SN-38-glucuronide which is transformed back to SN-38 during EHR, the active metabolite of irinotecan, resulting in greater toxicity[45].

We identified that *Blautia hydrogenotrophica* is inversely associated with three pharmacokinetic measures (MPA EHR, MPAG and acylMPAG ratios, and MPA concentration over the dosing interval (Css). Low abundance of the genus *Blautia* has previously been associated with aGVHD[46]. Our findings of lower MPA exposure in those with *Blautia* spp. are inconsistent with greater protection from GVHD. Others have found that the loss of *Blautia* spp. was associated with use of antibiotics active against anaerobic bacteria, and we observed that the low MPA EHR group received more antibiotics including those active against anaerobes.

Not all *Bacteroides* produce β-glucuronidase enzyme. We found that the non-β-glucuronidase producing *Bacteroides vulgatus*[47] was positively correlated with MPA EHR although the mechanism of this association is not clear. We did not identify associations with certain other β-glucuronidase producing bacteria such as *B. fragilis* (which is a major contributor to enzyme levels), *E. coli, P. merdae, C. perfringens* and *Enterococcus* spp. [28] although it does not exclude their effect since it may be obscured by more abundant species and observable in a larger study population.

We observed that 80% of the individuals in the low EHR group received a broad spectrum IV cephalosporins and/or vancomycin in the 72 hours prior to pharmacokinetics whereas in the high EHR group only 20% received a broad-spectrum antibiotic. Broad spectrum cephalosporins and vancomycin are active against Gram-negative anaerobes, which is consistent with a reduction in β-glucuronidase enzyme and subsequently lower EHR. Antibiotic selection may have a substantial influence on EHR in HCT although how much exposure or duration of antibiotic exposure is needed to alter MPA EHR is not known. In a previous study by our group in patients with acute leukemia and HCT, there was no change in bacterial alpha diversity and no major effects on abundance except for slightly lower *Parabacteroides* spp and higher *Blautia* in the levofloxacin group

compared to no antibiotics[48]. However, we found that greater *Blautia* spp is correlated with lower EHR and inconsistent with it being a more beneficial taxon. Naderer et al [49] showed that MPA AUCs were reduced by 33% when co-administered with norfloxacin and metronidazole, which is highly active against Gram negative anaerobes. In kidney transplant recipients, the use of oral ciprofloxacin or amoxicillin-clavulanic acid resulted in a 46% reduction in MPA trough within 72 hours of antibiotic initiation suggesting that only minimal antibiotics exposure alters MPA EHR and blood concentrations[50]. MPA concentrations increase over time and decrease by up to ~50% when MPA was administered with amoxicillin-clavulanate, metronidazole and ciprofloxacin, respectively[51]. In HCT, after reduced intensity conditioning MPA exposure has been noted to increase by nearly 40% in the first week and trended towards higher at one-month post-transplant[52].These longitudinal changes may be in part due to antibiotic prophylaxis, changing antibiotic treatment, and/or recovery of the microbiome posttransplant. In a recent mouse study, MMF altered the gut microbiome in mice by selecting for β-glucuronidase producing bacteria[53]. Vancomycin eliminated these bacteria, reduced MMF related gastrointestinal toxicity, increased stool MPAG concentrations but reduced stool MPA concentrations.

Most evaluations of mycophenolate EHR are with oral administration. In this study we evaluated IV MMF and showed a median of 22% EHR (range of 5-44%). Previous studies with oral MMF in normal volunteers showed a higher MPA EHR with a mean of 37% (range 10-61%) using cholestyramine as a probe[40]. Numerous drugs undergo EHR and in the case of morphine there is significantly greater EHR with oral administration vs IV[54]. We expect that EHR variability and the influence of the bacterial microbiome may be more profound with oral MMF[40]. In an older HCT study no MPA EHR was observed after oral administration, as secondary peaks were not seen; however, all patients were receiving concomitant cyclosporine use which is known to greatly diminish or eliminates MPA EHR[39,52].

Multiple bacteria may be involved in MPA EHR. In an in vitro microbiome model, MMF was one of the most highly metabolized drugs by bacteria with ~70 gut bacteria showing metabolic activity, including *Clostridium spp,* numerous *Bacteroides sp. including B.*

vulgatus, B. thetaiotaomicron and B. stercoris, E. Coli, Bifidobacterium adolescentis, Blatuia, and Ruminocuccus gnavsu[26]. Our study also found that *B. vulgatus, B. thletaiotaomicron and B. stercoris* were associated with MMF and specifically higher EHR. Javdan et al [25] found in a metabolism screen that gut microbiota were associated with ester hydrolysis of MMF forming mycophenolic acid. There are many different β-glucuronidase enzymes produced by bacteria. Data from the Human Microbiome Project from volunteer samples showed that there is a median of 23 (range 4-38) unique β-glucuronidase protein sequences per individual[28]. There may be even greater numbers in immunocompromised populations since they may receive antibiotics that select for a greater abundance of anaerobic populations. Some β-glucuronidases are substrate specific and therefore it is possible that not all bacteria producing this enzyme will affect MPA disposition[28]. Our analysis does not discern which enzyme(s) are responsible for MPAG deconjugation and EHR although it is likely that many are involved.

There are limitations to our study. We used the median MPA EHR (22%) as a convenient cutoff point to stratify patients into high or low EHR groups. The lowest and highest acceptable bounds for optimal EHR should be explored in a larger sample size. Clinical factors and drug-drug interactions known to impact MPA concentrations were not evaluated, but their clinical impact is generally considered low except for the interaction with cyclosporine, and all patients in our study received tacrolimus. Our data indicate that despite similar abundances of the β-glucuronidase gene in the stool of patients with high and low EHR, only a few *Bacteroides* spp. showed significant correlations and differences in abundance between these groups. This may mean that the gene does not express in all *Bacteroides* spp. or that only some β-glucuronidase enzymes produced by *Bacteroides* spp. are active toward MPA. Mycophenolate is reabsorbed in the small intestine but our stool samples are primarily reflective of the bacteria in the lower gastrointestinal tract but it is not most feasible to get samples from the small intestine[55].

We describe an association between the stool microbiome and MPA EHR after HCT. MMF is given as a fixed dose (i.e. 2-3 g/day)[56], and therapeutic drug monitoring is not routinely performed in HCT; therefore, low or high MPA exposure influenced by the

microbiome may go undetected. High EHR may be predictive of better immunosuppression, but it also may be related to MPA concentration dependent toxicities such as leukopenia and anemia. Testing of the microbiome may provide a tool to help guide MMF initial dose selection and dose adjustments.

Tables

Table 1.1: Patient Demographics (n=20)

Age median (IQR), years	46 (19.5)
Male n (%)	12 (60)
Race, n (%)	
American Indian	2 (10)
Caucasian	18 (90)
Diagnosis, n (%)	
Acute Leukemia	16 (80)
Lymphoma	2 (10)
Myelodysplastic syndrome	2 (10)
Preparative Regimen, n (%)	
Reduced intensity	5 (25)
Myeloablative	15 (75)
Height (m) on admission day, median (IQR)	1.75 (0.14)
Weight (kg) on admission day, median (IQR)	90.7 (27.1)
Laboratory values at time of PK, median (IQR)	
Serum creatinine, mg/dL	0.58 (0.16)
Total bilirubin, mg/dL	0.55 (0.32)

Blood urea nitrogen, mg/dL	9.5 (4.5)
Aspartate aminotransferase (AST), U/L	10.5 (4.75)
Alanine transaminase (ALT), U/L	21 (17)
Alkaline phosphatase, U/L	67 (19.75)

Table 1.2: Number of Participants in MPA Troughs by EHR Group

	High EHR (n=10) No. (%)	Low EHR (n=10) No. (%)
Trough <1 mcg/mL (below)	2 (20)	10 (100)
Trough 1-3.5 mcg/mL (within)	8 (80)	0 (0)
Trough > 3.5 mcg/ml (above)	0 (0)	0 (0)

Table 1.3: Significant Correlations of Species Relative Abundance with Mycophenolate Pharmacokinetics. Only species with at least one significant association are shown.

