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ARTICLE

Pharmacogenetic Analysis of the Model-Based Pharmacokinetics of Five Anti-HIV Drugs: How Does This Influence the Effect of Aging?

Jingxian Chen[1], Farida S. Akhtari[2,3], Michael J. Wagner[1], Oscar Suzuki[1], Tim Wiltshire[1], Alison A. Motsinger-Reif[3,4] and Julie B. Dumond[1,*]

Analysis of aging and pharmacogenetics (PGx) on antiretroviral pharmacokinetics (PKs) could inform precision dosing for older human HIV-infected patients. Seventy-four participants receiving either atazanavir/ritonavir (ATV/RTV) or efavirenz (EFV) with tenofovir/emtricitabine (TFV/FTC) provided PK and PGx information. Aging-PGx-PK association and interaction analyses were conducted using one-way analysis of variance (ANOVA), multiple linear regression, and Random Forest ensemble methods. Our analyses associated unbound ATV disposition with multidrug resistance protein (MRP)4, RTV with P-glycoprotein (P-gp), and EFV with cytochrome P450 (CYP)2B6 and MRP4 genetic variants. The clearance and cellular distribution of TFV were associated with P-gp, MRP2, and concentrative nucleoside transporters (CNTs), and FTC parameters were associated with organic cation transporters (OCTs) and MRP2 genetic variants. Notably, $p16^{INK4a}$ expression, a cellular aging marker, predicted EFV and FTC PK when genetic factors were adjusted. Both age and $p16^{INK4a}$ expression interacted with PGx on ATV and TFV disposition, implying potential dose adjustment based on aging may depend on genetic background.
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Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
✔ Previous studies determined an important role of aging on disposition of some ARVs. Gene polymorphisms, especially in metabolic enzymes, are related to variability in ARV PK, but their role in influencing the aging-PK relationship is unknown.

WHAT QUESTION DID THIS STUDY ADDRESS?
✔ This study asked whether gene polymorphisms alter the interpretation of the effect of aging on (1) unbound ATV, RTV, and EFV clearance and (2) clearance and cellular distribution of TFV and FTC.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE
✔ This study updates the previous aging studies, shedding light on the significant effect of the cellular aging biomarker, $p16^{INK4a}$, on unbound EFV disposition. We also present evidence of PGx-aging interaction in ATV and TFV PKs, implying potential dose adjustment of these two drugs based on aging may be dependent on gene polymorphisms.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE
✔ The $p16^{INK4a}$ might be an important biomarker to guide ATV dosing for older HIV-infected patients. Evaluating the role of aging in dose adjustment of ATVs should take into account the presence of genetic variants in metabolic enzymes and drug transporters.

Gene polymorphisms play an important role in drug disposition and individualized therapy, as they can contribute to variability in pharmacokinetics (PKs), and may alter the effect of nongenetic factors. In HIV treatment, pharmacogenetic-pharmacokinetic (PGx-PK) relationships have been demonstrated for many antiretrovirals (ARVs) in the general population.[1] Their roles in special populations, such as pregnancy[2] and pediatrics,[3] have also been evaluated. However, the PGx of ARVs in the older HIV-infected population has not been well studied.

Aging is a trend in the HIV-infected population.[4] The estimated proportion of patients aged 50 years old, which is the general definition of "older" in the HIV field, was 42% in 2013,[5] and is predicted to be 70% by the end of 2035.[6] Aging could change drug absorption, distribution, metabolism, and elimination,[7] which could alter efficacy and safety profiles. In our previous studies, we have investigated the effects of chronological age and aging surrogates on the PK of five selected ARVs.[8,9] Our analysis showed that the clearances of two renally eliminated drugs, tenofovir (TFV) and

[1]University of North Carolina Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA; [2]Department of Biological Sciences, North Carolina State University, Raleigh, NC, USA; [3]Bioinformatics Research Center, North Carolina State University, Raleigh, NC, USA; [4]Department of Statistics, North Carolina State University, Raleigh, NC, USA. *Correspondence: Julie B Dumond (jdumond@unc.edu)
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emtricitabine (FTC), demonstrated a significant association with creatinine clearance, which is strongly related to chronological age.[9] In addition, we found that the cellular clearances of their active metabolites, tenofovir diphosphate (TFV-DP) and emtricitabine triphosphate (FTC-TP), are associated with the expression of a cellular aging biomarker, $p16^{INK4a}$.[9] On the other hand, three hepatically metabolized drugs, atazanavir (ATV), ritonavir (RTV), and efavirenz (EFV), did not show statistically significant relationships with aging, in either total or unbound drug disposition,[8] although the sample size for these drugs was smaller than that of TFV/FTC.