	MPA $AUC_{4-8/}AUC_{0-8}$	MPAG $AUC_{4-8/}AUC_{0-8}$	AcylMPAG $AUC_{4-8/}AUC_{0-8}$	MPAG AUC_{0-8}	MPA Css
Bacteroides vulgatus	**ρ= 0.58****	N.S.	N.S.	N.S	N.S
Bacteroides thetaiotaomicron	**ρ= 0.46***	N.S.	N.S.	N.S	N.S
Blautia hydrogenotrophica	**ρ= -0.53***	**ρ= -0.57***	**ρ= -0.50***	N.S	**ρ= -0.50***
Parabacteroides distasonis	N.S.	**ρ=0.69****	N.S.	N.S	**ρ=0.53***
Bacteroides uniformis	NS	NS	NS	**ρ=0.48***	N.S

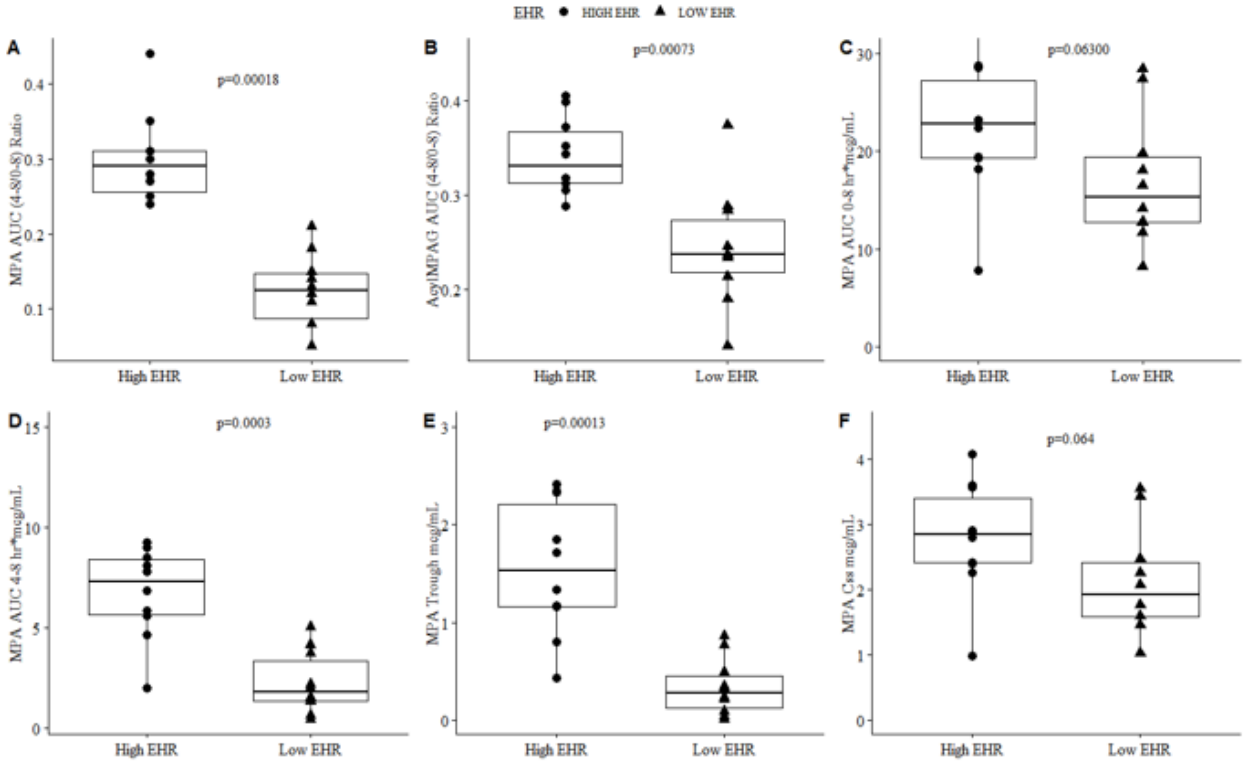
** p-value = or <0.01; *p-value <0.05; N.S., not significant

Table 1.4: Antibiotic Exposure 72 Hours Prior to MPA Pharmacokinetics by EHR Group

High EHR (no. of individuals)	**Low EHR (no. of individuals)**
Levofloxacin (8)	Levofloxacin (3)
Cefepime (2)	Cefepime (1)
	Vancomycin (1)
	Azithromycin/cefepime (1)
	Vancomycin/cefepime (3)
	Cefpodoxime (1)
	Cefdinir (1)

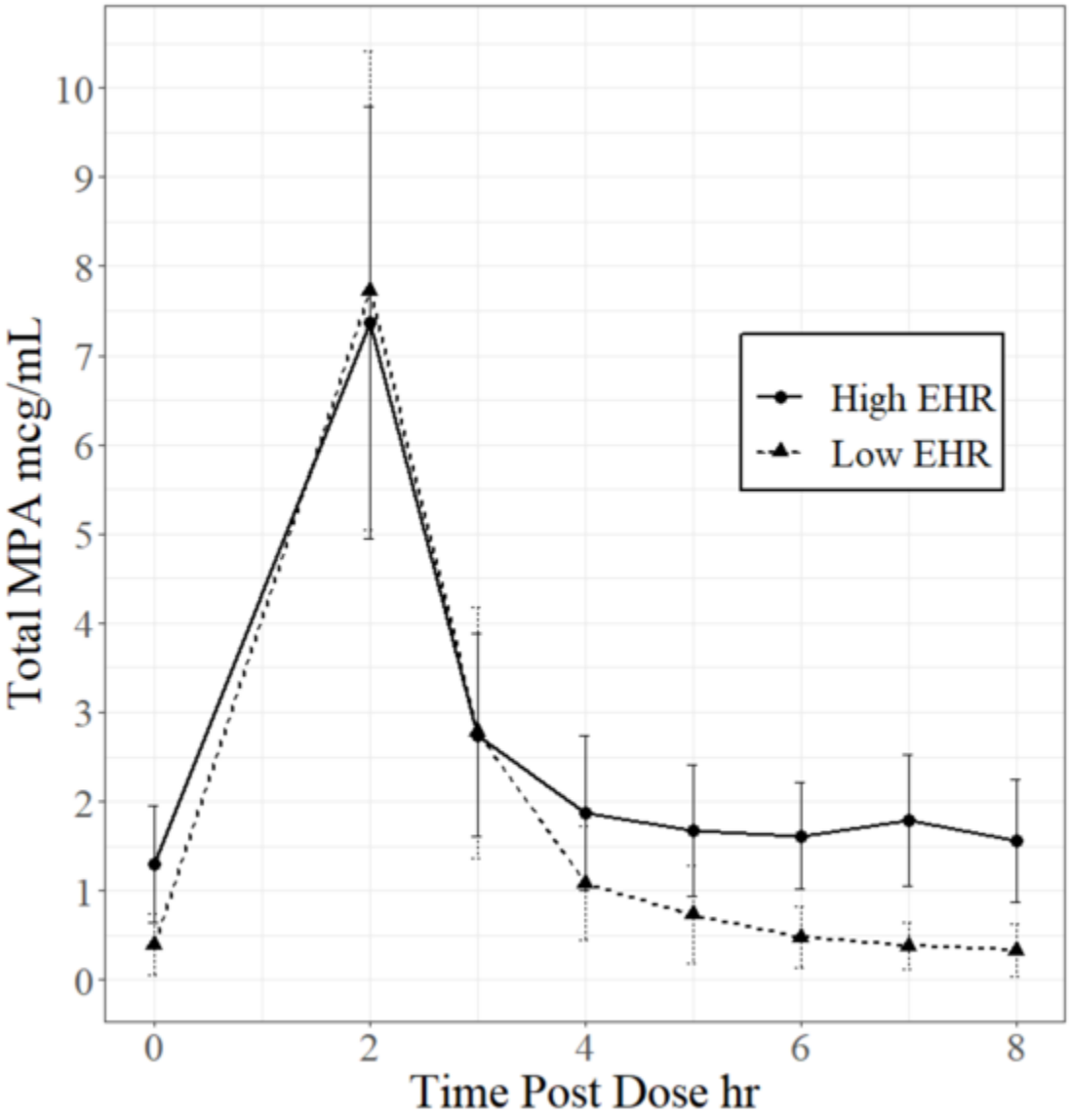
Figures

Figure 1.1: Mycophenolate Pharmacokinetics Measures by EHR Group



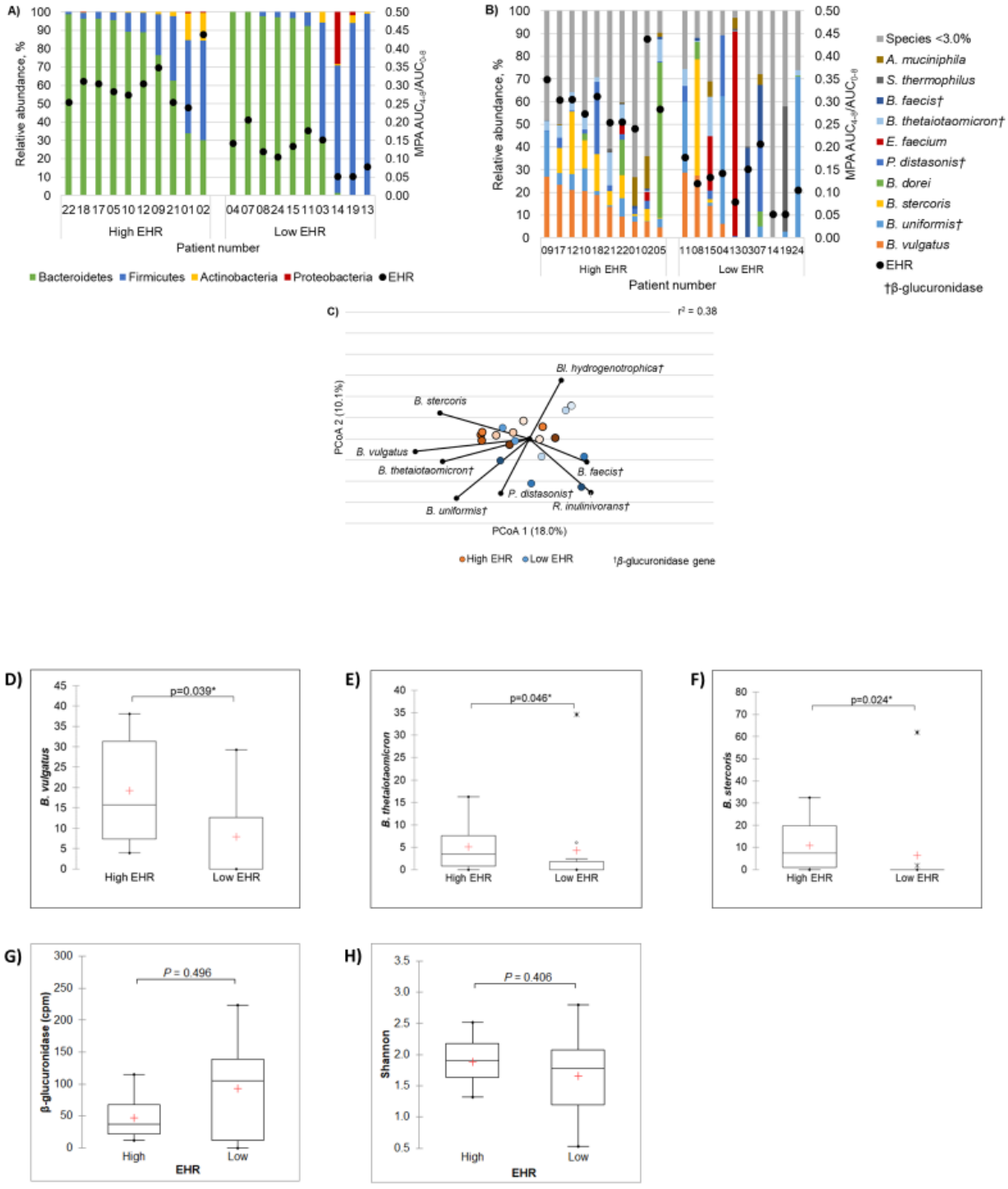
EHR = Enterohepatic Recirculation, AUC = Area Under the Concentration-versus-Time Curve, circles and squares represent individual values of high EHR and low EHR, respectively.

Figure 1.2: Total MPA Concentration-time profile in patients with high and low EHR



Data are the mean with standard deviation. The median $AUC_{0\text{-}8}$ hr*mcg/mL is 22.82 and 15.34 in the high and low EHR groups, respectively (p=0.06). The median MPA $AUC_{4\text{-}8}$ hr*mcg/mL is 7.33 and 1.79 hr*mcg/mL in the high and low EHR groups, respectively, (p=0.0003).

Figure 1.3: Microbial community composition and functional data.



A) Relative abundances of phyla; x axis represents patient number. Black dots represent the EHR values. B) Distribution of abundant species in individual patient samples. Species with a mean relative abundance <3.0% among all

samples were consolidated for clarity and species in which a β-glucuronidase gene was annotated are indicated in shades of blue, the x axis represents participant number. Black dots represent the EHR value in each individual. C) Principal coordinate analysis of Bray-Curtis dissimilarities. Species shown reflect predominant species that were significantly correlated with axis position (Spearman correlation $P < 0.05$, where distance from the origin indicates the strength of a strong correlation). Orange shading indicates individuals with high EHR values and the darker the orange the higher the EHR value. Blue shading indicates individuals with low EHR values and the darker the blue the higher the EHR value. D-F) *Bacteroides* spp. abundances that differed significantly by EHR group; G) β-glucuronidase abundances; H) Shannon index. Box and whisker plots show interquartile range ± standard error where + indicates the mean. Statistics reflect the Kruskal-Wallis test.

Supplemental Materials

Supplemental Table 1.1: Pharmacokinetics of MPA, MPAG, AcylMPAG and Ratios by High and Low EHR

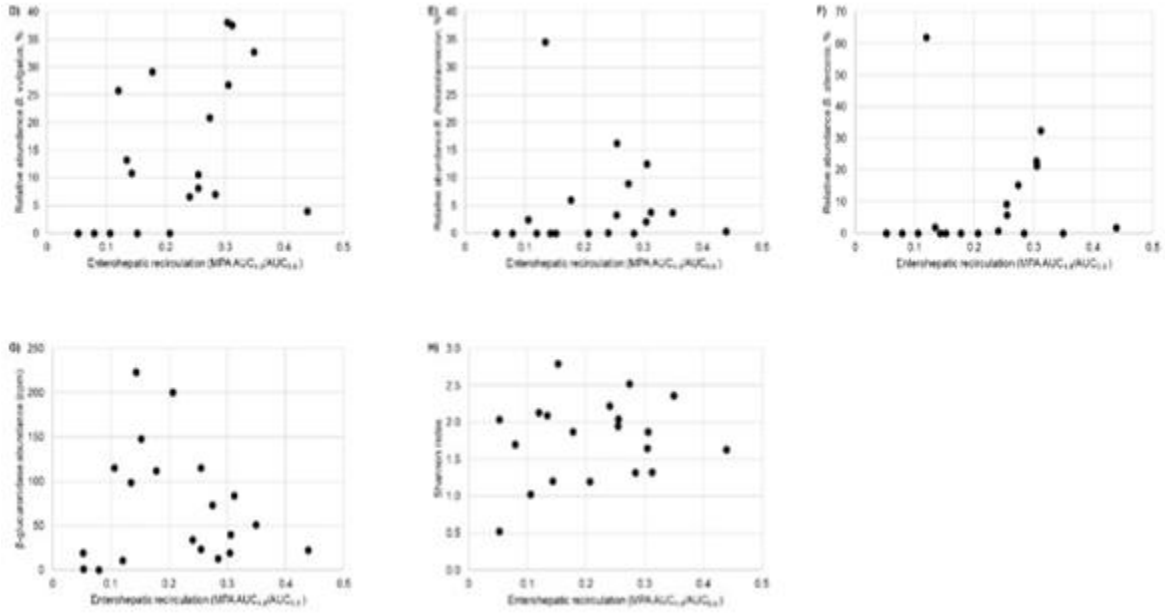
High EHR (n=10)				**Low EHR (n=10)**			
	Median (IQR)	Minimum	Maximum	Median (IQR)	Minimum	Maximum	p-value*
MPA EHR							
MPA AUC_{4-8}/AUC_{0-8}	0.29 (0.05)	0.24	0.44	0.12 (0.06)	0.05	0.21	**0.00018**
MPA							
MPA AUC_{0-8} hr*mcg/mL	22.82 (7.87)	7.84	32.65	15.34 (6.63)	8.12	28.47	0.06
MPA AUC_{0-4} hr*mcg/mL	15.35 (5.82)	5.84	23.39	13.22 (5.7)	7.7	23.39	0.58
MPA AUC_{4-8} hr*mcg/mL	7.33 (2.75)	2	9.26	1.79 (1.99)	0.43	5.05	**0.0003**
MPA trough at 8 hr mcg/mL	1.53 (1.05)	0.43	2.41	0.28 (0.34)	0	0.85	**0.0001**
MPA Cmax mcg/mL	7.62 (2.71)	2.77	10.25	6.86 (3.72)	4.37	12.2	0.97