Identification of influential single-nucleotide polymorphisms (SNPs) could provide PGx information for ARV dosing adjustment, and improve statistical analyses of aging by reducing variability in PK. Additionally, gene polymorphisms can modify the effect of aging on PK, and evidence for such interactions has been shown for non-ARV drugs. For example, less profound changes in systemic clearance of omeprazole between younger and older patients was observed in cytochrome P450 (CYP)2C19 poor metabolizers than in extensive metabolizers.[10] This implies that dose adjustment in older patients might be less important given the dysfunctional CYP2C19 genotype. In another example, an eightfold increase in the mean dose-adjusted plasma concentration of venlafaxine was seen in older compared with younger patients among the CYP2D6 poor metabolizers, but not in the other genotype groups.[10] This suggests that dose adjustment might be necessary in the presence of CYP2D6 dysfunction. Therefore, investigating aging in the context of PGx or aging-PGx interaction is essential to provide precise treatment for older patients.

In addition to the aging-PK associations, as outlined in our previous publications, our studied drugs also serve as reasonable models for aging-PGx-PK investigation, because their disposition profiles cover a broad spectrum of metabolic enzymes and drug transporters. ATV and RTV are CYP3A substrates,[11,12] and served as two model drugs to probe these enzymes. In addition, the upstream CYP3A expression regulator, pregnane X receptor (PXR), affects ATV clearance.[13,14] Uridine 5′-diphosphate glucuronosyltransferase (UGT)1A is responsible for ATV glucuronidation and is inhibited by ATV.[11] EFV has a complex metabolic profile, which involves CYP2B, CYP2A, CYP3A, CYP1A, and UGT.[15] It is also an inducer or inhibitor of CYP2B, CYP3A, and CYP2C.[16] Transporters in the gut, such as P-glycoprotein (P-gp) and multidrug resistance proteins (MRPs), are presumably important globally to ARVs, which are mostly orally administrated.[1] Organic anion-transporting polypeptides (OATPs), which are enriched in the liver, could be relevant to ATV, RTV, and EFV, and OATs or organic cation transporters (OCTs) in the kidney are important to TFV or FTC.[17] Nucleoside transporters, concentrative nucleoside transporters (CNTs) and equilibrative nucleoside transporters, are believed to transport nucleoside analogs,[1,17] which could be crucial to the disposition of TFV and FTC and the cellular accumulation of their active metabolites.

In the current study, we aim to re-evaluate the effect of aging on PK on five studied drugs in the context of PGx and investigate the aging-PGx interaction in PK. Using a candidate gene approach to select important SNPs, we also aim to provide new hypotheses for further PGx investigations of ARVs. For phenotype, we have focused on the clearance of TFV, FTC and unbound ATV, RTV and EFV, as well as cellular clearance of TFV-DP and FTC-TP, using the associated individual parameters derived from the previous established population PK models.

MATERIALS AND METHODS

Clinical trial

Study conduct has been described in previous publications.[8,9] Briefly, HIV-infected patients were enrolled from the University of North Carolina HealthCare Infectious Diseases Clinic (Chapel Hill, NC) and the Cone Health Regional Center for Infectious Diseases (Greensboro, NC). All the subjects received steady-state tenofovir disoproxil fumarate (TDG)/FTC 300/200 mg with either EFV 600 mg or ATV/RTV 300/200 mg daily. Blood samples were collected either sparsely at four time points (predose, and then 2 h, 4–6 h, and 10–14 h postdose) or intensively at 11 time points for PK analysis. An extra blood sample was collected for those who consented to be involved into the PGx analysis. The study protocol was approved by the institutional review boards of both institutions (Clinicaltrials.gov NCT01180075).

Genotyping

Samples for this test were obtained from patients who consented to genotyping assays at screening. Genomic DNA was extracted from whole blood using QIAamp DNA Blood Mini Kit (QIAGEN). Genotyping was conducted for selected metabolic enzymes and drug transporters using DMET Plus microarray (Affymetrix, CA). Samples with a call rate $<95\%$ were excluded from analysis. The SNPs were excluded if its minor allele frequency was $<4\%$ and its missing genotypes were $>30\%$. Deviations from expected proportions based on Hardy-Weinberg equilibrium (exact test, $\alpha > 0.05$) and linkage disequilibrium ($R^2 > 0.2$) analysis were performed in PLINK software version 1.07.[18]

Phenotypes

The phenotypes in this study were the post hoc PK parameters. The PK analysis was performed as previously described.[8,9] Briefly, drug concentrations were measured in the University of North Carolina Center for AIDS Research Clinical Pharmacology and Analytical Chemistry Laboratory. Total and intracellular drug concentrations were measured by validated liquid-chromatography tandem mass spectrometry. Unbound EFV, ATV, and RTV concentrations were analyzed using rapid equilibrium dialysis followed by liquid-chromatography tandem mass spectrometry. Population PK models were developed for each drug using nonlinear mixed effects modeling program NONMEM version 7.3 (ICON Development Solutions, Hanover, MD). For the current study, individual PK parameters of interest included clearance of the unbound drug for EFV, ATV, and RTV, clearance of the parent drug for TFV and FTC, and the rate constant of intracellular metabolite conversion and elimination for TFV-DP and FTC-TP. These estimates were obtained using the Bayesian post hoc estimation method based on the final models, including the covariate effects of creatinine

clearance on TFV and FTC clearance and $p16^{INK4a}$ expression on TFV-DP and FTC-TP disposition.