MPA Css mcg/mL	2.85 (0.98)	0.98	4.08	1.92 (0.83)	1.02	3.56	0.06
MPAG							
MPAG AUC_{4-8}/AUC_{0-8}	0.43 (0.04)	0.35	0.47	0.38 (0.05)	0.29	0.48	0.12
MPAG AUC_{0-8} hr*mcg/mL	211.35 (95.99)	150.0	340.6	183.47 (119.87)	64.59	418.47	0.39
MPAG AUC_{0-4} hr*mcg/mL	125.72 (49.46)	80.87	208.43	113.62 (44.73)	46.15	245.85	0.48
MPAG AUC_{4-8} hr*mcg/mL	89.69 (40.23)	69.13	148.8	67.69 (72.9)	18.43	172.62	0.22
MPAG trough mcg/mL at 8 hr	19.34 (7.99)	11.99	29.56	10.99 (17.48)	1.86	28.59	0.19
MPAG Cmax mcg/mL	39.13 (15.78)	24.83	67.77	34.88 (10.6)	16.9	70.49	0.58
AcylMPAG							
AcylMPAG AUC_{4-8}/AUC_{0-8}	0.33 (0.05)	0.29	0.41	0.24 (0.06)	0.14	0.37	**0.0007**
AcylMPAG AUC_{0-8} hr*mcg/mL	2.1 (1.34)	1.21	8.12	1.99 (1.51)	0.74	4.68	0.44

AcylMPAG AUC_{0-4} hr*mcg/mL	1.4 (0.73)	0.84	5.54	1.48 (1.07)	0.57	3.36	0.97
AcylMPAG AUC_{4-8} hr*mcg/mL	0.69 (0.55)	0.36	2.58	0.52 (0.43)	0.15	1.39	0.14
AcylMPAG trough mcg/mL at 8 hr	0.17 (0.13)	0.07	0.58	0.07 (0.16)	0	0.25	0.07
AcylMPAG Cmax (mcg/mL)	0.54 (0.2)	0.34	2.22	0.6 (0.38)	0.23	1.2	0.97

The p-value was calculated using Wilcoxon test at level of significance = 0.05
*p-value is the comparison of the high and low EHR groups

Supplemental Figure 1.1: Pharmacokinetics by EHR group



Chapter 2 :
Minimal Steroid-Tacrolimus Drug-Drug Interaction in Patients Who are CYP3A5 Non-Expressor

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Ethics approval: Approval was obtained from the Institutional Review Board of the University of Minnesota. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Consent to participate: Informed consent was obtained from all individual participants.

Consent for publication: Patients signed informed consent for use of their data.

Overview

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Purpose: Tacrolimus (TAC) is the primary immunosuppressant used in organ transplantation. TAC is a substrate for CYP3A4 and CYP3A5. These enzymes are inhibited or induced by multiple concomitantly administered drugs leading to high variability in blood concentrations and need for therapeutic drug monitoring. It is well-known that concomitant steroid use induces tacrolimus clearance (CL); however, the extent of the induction varies among individuals. We hypothesized that induction of CYP enzymes by steroids and a resulting increase in TAC CL was greater in individuals carrying functional CYP3A4 and CYP3A5 alleles than those with nonfunctional alleles.

Methods: Data was obtained from a multicenter observational GWAS study [Deterioration of Kidney Allograft Function (DeKAF); development cohort, Genomics of Kidney Transplantation (GEN03); validation cohort]. Participants who were 18 years old or older, received immediate release tacrolimus as a maintenance immunosuppression and had CYP3A4 and CYP3A5 genotyping information available were identified and eligible for this analysis. A total of 44546 TAC troughs and doses (28536 with steroids and 15977 without steroids) from 2462 kidney transplant recipients (89 Asians, 436 African Americans, 70 Native Hispanic, 1867 Caucasians) with CYP3A genotype data (CYP3A5 loss of function alleles [LOF] **3, *6, *7* and CYP3A4 LOF **22*) available from a GWAS chip were included. Induction and maintenance immunosuppression with TAC, mycophenolate and steroids were based on center-specific standard of care protocol, and the TAC dose was adjusted according to the center's therapeutic target range and tolerability. Individuals were categorized according to the number of LOF they carried into four groups: zero LOF (0-LOF), one LOF (1-LOF), two LOF (2-LOF), three or more LOF (+3-LOF). Presence or absence of a concomitant steroid was obtained for each TAC trough. A population pharmacokinetic model (PopPK) model was built using NONMEM to evaluate the effect of steroids as a time-varying covariate on TAC

clearance (CL) across the *CYP3A* LOF alleles groups while accounting for other clinical factors. Recipients with two LOFs and not receiving steroids were assigned as a reference group to which we compared the change in TAC CL across the other LOF groups.

Results: The final PopPK analysis from the development cohort showed that TAC CL/F decreased by 10 % on average if a recipient was diabetic,7% if receiving a calcium channel blocker, and 4% if receiving an antiviral drug. TAC CL/F was higher by 21% in the first 9 days post-transplant, and by 4% if receiving an angiotensin converting enzyme inhibitor 0-, 1-, 2- and +3- LOF alleles were present in 116, 486, 1648 and 191 recipients, respectively. The PopPK analysis showed that individuals with 0-LOF had 137% higher TAC CL without steroids use and 165% higher TAC CL with steroids use relative to the reference group. Individuals carrying 3 LOF had lower TAC CL than the reference group by 32% and 25% in the absence and presence of steroids, respectively. The induction effect of steroids on TAC CL within each LOF allele group was 11.8% and 2.6% in individuals with 0- and 2 LOF alleles, respectively.

Conclusion: The current study identifies a drug-drug-gene interaction with steroids and tacrolimus and explains why some individuals may have significant drug-drug interactions (DDI) with steroids and others do not. The combined effect of steroids and genotype on TAC CL is greater than the effect of each alone. Noncarriers of LOFs are more affected by the TAC-steroids drug-drug interaction than the others. The extent of the drug-drug interaction between TAC and steroids is not pronounced in individuals carrying 2 LOF. Individuals of African ancestry may be at higher risk of the DDI since they may be more likely to carry functional CYP3A4 and CYP3A5 enzymes. Steroid doses should be evaluated for the effect on this interaction as a future direction since higher doses may result in a greater effect.

Introduction

Tacrolimus (TAC) in combination with mycophenolate is a commonly used immunosuppressive regimen in solid organ transplantation[57]. Steroids are also commonly used as part of the induction regimen, may be added to the maintenance regimen in high-risk transplants and are the treatment of choice for acute rejection. Tacrolimus is a calcineurin inhibitor used for maintenance immunosuppression and is highly effective in reducing the risk of acute rejection and improving graft survival. Tacrolimus use is complicated by its narrow therapeutic index, high pharmacokinetic (PK) variability and numerous drug-drug interactions (DDIs)[58]. Therapeutic drug monitoring is required[59] to avoid tacrolimus under- and over-exposure which place patients at risk of graft rejection and drug toxicity, respectively[60–67]. Overexposure is associated with serious problems including new onset diabetes[68–70], acute kidney injury[71], hypertension, tremors affecting quality of life, and malignancies with chronic overexposure[72,73]. Fluctuations in tacrolimus troughs over time are strongly associated with acute rejection and graft loss[74,75]. Problems in maintaining safe and effective tacrolimus troughs are closely associated with the DDIs and nonadherence.

Tacrolimus is a substrate of CYP3A5 and CYP3A4 enzymes[76]. It is well known that interpatient variability in tacrolimus PK and dose requirements is strongly associated with genetic variation in these genes[77,78]. Intrapatient tacrolimus PK variability is strongly associated with DDIs which occur mainly through CYP3A inhibition and induction[79,80] Steroids have a known interaction with tacrolimus[81–84]. The total tacrolimus daily dose required to achieve target trough concentrations is higher in kidney recipients receiving high doses of prednisone, a CYP3A inducer, than those taking low doses but a wide interindividual variability has been detected (0.05-0.39 mg/kg/d)[85]. In another study, prednisolone discontinuation was associated with an average increase in tacrolimus dose-normalized troughs by 36% but the effect was highly variable (-40% to +200%)[86]. Reasons for high variability are not known, although it may be that induction of tacrolimus clearance is dependent on the CYP3A genetic background. We hypothesized that individuals who express CYP3A5 protein (CYP3A5*1/*1 or

CYP3A5*1/*3) may be more susceptible to CYP3A induction relative to those (CYP3A5*3/*3) who have greatly reduced or do not produce CYP3A5 protein. Differences in the strength of DDIs due to *CYP2C9* genetic variation has been previously described for the simvastatin-warfarin[87] and the rifampin-tolbutamide DDI[88]. The purpose of our study was to quantify the magnitude of the DDI between steroids and tacrolimus across *CYP3A4* and *CYP3A5* diplotypes.

Methods

Study design

This is an analysis of kidney transplant recipients enrolled from two multicenter observational studies (Deterioration of Kidney Allograft Function [DeKAF] and Genomics of Kidney Transplantation [GEN03]). Signed informed consent was approved by local institutional review boards. The DeKAF study (www.clinical trials.gov NCT00270712) involved seven enrolling transplant centers, and participants were recruited from 2005 to 2011. The GEN03 study (www.clinicaltrials.govNCT01714440) includes patients recruited from 2012 to 2016 from five enrolling centers. Participants from these studies who were 18 years old or older, received immediate release tacrolimus as a maintenance immunosuppression, and had CYP3A4 and CYP3A5 genotyping information available were identified and eligible for this drug-drug-gene interaction analysis.

Immunosuppression

Induction and maintenance immunosuppression with tacrolimus, mycophenolate, steroids and Thymoglobulin were based on center-specific standard of care protocols. In general, patients on early steroid withdrawal protocols received steroids over 3 to 5 days beginning on day 0, those receiving steroid avoidance protocols were given no steroid induction or maintenance steroids, and late steroid withdrawal protocols were given to high-risk patients (5-10mg daily in the first 6 months). Steroids were also given continuously after the treatment of an acute rejection event.

Tacrolimus Troughs

Tacrolimus trough concentrations were measured before an oral dose as a part of routine clinical care. Two tacrolimus trough concentrations per week were obtained from the medical record for the first 8 weeks and then twice a month from months 3-6 posttransplant for a maximum of 24 trough measurements per patient, if available. The tacrolimus dose was adjusted according to the center's therapeutic target range and tolerability. Trough concentrations were measured at each center and, in general, concentrations of 8–12 ng/mL were targeted for the first 3 months and 6–10 ng/mL for 3–6 months posttransplant. A median of 10 of TAC trough concentrations were obtained from each participant in the first 6 months posttransplant.

Genotyping

A blood sample was collected from each participant and DNA was extracted at a central laboratory at the University of Minnesota as previously described[88]. A customized Affymetrix Axiom Transplant genome-wide chip was used for genotyping[89,90], and CYP3A5*3 (rs776746), CYP3A5*6 (rs10264272), CYP3A5*7 (rs41303343) and CYP3A4*22 (rs35599367) were taken from the genotyping results and used in this analysis.

Population pharmacokinetic modeling

The steroid-tacrolimus DDI across CYP3A4 and CYP3A5 diplotypes was studied using a population PK modeling approach to develop a tacrolimus apparent oral clearance (CL/F) model. Data from the DeKAF study was used for model development and data from the GEN03 study was used for model validation. The model was developed and validated using nonlinear mixed-effect modeling software (NONMEM version 7.5; ICON Development Solutions, Ellicott City, MD, USA) by first-order conditional estimation (FOCE) method with interaction. Exploratory analyses and diagnostic graphics were performed with RStudio (version 2022.02.0; RStudio, Inc., The R Development Core Team) and Perl-speaks-NONMEM (PsN 2.9.7, Uppsala University, Uppsala, Sweden) under the Pirana® interface.

Our previously developed pharmacokinetic base model was used[91,92]. In this model, we created a steady-state infusion model to build the PK structural model via the $PRED library in NONMEM. We used tacrolimus CL/F, the quotient of total clearance (CL) and bioavailability (F), to regress the average daily tacrolimus steady state concentration (C_{ss}) to the dose given. Since tacrolimus half-life is long [93], we assumed that the steady-state trough concentrations were approximately equivalent to the average C_{ss} with minimal peak-trough fluctuation, and the difference between the actual and approximated CL/F is negligible (Eq 1 and 2).

$$Ctrough = C_{ss} \quad \text{Eq 1}$$

$$C_{ss} = \text{total daily dose}_{tacrolimus} / [(CL/F) \times 24] \quad \text{Eq 2}$$

Each transplant center had slight differences in immunosuppression treatment protocols and target tacrolimus troughs. Therefore, we included two-level nested random effects in our model: inter-individual variability (IIV) and inter-site variability (ISV), and we used an exponential error model to describe both as expressed in Eq3. We used the $LEVEL record to account for the ISV. Each transplant center was identified by a specific number in the dataset. Participants from the same transplant center shared a similar random effect (ηISV) nested with ηIIV.

$$CL/F_i = TVCL/F \times \exp^{(\eta IIV + \eta ISV)i} \quad \text{Eq 3}$$

Where CL/F_i is a function of the typical value of apparent oral clearance (TVCL/F) and the individual parameter for the i^{th} participant. ηIIV and ηISV are the estimates of individual deviation and the center deviation from TVCL/F, respectively. Both ηIIV and ηISV are assumed to be normally distributed with a mean of 0 and a variance of ω^2, and they are expressed in our results as coefficient of variance (CV%) (Eq 4). We used a combined error model to describe the residual unexplained variability (RUV) (Eq 5).

$$CV\% = \sqrt{e^{\omega}}-1 \quad \text{Eq 4}$$

$$C_{ij} = C_{pred,ij} * (1 + \varepsilon_{(prop)ij}) + \varepsilon_{(add)ij} \quad \text{Eq 5}$$

where C_{ij} and C_{predij} are the observed concentration and the predicted concentration in the i^{th} individual at the j^{th} occasion, respectively. ε(prop)ij and ε(add)ij are the proportional error and the additive error, respectively. ε(prop)ij and ε(add)ij are assumed to be normally distributed with a mean of 0 and variance σ^2.

Covariate Analysis

To identify the potency of the tacrolimus, steroid DDI across diplotypes, steroids in combination with CYP3A LOFs were tested as a covariate at time of each tacrolimus trough as described below. To identify and account for other factors that affect tacrolimus clearance (TVCL/F) we tested the effect of other concomitant medications use [calcium channel blockers (CCB) , angiotensin converting enzyme (ACE) inhibitors, prophylactic antiviral agents], body weight, serum creatinine, glomerular filtration rate calculated by the Modification of Diet in Renal Disease equation at each trough concentration measurement as time-varying covariates. Recipients' age effect was tested as continuous covariates using power relation. The effect of diabetes at time of transplant, previous kidney transplant, donor type (living or deceased), time post-transplant (before day 9 or after day 9) on TAC TVCL/F were tested as categorical covariates using linear relation.

The CYP3A5*3, *6, *7 and CYP3A4*22 loss of function (LOF) alleles were combined, and each patient was categorized according to the number of LOFs (zero [0-LOF], one [1-LOF], two [2-LOF], and three/four [+3-LOF]). Only 4 participants with 4-LOF whose diplotype was *CYP3A5*3/*3* with *CYP3A4*22/*22*; and was combined with the 3-LOF group in one group +3-LOF. At each trough measurement, these groups were designated by the number of LOFs (0, 1, 2 or +3) and the presence of steroids (yes/no). The effect of the LOF and steroid composite on TAC TVCL/F was tested as a categorical time-varying covariate. We used stepwise covariate modeling in the PsN tool kit. The significance of inclusion and elimination of each covariate was tested based on the likelihood ratio test that follows the χ^2 distribution. In the forward inclusion step, covariates improving the

objective function (OFV) by 6.6 or more ($p<0.01$) were considered significant and included in the full forward model. The full forward model was further tested by the backward elimination step. Covariates increasing the OFV by 10.8 or more ($p<0.001$) were considered significant and retained in the final covariate model.

Model Evaluation and External Confirmation

To evaluate the precision and robustness of the final model parameter estimates, we performed a non-parametric bootstrap. The bootstrap was repeated 1,000 times using random sampling with replacement to generate 1,000 bootstrapped datasets while stratifying on the LOF alleles covariate to ensure representation of each of the LOF alleles groups in each bootstrapped dataset. The bootstrap results including the median of each parameter estimate with 95% confidence intervals (CIs) were compared to the estimated values from the original data in this study.

The confirmation cohort (GEN03) was used to evaluate the predictive ability of the final model, significant covariates and the final model parameters were fixed to predict TAC trough concentrations in the validation cohort. We used median prediction error (MPE) and median percentage error (MPPE) to compute the bias in the model predictions, and root median squared error (RMSE) was used to compute the model precision of population predicted trough concentrations (PRED) (Eq 5-7).

$$\text{MPE} = \text{Median}\,(\text{PRED} - \text{DV}) \qquad \text{Eq 5}$$

$$\text{MPPE} = \text{Median}\,[(\text{PRED} - \text{DV})/\text{DV} \times 100] \qquad \text{Eq 6}$$

$$\text{RMSE} = \sqrt{}\,\text{Median}\,[(\text{PRED} - \text{DV})^2] \qquad \text{Eq 7}$$

where DV is the observed tacrolimus trough concentration; PRED is the population predicted tacrolimus trough concentration.

Results

We evaluated 2462 kidney transplant patients (1608 in the development cohort and 854 in the confirmatory cohort) with 44546 troughs (28718 in the development cohort and 15828 in the confirmatory cohort). Demographic data are summarized in Table 2.1. In all participants the median tacrolimus trough and total daily dose were 8.3 ng/mL (range: 1 - 38) and 6 mg/d (range: 0.3 - 33), respectively, in the first 6 months post-transplant. The median TAC trough in 0-LOF, 1-LOF, 2-LOF and +3-LOF were 6.6 ng/mL [range: 1-11.7], 7.6 ng/mL [range: 2.1-11.7], 8.5 ng/mL [range: 2.4-16.8] and 8.7 ng/mL [range: 4.9-38], respectively (Figure 2.1).