Statistical analysis

A candidate gene approach was used to perform this analysis. The candidate genes were chosen if shown to be relevant to studied drug disposition pathways. Variables were log-transformed to achieve normality when appropriate. In the univariate analysis, the association analysis between genotypes and PK parameters was conducted using one-way analysis of variance (ANOVA). Then, SNPs with a nominally significant *P* value were included in multivariate analysis using the stepwise selection procedure in both forward and backward directions. Model selection was based on Akaike information criteria, involving demographic covariates of interest, including chronological age, $p16^{INK4a}$ expression level, race, sex, and body mass index (BMI). Missing genotypes were imputed using IMPUTE2 with the 1000 Genomes Phase III panel.[19,20] For race, patients who were other, unknown, and nonreported races were grouped as one category ("others") due to low counts. Additionally, the effect of creatinine clearance and treatment arms were evaluated for TFV and FTC. Comparison of demographics between two treatment arms was conducted using Mann-Whitney U test or Kruskal-Wallis test. These statistical analyses were performed using R version 3.2 (R-project.org). A P value < 0.05 was considered statistically significant.

To investigate the interaction between aging and gene polymorphism, we used distinct linear regression models (R function *lm*, package stats version 3.4.0; www.r-project.org)[21] to determine if there is an effect on drug clearance due to the interaction between chronological age and gene polymorphisms (SNPs), and between $p16^{INK4a}$ expression and gene polymorphisms. An F-test (R function ANOVA, package stats version 3.4.0)[21] was used to compare the model without interaction effects to the one with interaction effects. Only significant predictors from the best fitting model were retained in the final model, with the cutoff for significance being $P < 0.05$ for each predictor. The factors involved in each interaction effect were always included in the model. Multiple testing corrections were performed across all drugs using the Benjamini-Hochberg method with a false discovery rate of $q < 0.1$ (R function p.adjust, package stats version 3.4.0).[21]

In order to determine variable interactions and variable importance of all available variables, we used the Random Forest ensemble method (R function randomForest(ntree = 5000), package randomForest version 4.6-12)[22] for each drug, with the PK parameter as the response, after removing all incomplete cases. We used the percent increase in mean squared error to determine the three most important predictors for each drug.

The effects of significant SNPs on corresponding proteins were evaluated based on literature and database (e.g., dbSNP, PharmGKB) searching. The SIFT and PolyPhen algorithms were used to make predictions for SNPs with no current information on how they influence the protein. If still no information is available, those SNPs in linkage disequilibrium with $r^2 > 0.8$ were also investigated. Protein function prediction was conducted using SNPnexus.[23–25] Linked SNPs were obtained through HaploReg version 4.1.[26]

RESULTS

Population

In 91 patients enrolled in the original clinical study, 74 consented to the genetic tests; 25 were in the ATV/RTV/TFC/FTC arm and 49 in the EFV/TFV/FTC arm. The median age of the 74 patients was 48 years. The self-reported race includes the following categories: white (24/74); Black/African American (45/74); other (3/74); unknown (1/74), and not reported (1/74). Fifty-two percent of the patients were men. Three patients demonstrated the Fried frailty phenotype.[27] The characteristics between the two treatment arms were not significantly different. The studied PK parameters included apparent unbound drug clearance (CL_u/F) of ATV, RTV, and EFV, apparent clearance (CL/F) of TFV and FTC, and clearance of TFV and FTC metabolites (TFV-DP and FTC-TP, respectively) into (CL_{in}) and out of (CL_{out}) of peripheral blood mononuclear cells. In total, 22 genes were selected for investigation. Patient demographics, PK parameters, and studied genes and SNPs are summarized in **Table 1**.

Linear regression analysis

The *P* values for univariate analysis are shown in **Table 2**. Twenty-one SNPs were found to be significantly associated with the PK parameters. The SNP basic information, genotype frequency, effects on phenotypes, and SNP function are summarized in **Table 3**.[15,28–31] No significant SNPs were found for the TFV-DP CL_{out}. The FTC-TP CL_{in} showed the same statistical results as the FTC CL/F, because during the model construction the FTC-TP CL_{in} was described as a fixed fraction of FTC CL/F.[9] Therefore, TFV-DP CL_{out} and FTC-TP CL_{in} were excluded from further analysis. Associations between demographics and PK parameters were consistent with the previous population PK analysis. Briefly, BMI was associated with RTV clearance. Age and creatinine clearance were correlated, and shown to be related to CL/F of both TFV and FTC. The $p16^{INK4a}$ expression was associated with FTC CL/F and FTC CL_{out}.

In the multiple linear regression, *ABCC4*_c.*879T>C (rs1059751) was associated with increased ATV CL_u/F, with no demographics being statistically significant. Decreased RTV CL_u/F was associated with the mutation at *ABCB1*_c.3435 (rs1045642). BMI remained significant with RTV CL_u/F. In addition, race was found to be predictive of RTV CL_u/F; African Americans had significantly lower apparent clearance of RTV than the other populations. Decreased EFV CL_u/F was related to variants *CYP2B6*6*_15631G>T (rs3745274), *ABCC4*_c.3348A>G/Del (rs1751034), and *ABCC4*_c.*38T>G (rs3742106). Notably, after adjusting for the effects of these SNPs, $p16^{INK4a}$ expression was found to be significantly associated with EFV CL_u/F. A doubling of $p16^{INK4a}$ expression was shown to be related to 315 L/h (31% of the median) decrease in EFV unbound CL_u/F.