Tacrolimus Population Clearance Model and Clinical Covariates Effect

The final model parameters estimates including the effect of significant covariates on TAC CL/F in the development cohort model and in the bootstrap analysis are shown in Table 2.2. The typical value estimate of tacrolimus apparent clearance (TVCL/F) was 27.2 L/hr. The intersite variability (ISV) and interindividual variability (IIV) estimates were 18.9% and 44.9%, respectively.

The following covariates showed significant influence on TAC CL/F: the number of LOF alleles, diabetes at time of transplant, time post-transplant, CCB use, prophylactic antiviral use, ACE inhibitor use and recipient age. The final population pharmacokinetic model from the development cohort showed that TAC CL/F decreased by 10%, 7% and 4% if individuals had diabetes at the time of transplant, had concomitant CCB or antiviral medications at time of trough measurement, respectively. TAC CL/F was 21% and 4% higher in the first 9 days after transplantation and in the presence of ACE inhibitor, respectively (Table 2.3). The effect of age on TAC CL/F was described by a power function and TAC CL/F decreased by 0.67% for every year above the median age (51.41 years old).

Induction of TAC Clearance by Steroids Across LOF Groups

TAC TVCL/F differed according to the number of LOF alleles (Table 2.2, Figure 2.2). Individuals with 0-LOF alleles had the highest CL increase in the absence and presence of steroid (without steroids 164.5 L/hr, 95% CI (54.7, 75.6); 137% higher than the reference, and with steroids: 72.1 L/hr, 95% CI (64.7, 80.5); 165% higher than the

reference) as shown in Table 2.2 and Figure 2.2. The effect of carrying three or more LOF alleles (+3-LOF) on the TAC CL is a reduction by 32% without steroids and by 25% with steroids relative to the reference group. (Figure 2.2).

Concomitant steroid use resulted in increasing TAC CL across all LOF allele groups; however, the magnitude of the steroids effect on the TAC CL varied by the number of LOF alleles (range: 2.6% - 11.8%, Figure 2.2). The 0-LOF group showed the highest percentage increase (11.8%) in TAC CL in the presence of steroids, the 1-LOF group had a modest increase (5.5%) in TAC CL, and those with 2-LOFs had a negligible increase (2.6%) in TAC CL. Interestingly, those with +3-LOFs had an increase (10.8%) in TAC CL similar to the 0-LOF group. The +3-LOF group was primarily those with the CYP3A5*3/*3 and CYP3A4*1/*22 diplotypes.

Model Evaluation and External Validation

The diagnostic scatter plots showed an acceptable overall goodness-of-fit of the final model (supplementary figure 2.1). We obtained 1000 non-parametric bootstrap runs with 99% successful minimization. The final model parameter estimates were comparable to bootstrap median and within bootstrap 95% CIs as shown in Table 2.3, indicating that the model is robust and reproducible.

The predictive performance of the final mode is illustrated in Table 2.3. The MPE and the MPPE for TAC troughs in the validation cohort were -1.42 ng/mL and -17.8%, respectively. The RMSE was 2.6 ng/mL. This suggests that, on median, the model underpredicted the trough concentrations relative to the observed concentrations.

Discussion

Drug-drug-gene interactions (DDGIs) and DDIs are factors affecting drug pharmacokinetics and subsequently responses which may result in adverse drug reactions or treatment failure[94–97]. The pharmacokinetic variability of tacrolimus is partly attributed to DDIs through CYP3A metabolism inhibition and induction by other drugs such as steroids[98] and *CYP3A* genetic variation[99,100]. The complex superimposition of the TAC-steroids DDI and *CYP3A* genetic variation, which controls CYP3A protein,

on the pharmacokinetics of TAC has not been investigated. In the current analysis, a nonlinear mixed effects modeling approach was applied to this DDGI while accounting for other known influences on TAC CL.

Pharmacogenomic evidence for the effect of genetic variation on TAC pharmacokinetics is robust. Pharmacogenetics guidelines are available and include dose recommendations for patients with *CYP3A5* variants (**3, *6* and **7* alleles)[101]. Further evidence has emerged for the importance of CYP3A4 genetic variation[102–104]. In our previous Genome-Wide Association Study (GWAS) in individuals of European ancestry[103], we identified that the CYP3A4*22 allele was important toward TAC clearance even after accounting for CYP3A5. Elnes et al[105] found significantly higher dose-normalized TAC concentrations in kidney transplant recipients whose genotype was *CYP3A5*3/*3* and *CYP3A4*1/*22* compared with *CYP3A4*22* allele non carriers or CYP3A5 expressors. In our recent analysis we found that *CYP3A4*22* homozygous and heterozygous carriers had 72% and 22% reduction in TAC CL, respectively relative to noncarriers[92].

The *CYP3A4*22* genetic variant results from a splicing defect in intron 6 due to C > T substitution[106]. The *CYP3A4*22* variant showed a 20% and 40% reduction in the enzymatic activity on the CYP3A substrates, midazolam and erythromycin, respectively[107]. The *CYP3A5*3* polymorphism results from an aberrant defect at the splicing site in intron 3 of *CYP3A5* gene resulting in formation of multiple premature stop codon between exons 3 and 4. Consequently, CYP3A5 protein expression is reduced due to rapid degradation of the mRNA[108]. Studies showed that *CYP3A5*1/*3* individuals had higher CYP3A5 protein content and 1.7-fold higher midazolam clearance than *CYP3A5*3/*3* individuals[109,110].

There is a known DDI between steroid and tacrolimus although the extent of the interaction is highly variable. Tapering steroids in kidney transplant recipients resulted in a wide range of change in dose-normalized TAC concentrations (-40% to +200%)[84]. Along with 0.17 mg/kg/d of prednisone, a wide range of TAC daily doses (range: 0.03-0.38 mg/kg) required to achieve target therapy was required[83]. *CYP3A* induction mechanism by steroids is mediated via glucocorticoid and nuclear receptors[98,111].

Mourad el et al[82] found that weight-adjusted TAC doses required to achieve TAC target concentration were significantly and positively correlated to weight-adjusted prednisone dose; however, the number of CYP3A5 expressors in the study was low. Prednisone dose has also been associated with TAC troughs. High (>0.25 mg/kg) and low (<0.15 mg/kg) daily prednisone doses resulted in significant differences in dose-normalized TAC concentrations and TAC doses required to achieve therapeutic target concentration where higher TAC doses were needed with higher prednisone doses[83]. Stifft et al[112] showed a dose effect of prednisone on dose-normalized TAC levels in homozygous and heterozygous *CYP3A5*3* carriers where *CYP3A5*3* homozygotes had additional 0.3 mcg/L increase in their dose-normalized TAC concentration compared to *CYP3A5*1/*3*.

Our analysis identified a negative relationship between the number of *CYP3A* LOF alleles and the TAC CL/F where the higher the LOF alleles carried by an individual, the lower the TAC CL. The composite effect of LOFs and steroids on TAC CL/F was greater than the individual effect of each. As the number of CYP3A LOFs increased from 0, 1 to 2 the increase in TAC clearance declined in the presence of steroids. This suggests a possible lower induction effect by steroids on the CYP3A5 gene when the amount of functional protein is low. Interestingly, the induction effect of steroids on TAC CL was similar in the +3-LOF and the 0-LOF group (10.6% vs 11.8%). We hypothesize that the presence of both *CYP3A5* and *CYP3A4* variants and particularly *CYP3A4*22*, also reduces steroid metabolism and prolongs steroid exposure, this subsequently maintains a robust steroid induction effect on TAC. In a group of healthy volunteers, the concomitant use of ketoconazole, a CYP3A4 inhibitor, with the steroid budesonide, a 50% reduction in budesonide elimination and a 6.5-fold increase in exposure[113] was observed. Skauby et al[114] showed that the intrinsic clearance of prednisolone, the pharmacologically active metabolite of prednisone, was more dependent on CYP3A4 than CYP3A5 enzyme for metabolism.

We previously showed that African American (AA) ancestry had larger variability in their TAC trough concentrations than those of European ancestry[115]. Individuals of AA ancestry are more likely not to carry LOFs while the majority of European ancestry

individuals carry 2-LOFs. It is possible that the greater induction effect by steroids on TAC CL in the 0-LOF group, with fully functional CYP3A5 protein, leads to higher intrapatient variability in TAC troughs compared in African Americans.

Other variables affecting TAC pharmacokinetics and causing between-patients variability are reviewed elsewhere[58,93,116,117]. We identified the composite variable (CYP3A LOF + steroids), ACE inhibitor use, CCB use, antiviral use, history of diabetes at time of transplant, recipient's age and time post-transplant as significant covariates affecting TAC CL/F. The effect of significant covariates in our analysis is in agreement with previous findings[118–121].