The polymorphisms at *ABCB1*_c.-129 (rs3213619) and *SLC28A1*_c.1561 (rs2242046) were significantly associated with decreased TFV CL/F, whereas *SLC28A2*_c.531T>C (rs8023604) showed the opposite effect. Creatinine

Table 1 Overview of demographics of studied population and the pharmacokinetics and pharmacogenetics of the studied drugs

		ATV	RTV	EFV	TFV	FTC
No. of subjects		25		49	74	
Age (years)		49 (24, 61)		48 (22, 73)	48 (22, 73)	
Race	African American	15 (60%)		30 (61%)	45 (61%)	
	White	8 (32%)		16 (33%)	24 (32%)	
	Others	2 (8%)		3 (6%)	5 (7%)	
Sex	Female	10 (40%)		12 (24%)	22 (30%)	
	Male	15 (60%)		37 (76%)	52 (70%)	
CrCL (L/h)		96.6 (66.8, 228)		112 (43.3, 200)	109 (43.3, 228)	
BMI (kg/m^2)		29.8 (20.2, 40.4)		27.1 (17.6, 44.3)	28.6 (17.6, 44.3)	
Frailty status	Nonfrail	16 (64%)		37 (76%)	53 (72%)	
	Pre-frail	8 (32%)		10 (20%)	18 (24%)	
	Frail	1 (4%)		2 (4%)	3 (4%)	
p16^{INK4a} expression[a]		2.20 (0.158, 3.91)		1.92 (-1.14, 2.81)	2.01 (-1.14, 3.91)	

					TFV CL/F: 46.8 (16.6, 115)	**FTC CL/F: 17.9 (4.54, 27.4)**
					TFV-DP CL_{in}: 0.00235 (0.000814, 0.00874)	**-**
PK parameters and medians (ranges)		**CL_u/F: 93.6 (18.2, 355)**	**CL_u/F: 965 (69.5, 2380)**	**CL_u/F: 1010 (417, 3280)**	**TFV-DP CL_{out}: 0.00214 (0.000869, 0.00511)**	**FTC-TP CL_{out}: 0.0292 (0.0978, 0.141)**
No. of SNPs		40	37	44	52	60
Studied genes	**Genes for metabolic enzymes**	*PXR*	*PXR*	*PXR*	-	-
		CYP2C9	*CYP1A2*	*CYP2A6*		
		CYP3A4	*CYP2B6*	*CYP2B6*		
		CYP3A5	*CYP3A4*	*CYP3A4*		
		UGT1A1	*CYP3A5*	*CYP3A5*		
			CYP3A7	*UGT2B7*		
	Genes for drug transporters	*ABCB1*	*ABCB1*	*ABCB1*	*ABCB1*	*ABCC1*
		ABCC4	*SLCO1B1*	*ABCC4*	*ABCC2*	*ABCC2*
		SLCO1B1	*SLCO1B3*		*ABCC4*	*ABCC3*
					ABCG2	*ABCC4*
					SLC22A11	*ABCC5*
					SLC22A2	*ABCG2*
					SLC22A7	*SLC22A2*
					SLC22A8	*SLC22A3*
					SLC28A1	*SLC28A1*
					SLC28A2	*SLC28A2*
					SCL28A3	*SLC28A3*
					SLC29A2	*SLC29A2*

ATV, atazanavir; BMI, body mass index; CL/F, apparent clearance; CL_{in}, clearance into the cell; CL_{out}, clearance out of the cell; CL_u/F, apparent unbound drug clearance; CrCL, creatinine clearance; CYP, cytochrome P450; EFV, efavirenz; F, undetermined bioavailability; FTC, emtricitabine; FTC-TP, emtricitabine triphosphate; PK, pharmacokinetic; PXR, pregnane X receptor; RTV, ritonavir; SNP, single-nucleotide polymorphism; TFV, tenofovir; TFV-DP, tenofovir diphosphate; UGT, uridine 5′-diphosphate glucuronosyltransferase.
Clearance is in liter/hour (L/h).
[a]The expression of p16^{INK4a} is presented as logarithm (base 2) of gene expression. Continuous variables are in median (range). Categorical variables are in count (percentage).

clearance remained significant, whereas age was removed from the model. Sex was related to TFV CL/F after adjusting for gene polymorphism, with women demonstrating lower TFV clearance than men. The *ABCB1*_c.1725+38G>A (rs2235013), *SLC28A2*_c.734G>C (rs10519020), and *ABCC2*_c.3563T>A (rs17222723) were retained in the model for TFV-DP CL_{in}. For FTC CL/F, the significant predictors were *SLC22A3*_c.360C>T>G (rs668871), *SLC22A2*_c.1506A>G (rs316003), *ABCC2*_c.4488C>T (rs8187707), creatinine clearance, and p16^{INK4a} expression; the effect of age was eliminated. The *ABCC2*_c.1249G>A (rs2273697) and p16^{INK4a} expression were the two significant predictors in the FTC CL_{out} linear model. Results from the multiple linear regressions are summarized in **Table 4**.

Interaction analysis

To analyze interactions, we used two distinct linear regression models to determine if there is an interaction effect

Table 2 The *P* values in the univariate analysis

Factors	ATV CL_u/F	RTV CL_u/F	EFV CL_u/F
Age	0.74	0.64	0.55
p16 expression	0.20	0.43	0.57
Race	0.40	0.95	0.22
BMI	0.66	0.025*	0.64
Sex	0.10	0.75	0.07
Frailty status	0.074	0.21	0.69
CrCL	–	–	–
Treatment arm	–	–	–
SNP	*SLCO1B1*_c.571T>C: 0.036 *ABCC4*_c.*879T>C: 0.040	*ABCB1*_c.3435C>T: 0.0021 *CYP1A2**1C*_-3860G>A: 0.011 *CYP2B6*_14593C>G: 0.047	*CYP2B6**6*_15631G>T: 0.00025 *ABCC4*_c.3348A>G/Del: 0.0056 *ABCC4*_c.*311G>A: 0.0055 *CYP2B6**4*_18053A>G(K262R): 0.013 *ABCC4*_c.*38T>G: 0.013

Factors	TFV CL/F	TFV-DP CL_{in}	FTC CL/F	FTC-TP CL_{out}
Age	<0.001***	0.43	<0.001***	0.50
p16 expression	0.78	0.86	0.020*	<0.001***
Race	0.34	0.49	0.14	0.47
BMI	0.11	0.26	0.19	0.83
Sex	0.20	0.17	0.54	0.23
Frailty status	0.58	0.30	0.11	0.22
CrCL	<0.001***	0.28	<0.001***	0.63
Treatment arm	0.11	0.28	0.15	0.86
SNP	*ABCB1*_c.-129T>C: 0.013 *SLC28A1*_c.1561G>A: 0.015 *SLC28A2*_c.531T>C: 0.0060	*ABCB1*_c.1725+38A>G: 0.0064 *SLC28A2*_c.734G>C: 0.0090 *ABCC2*_c.3563T>A: 0.038	*SLC22A3*_c.360C>T>G: 0.032 *SLC22A2*_c.1506G>A: 0.018 *ABCC2*_c.4488C>T: 0.020	*ABCC2*_c.1249G>A: 0.0046 *SLC28A1*_c.1149A>G: 0.038

ATV, atazanavir; BMI, body mass index; CL/F, apparent clearance; CL_{in}, clearance into the cell; CL_{out}, clearance out of the cell; CL_u/F, apparent unbound drug clearance; CrCL, creatinine clearance; EFV, efavirenz; F, undetermined bioavailability; FTC, emtricitabine; FTC-TP, emtricitabine triphosphate; RTV, ritonavir; SNP, single-nucleotide polymorphism; TFV, tenofovir; TFV-DP, tenofovir diphosphate.
*$P < 0.05$; ***$P < 0.001$.

between chronological age and genetic polymorphisms, and between $p16^{INK4a}$ expression and genetic polymorphisms. Most of the significant SNPs in the previous multiple linear regressions were confirmed in this analysis, which was without other demographic variables (**Table 5**). Notably, we found significant evidence of the interaction effects of age and *ABCC4*_c.*879T>C (rs1059751) with ATV CL_u/F. This effect was also found in the model with $p16^{INK4a}$ expression. In addition, both models showed very strong significant evidence of the interaction between age or $p16^{INK4a}$ expression and *SLC28A2*_c.734G>C (rs10519020) with TFV-DP CL_{in}.

In addition to the analysis with traditional statistical approaches, we performed machine learning analysis to evaluate the variable interactions and the overall importance of each of the variables with the PK parameters. Although the Random Forest method did not find consistent interactions, we used it to determine variable importance. The three most important predictors for ATV CL_u/F were *ABCC4*_c.*879T>C (rs1059751), *SLCO1B1*_c.571T>C, (rs4149057), and sex of the individual. For RTV CL_u/F, they were *ABCB1*_c.3435C>T (rs1045642), *CYP1A2*1C*_-3860G>A (rs2069514), and BMI. For EFV CL_u/F, the three most important variables were *CYP2B6*6*_15631G>T (rs3745274), *ABCC4*_c.3348A>G/Del (rs1751034), and *ABCC4*_c. 311G>A (rs4148551). For TFV they were creatinine clearance, age, and *SLC28A1*_c.1561G>A (rs2242046). For TFV-DP CL_{in}, they were *ABCB1*_c.1725+38G>A (rs2235013), sex, and BMI. For FTC CL/F, they were creatinine clearance, $p16^{INK4a}$ expression, and age. For FTC-TP CL_{out}, the three most important predictors were $p16^{INK4a}$ expression, *ABCC2*_c.1249G>A (rs2273697), and *SLC28A1*_c.1149A>G (rs2305367). The three most important predictors for each drug are listed in **Table 6**.