There are several limitations to our study. We evaluated the effect of steroids based on steroid use status (yes/no) and did not evaluate steroid dose or duration of use. As the safety of systemic steroids was associated with the time course of steroid treatment[122], we think that the CL induction of effect of steroids could be related to the treatment duration. Our analysis lacks the type of steroid used (methylprednisolone vs prednisone), the start and stop date which may affect the degree of CYP3A induction. We also do not have hematocrit , antifungal drug use and other variants such as *CYP3A4* and *PXR* which may affect TAC clearance[112,123].

Our model underpredicts TAC concentration by 1.42 ng/mL with an error distribution by 2.6 ng/mL. This model can help predict TAC troughs in kidney transplant recipients with a certain degree of uncertainty; however, the outcomes of this model must be used with caution coupled with clinical judgment. The model can benefit from further validation and optimization by future studies.

Future directions:

In our study, we showed a DDGI between TAC-steroids and *CYP3A*. We suggest a different perspective when examining DDIs by incorporating the effect of gene variants, particularly, if these interactions are mediated through the CYP450 induction or inhibition. In future studies, we intend to investigate the effect of steroids dose on this DDGI, and we also intend to investigate the time effect of steroids on TAC CL. We recommend future studies that explore the molecular mechanism of *CYP3A5* induction by

steroids and the effect of different polymorphisms (*CYP3A5*3, *6* and **7*) on the induction effect. In addition, we suggest a study that investigates the DGI between *CYP3A* and steroids and incorporate this into the DDGI between TAC-steroids and *CYP3A* in solid organ transplant recipients.

Conclusion

We studied for the first time the DDGI between TAC-steroids and *CYP3A4* and *CYP3A5* using a pharmacokinetic model that estimates TAC CL in a large cohort of kidney transplant recipients. We determined 8 covariates that significantly affected TAC CL. We showed that the effect of steroids on TAC CL varied according to the number of *CYP3A* LOF alleles. Simultaneous accounting for the gene variants effect and steroid effect resulted in greater change in TAC CL in comparison to the sole effect of each.

Tables

Table 2.1: Study Population Demographics

Cohort	Development Cohort (DeKAF)	Validation Cohort (GEN03)
median (range)		
Age, years	51.41 (18 - 84)	51 (18 - 81)
Tacrolimus daily dose, mg	6 (0.3 - 26)	6 (0.5 - 33)
Tacrolimus trough, ng/mL	8.4 (1 - 38)	8.1 (1 - 13.4)
Tacrolimus trough dose-normalized, ng/mL.mg.day^{-1}	1.4 (0.3 - 41.8)	1.4 (0.2 - 20.2)
Weight at time of transplant, kg	81.6 (37.7 - 158)	80.6 (37.8 - 161)
eGFR by MDRD, ml/min/1.73 m^2	50.6 (9.7 - 156.7)	52.7 (6.3 - 160.6)
Creatinine level, mg/dl	1.4 (0.5 - 7)	1.3 (0.5 - 10.1)
n (%)		
Participants	1608	854
Male Recipient	1018 (63.3)	512 (60)
Female Recipient	590 (36.7)	342 (40)
Living donor	955 (59.4)	539 (63.1)
Diabetes at transplant	620 (38.6)	262 (30.7)
Prior kidney transplant	251 (15.6)	122 (14.3)
Race		
Asian	51 (3.2)	38 (4.4)
African Americans	267 (16.6)	169 (19.8)
Hispanics	34 (2.1)	36 (4.2)
Caucasians	1256 (78.1)	611 (71.5)
Total no. of tacrolimus trough measurements	28718	15828
No. of troughs with CCB	10869 (37.8)	6996 (44.2)

No. of troughs with antiviral drug	16279 (56.7)	8181 (51.7)
No. of troughs with ACE inhibitor	3888 (13.5)	1602 (10.1)
No. of troughs with steroids	18252 (63.6)	10284 (65)
No. of troughs without steroids	10443 (36.4)	5544 (35)
No. of troughs with missing steroids status	23 (0.1)	0 (0)
No. of patients with 0-LOF alleles	70 (4.4)	46 (5.4)
No. of patients with 1-LOF alleles	316 (19.7)	170 (19.9)
No. of patients with 2-LOF alleles	1079 (67.1)	569 (66.6)
No. of patients with 3+-LOF alleles	130 (8)	61(7.1)
No. of patients with all missing genotype	12 (0.8)	8 (1)

Table 2.2: Final Tacrolimus PK Model Estimates, Precision, and Shrinkage in Development Cohort

Parameter/ Covariate	Estimate (RSE%)	Bootstrap analysis median (95% CI)	Shrinkage[1] (%)
TVCL/F (L/hr)	27.2 (2)	27.2 (25.82,28.77)	
Effect of LOF alleles on tacrolimus CL/F compared with 2-LOF without steroids			
0 LOF without steroids	2.37 (14.6)	2.37 (2.01,2.78)	
0 LOF with steroids	2.65 (10.7)	2.65 (2.38, 2.96)	
1 LOF without steroids	1.78 (8.7)	1.77 (1.6, 1.98)	
1 LOF with steroids	1.87 (7.1)	1.87 (1.73, 2.03)	
2 LOF with steroids	1.03 (64.5)	1.03 (0.96, 1.09)	
3+ LOF without steroids	0.68 (11.5)	0.67 (0.56, 0.82)	
3+ LOF with steroids	0.75 (13.8)	0.75 (0.67,0.84)	
Diabetes at time of tx	0.90 (21.7)	0.90 (0.86, 0.95)	
Before day 9 post-transplant	1.21 (5.2)	1.22 (1.18, 1.25)	
CCB use	0.93 (9.6)	0.93 (0.90, 0.96)	
Antiviral use	0.96 (15.1)	0.96 (0.94, 0.98)	
ACE inhibitor use	1.04 (25.1)	1.04 (1,1.07)	
Age	$(Age/ 51.41)^{-0.35}$ (11.4)	-0.35 (-0.42, -0.27)	
Variability (CV%)			
IIV	0.18 (44.9)	0.18 (0.16, 0.2)	2.6
ISV	0.04 (18.9)	0.04 (0.03, 0.05)	0.1
Proportional residual unexplained variability	0.07 (26.9)	0.07 (0.06, 0.08)	2.7
Additive residual unexplained variability	4.28 (2.07)*	4.29 (3.69, 4.93)	2.7

TAC TVCL/F= tacrolimus typical value of apparent oral clearance in reference group of 2-LOF without steroids, CCB=calcium channel blockers, ACE=angiotensin-converting enzyme, IIV = Interindividual variability, ISV= Inter-site variability, S= steroids,

[1] shrinkage was calculated for both η and ε
[*] The magnitude of additive error is expressed as standard deviation (SD).

Table 2.3: Validation

Predictive Performance Measure	Estimate
Median Prediction Error (MPE)	-1.42 ng/mL
Median Percentage Error (MPPE)	-17.8%
Root Median Squared Error (RMSE)	2.6 ng/mL