DISCUSSION

In this study, we investigated the effects of aging and gene polymorphisms on key PK parameters of five ARVs. Some participant characteristics were significant predictors after adjusting for genetic factors, demonstrating the importance and potential of accounting for PGx in PK analysis. The results from the interaction analysis brought to light interactions between genotype and aging (both chronological and cellular $p16^{INK4a}$ expression). The multiple analyses methods used reinforced several of our findings.

Our analysis showed that increased apparent clearance of unbound ATV is associated with *ABCC4*_c.*879T>C. ATV is highly metabolized in the liver; this variant might lead to decreased efflux[32] of ATV out of the hepatic cells, thus increased ATV metabolism due to accumulation. There is a nominal association with rs4149057 in *SLCO1B1*, the gene that also showed marginal significance in a genomewide association study of ATV PK.[33] Possibly due to limited

Table 3 Summary of the SNPs showing statistical significance in the univariate analysis

Studied drug	dbSNP RS	Associated gene/protein	c.	Base change	Function	Genotype frequency	Phenotype	Previously reported phenotype	Physiological reasonable?	Physiological reason
ATV	rs1059751	*ABCC4*/MRP4	13	879T>C	3'-UTR	T/T+T/C: 23 (92%) C/C: 2 (8%)	↑ CL/F	Decreased function	Y	Decreased efflux from liver to system
	rs4149057	*SLCO1B1*/OATP1B1	12	571T>C	L191L	T/T+T/C: 21 (84%) C/C: 4 (16%)	↑ CL/F	–	–	–
RTV	rs1045642	*ABCB1*/P-gp	7	3435C>T	I1145I	C/C: 14 (56%) C/T+T/T: 11 (44%)	↓ CL/F	Decreased expression	Y	Increased bio-availability
	rs2069514	*CYP1A2*/CYP1A2	15	-3860G>A	Promoter	G/G+G/A: 22 (88%) A/A: 3 (12%)	↓ CL/F	1C,[a] decreased function	Y	Decreased metabolism
	rs4803418	*CYP2B6*/CYP2B6	19	14593C>G	Intronic	C/C: 19 (76%) C/G: 6 (24%)	↑ CL/F	1C,[a] increased function	Y	Increased metabolism
EFV	rs3745274[b]	*CYP2B6*/CYP2B6	19	15631G>T	Q172H	G/G+G/T: 41 (84%) T/T: 6 (12%) Missing: 2 (4%)	↓ CL/F	6,[a] decreased function	Y	Decreased metabolism
	rs2279343	*CYP2B6*/CYP2B6	19	18053A>G	K262R	A/A+A/G: 39 (80%) G/G: 7 (14%) Missing: 3 (6%)	↓ CL/F	4,[a] decreased function	Y	Decreased metabolism
	rs1751034	*ABCC4*/MRP4	13	3348A>G/Del	K1116K/X	A/A+A/G: 45 (92%) G/G: 4 (8%)	↓ CL/F	Decreased function	Y	Increased bio-availability
	rs4148551	*ABCC4*/MRP4	13	311G>A	3'-UTR	G/G: 11 (22%) G/A+A/A: 38 (78%)	↑ CL/F	–	–	–
	rs3742106	*ABCC4*/MRP4	13	38T>G	3'-UTR	T/T+T/G: 46 (94%) G/G: 3 (6%)	↓ CL/F	Decreased function	Y	Increased bio-availability
TFV	rs3213619	*ABCB1*/P-gp	7	-129T>C	5'-UTR	T/T: 61 (82%) T/C: 13 (18%)	↓ CL/F	Decreased function	Y	Increased bio-availability
	rs2242046	*SLC28A1*/CNT1	15	1561G>A	D521N	G/G: 49 (66%) G/A+A/A: 19 (26%) Missing: 6 (8%)	↓ CL/F	Increased function	Y	Decreased renal excretion
	rs8023604	*SLC28A2*/CNT2	15	531T>C	F177F	T/T+T/C: 58 (79%) C/C: 15 (20%) Missing: 1 (1%)	↑ CL/F	–	–	–
TFV-DP	rs2235013	*ABCB1*/P-gp	7	1725+38G>A	Intronic	G/G: 24 (27%) G/A+A/A: 50 (73%)	↓ CL_{in}	–	–	–
	rs10519020	*SLC28A2*/CNT2	15	734G>C	S245T	G/G+G/C: 72 (97%) C/C: 2 (3%)	↑ CL_{in}	–	N[a]	Presumably decreased cellular uptake
	rs17222723	*ABCC2*/MRP2	10	3563T>A	V1188E	T/T: 64 (86%) T/A: 10 (14%)	↑ CL^{in}	Decreased function[c]	Y	Decreased efflux

(*Continued*)

Table 3 Continued

Studied drug	dbSNP RS	Associated gene/protein	c.	Base change	Function	Genotype frequency	Phenotype	Previously reported phenotype	Physiological reasonable?	Physiological reason
FTC	rs668871	*SLC22A3*/OCT3	6	360C>T/G	R120R	C/C: 14 (19%) C/T+T/T: 57 (77%) Missing: 3 (4%)	↑ CL/F	Increased expression[d]	Y	Increased renal excretion
	rs316003	*SLC22A2*/OCT2	6	1506A>G	V502V	A/A+A/G: 60 (81%) G/G: 14 (19%) Missing: 1 (1%)	↑ CL/F	–	–	–
	rs8187707	*ABCC2*/MRP2	10	4488C>T	H1496H	C/C: 67 (91%) C/T: 7 (8%)	↑ CL/F	Decreased function[c]	N	Presumably decreased renal excretion or increased bioavailability
FTC-TP	rs2273697	*ABCC2*/MRP2	10	1249G>A	V417I	G/G: 45 (61%) G/A+A/A: 29 (39%)	↑ CL_{out}/F	Increased function	Y	Increased efflux
	rs2305367	*SLC28A1*/CNT1	15	1149A>G	K383K	A/A+A/G: 44 (60%) G/G: 27 (36%) Missing: 3 (4%)	↑ CL_{out}/F	–	–	–

ATV, atazanavir; c., chromosome number; CL/F, apparent clearance; CL_{in}, clearance into the cell; CL_{out}, clearance out of the cell; CNT, concentrative nucleoside transporter; CYP, cytochrome P450; dbSNP, database-single-nucleotide polymorphism; EFV, efavirenz; F, undetermined bioavailability; FTC, emtricitabine; FTC-TP, emtricitabine triphosphate; MRP, multidrug resistance protein; OATP, organic anion-transporting polypeptide; P-gp, P-glycoprotein; RS, reference single-nucleotide polymorphism cluster ID; SNP, single-nucleotide polymorphism; TFV, tenofovir; TFV-DP, tenofovir diphosphate; UTR, untranslated region.

[a]The finding is inconsistent with the predicted phenotypic effects.

[b]The rs8187710 ($r^2 = 0.98$ with rs17222723 and rs8187707) was shown to decrease MRP2 function (ref. 30 and 31).

[c]The rs555754 ($r^2 = 0.99$ with rs668871) was shown to increase OCT3 messenger RNA expression (ref. 29).

[d]The rs3745274 is a well-identified SNP influencing EFV elimination (ref. 15 and 28).

sample size, our study was not able to detect the UGT1A1 genotype, such as UGT1A1 *28, as a significant predictor of ATV PK.

The PGx profile of RTV is poorly studied. In our analysis, we found that *ABCB1*_c.3435C>T is significantly associated with decreased CL_u/F, as previously reported.[34] RTV is a substrate of P-pg;[12] the presence of variant might increase the bioavailability of RTV, thus decreases the CL_u/F. In the univariate analysis, *CYP1A2*1C*_-3860G>A (CYP1A2 *1C, decreased protein function[35]) is associated with decreased RTV CL_u/F, whereas *CYP2B6*_14593C>G (CYP2B6 *1C, conflicting protein function[28,35]) shows the opposite effect. BMI remains a significant predictor and is associated with higher RTV CL_u/F. Additionally, we found that race plays a role, with African Americans showing the lower RTV clearance than other races. However, the effect of "other" races is not interpretable due to the lack of information of the actual racial background. We found no association between race and the three SNPs mentioned above (data not shown), implying that race might predict RTV CL_u/F independent of these three SNPs. Our finding suggests that RTV drug disposition is influenced by several factors in a mixed manner, potentially explaining the RTV observed high-PK variability.

Our analyses replicated the demonstrated effect of *CYP2B6*6*_15631G>T (rs3745274) to decrease EFV clearance. In addition, we found *ABCC4*_c.3348A>G and *ABCC4*_c.*311G>A, both of which decrease MRP4 function,[37–39] were associated with decreased EFV CL_u/F. This might be due to increased bioavailability from MRP4 dysfunction. Interestingly, we found that after accounting for these SNPs, decreased EFV CL_u/F was significantly related to higher $p16^{INK4a}$ expression, suggesting that elimination of EFV is associated with cellular aging. The underlying mechanism still needs to be elucidated, although previous work demonstrated that the expression of CYP2B6 is negatively regulated through a CDK2 signaling pathway involving $p16^{INK4a}$ in a hepatic carcinoma cell line, HepG2.[40] Our analysis shows that EFV CL_u/F decreases by 31% of the median with twofold increase in $p16^{INK4a}$ expression. Further analysis with larger sample size is needed to validate this finding and inform the potential application of this biomarker to dosing guidance in older HIV-infected patients.