Figures

Figure 2.1: Tacrolimus Trough Levels by CYP3A Loss of Function Alleles

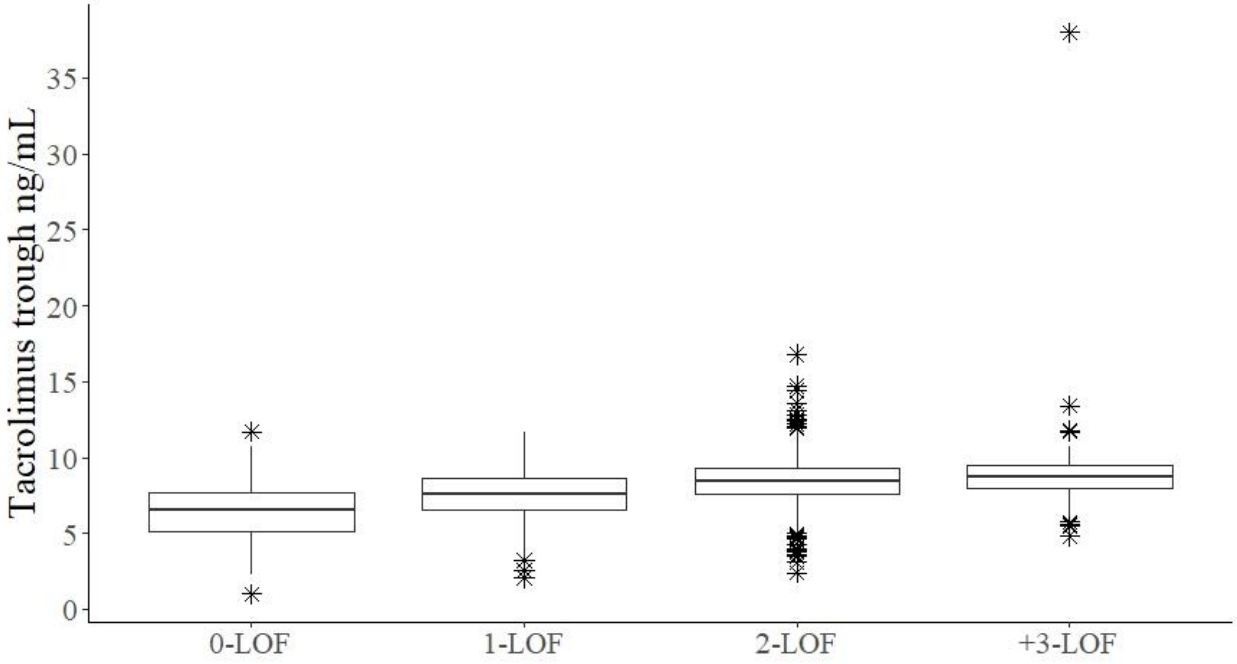
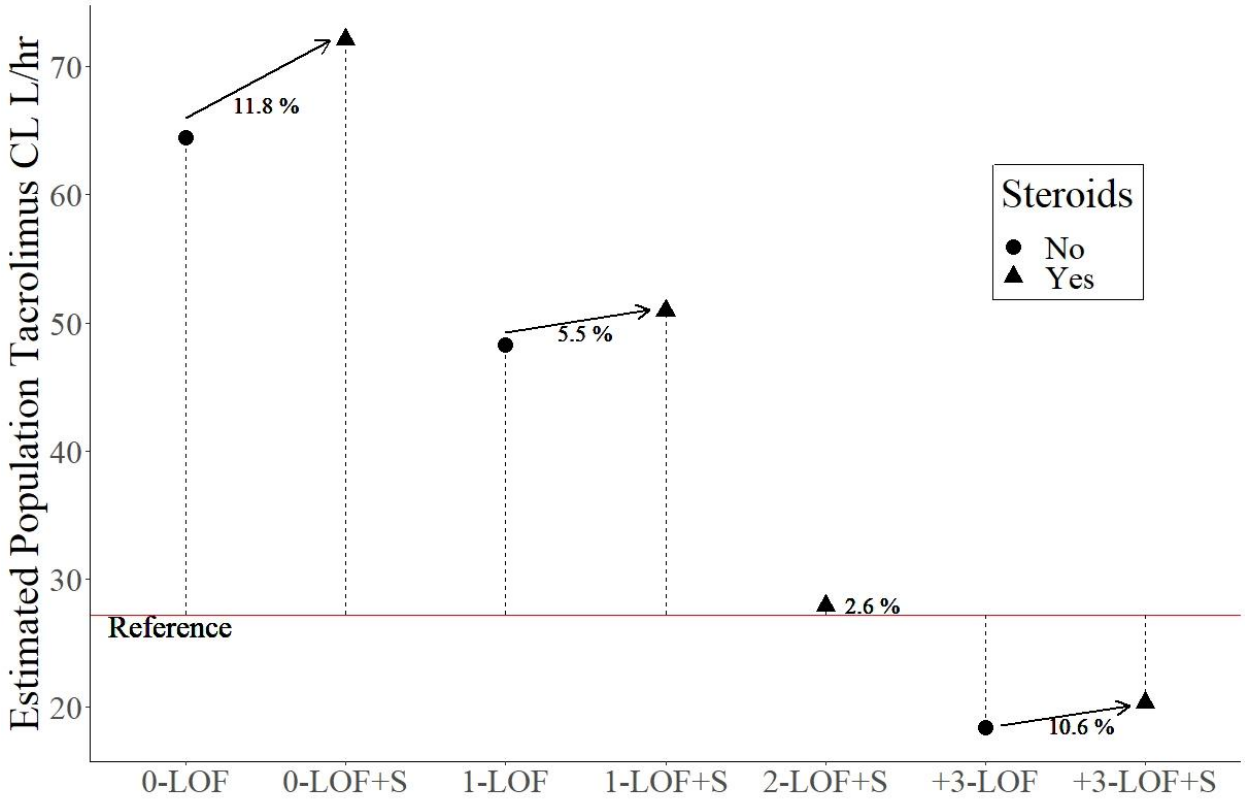


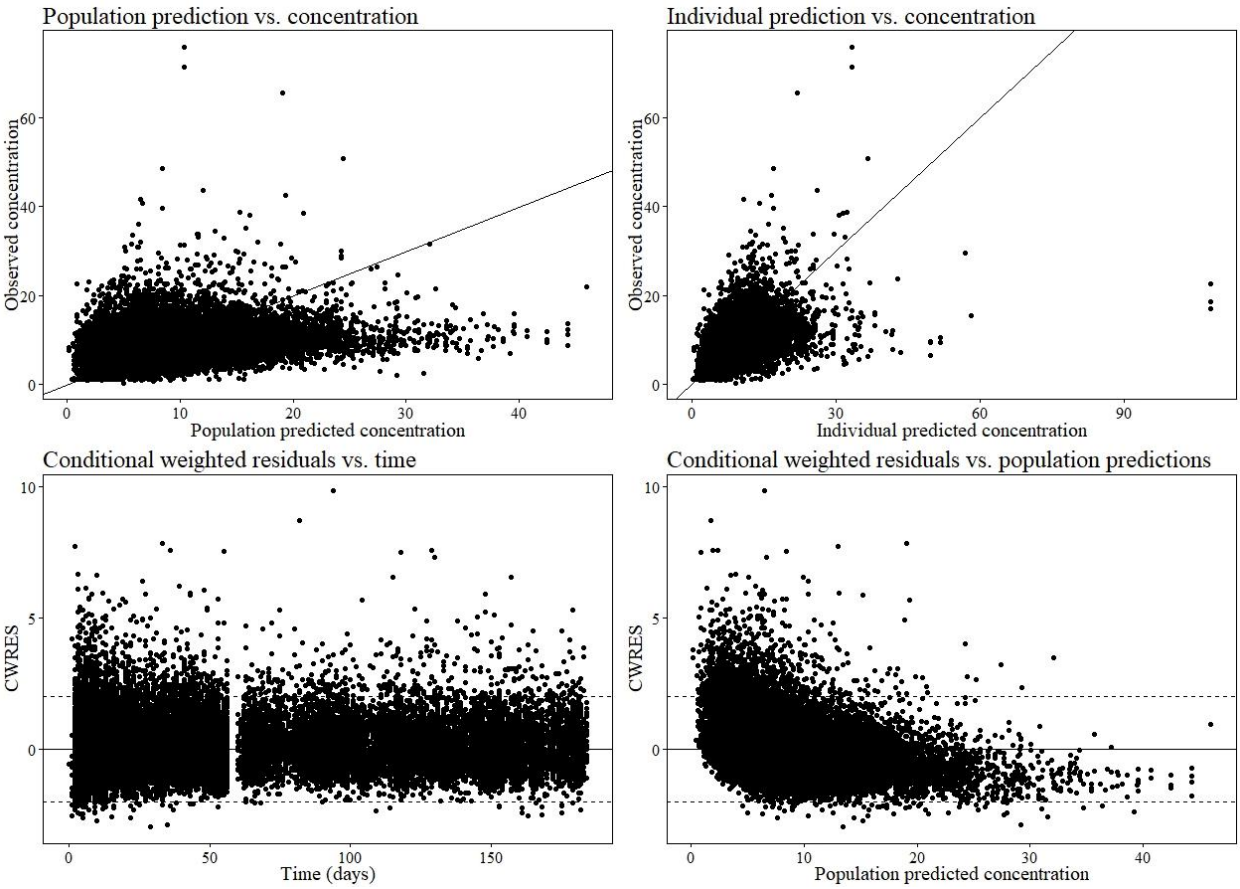
Figure 2.2: Estimated Population Tacrolimus Clearance by CYP3A Loss of Function Alleles and the Effect of Steroids After Accounting for Other Clinical Covariates



Reference = 2 LOF (CYP3A5*1/*3-CY3A4*1/*22 n=7 - CYP3A5*3/*3-CYP3A4*1/*1, n=387 - CYP3A5*3/*6-CYP3A4*1/*1, n=11, CYP3A5*3/*7-CYP3A4*1/*1, n= 8 - CYP3A5*6/*6,*7 or *7/*7 - CYP3A4*1/*1, n=5)
Arrows= Effect of Steroids Withing the Same Genotype, S=Steroids, n= number of patients

Supplemental Materials

Supplemental Figure 2.1: Goodness of Fit



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