The significant SNPs in the univariate analysis independently predict TFV and FTC CL/F, given that all of them are retained in the final model. We found that TFV CL/F is associated with three variants on P-gp, CNT1, and CNT2, respectively. The *ABCB1*_c.-129T>C (rs3213619) is shown to decrease CL/F, which might be associated with increased bioavailability through the gut due to decrease P-gp function.[41] The *SLC28A1*_c.1561G>A (rs2242046) also decreases CL/F, and this may be related to the decreased renal excretion of TFV resulting in increased CNT1 function,[42] which transports more drugs back to the nephrons. There is no information about *SLC28A2*_c.531T>C (rs8023604) function in the literature, but we hypothesize this SNP might decrease CNT2 function, in turn increasing drug excretion to the urine. The three SNPs associated with FTC elimination all increase CL/F. The *SLC22A3*_c.360C>T might be associated with increased OCT3 expression,[29] which increases renal excretion of drugs. The *ABCC2*_c.4488C>T

Table 4 Stepwise linear regression analysis

PK parameters	Factor	*P* value	Effect size
ATV CL_u/F	*ABCC4*_c.*879T>C (MRP4)	0.040*	151.4
RTV CL_u/F	*ABCB1*_c.3435C>T (P-gp)	<0.001***	−855.6
	BMI	<0.001***	70.21
	Caucasian[a]	0.0027**	603.1
	Others[a]	0.0036**	756.9
EFV CL_u/F	*CYP2B6*6*_15631G>T (CYP2B6)	<0.001***	−710.2
	*ABCC4*_c.3348A>G/Del (MRP4)	0.0020**	−768.7
	*ABCC4*_c.*38T>G (MRP4)	<0.001***	−845.8
	$p16^{INK4a}$ expression	0.0083**	−315.2
TFV CL/F	*ABCB1*_c.-129T>C (P-gp)	<0.001***	−15.22
	*SLC28A1*_c.1561G>A (CNT1)	0.0047**	−12.48
	*SLC28A2*_c.531T>C (CNT2)	<0.001***	19.18
	CrCL	<0.001***	0.3220
	Sex – female	0.049*	−6.660s
TFV-DP CL_{in}	*ABCB1*_c.1725+38G>A (P-gp)	0.0031**	−0.0006431
	*SLC28A2*_c.734G>C (CNT2)	0.028*	0.0026217
	*ABCC2*_c.3563T>A (MRP2)	0.033*	0.0006769
FTC CL/F	*SLC22A3*_c.360C>T>G (OCT3)	0.013*	3.882
	*SLC22A2*_c.1506A>G (OCT2)	0.019*	3.860
	*ABCC2*_c.4488C>T (MRP2)	0.0063**	6.026
	CrCL	<0.001***	0.07060
	$p16^{INK4a}$ expression	0.034**	−2.006
FTC-TP CL_{out}	*ABCC2*_c.1249G>A (MRP2)	0.022*	0.01012
	$p16^{INK4a}$ expression	0.0090**	0.008336

ATV, atazanavir; BMI, body mass index; c., chromosome number; CL/F, apparent clearance; CL_{in}, clearance into the cell; CL_{out}, clearance out of the cell; CL_u/F, apparent unbound drug clearance; CNT, concentrative nucleoside transporter; CrCL, creatinine clearance; EFV, efavirenz; F, undetermined bioavailability; FTC, emtricitabine; FTC-TP, emtricitabine triphosphate; MRP, multidrug resistance protein; OCT, organic cation transporter; P-gp, P-glycoprotein; PK, pharmacokinetic; RTV, ritonavir; TFV, tenofovir; TFV-DP, tenofovir diphosphate.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

[a]Reference race: African American; "others" includes other races, unknown races, and unreported races.

Note: Only factors that are retained in the final linear regression model are shown in this table. Drug clearance was modeled on the log scale. The expression of $p16^{INK4a}$ is presented as logarithm (base 2) of gene expression. Protein names related to the genes are presented in the parentheses.

was implicated in one previous study with decreased MRP2 function,[30,31] presumably resulting in decreased renal excretion or increased bioavailability, whereas our results indicate the opposite. Additional work will be needed to clarify this finding; no functional information for the *SLC22A2*_c.1506A>G polymorphism is available. In terms of demographics, creatinine clearance is a significant predictor for both TFV and FTC CL/F, and, expectedly, the effect of age was eliminated in multivariate analysis, due to the collinearity of the two variables. However, $p16^{INK4a}$ expression was significantly associated with FTC CL/F, but not TFV CL/F; potentially, pathways sensitive to cellular aging might play a role in FTC elimination.

Cellular uptake of TFV-DP (CL_{in}) is significantly associated with three SNPs on P-gp, CNT2, and MRP2 genes, respectively. TFV is not a substrate of P-gp, thus this association might be due to the bioavailability of tenofovir disoproxil fumarate, the prodrug of TFV, during the absorption phase rather than the cellular distribution. The *SLC28A2*_c.734G>C (rs10519020) is shown to increase TFV-DP CL_{in}, implying this SNP may increase CNT2 function. However, there is no known information about this SNP, and the function prediction is the opposite of our finding. The *ABCC2*_c.3563T>A (rs17222723) is related to increased CL_{in}, which makes some sense given that this SNP is associated with decreased function of MRP2.[30,31] There are some discordant findings regarding the relationship between TFV and MRP2. The study by Ray *et al.*[43] showed that TFV was not substrate of MRP2 using transporter-overexpressing cell lines. However, some gene association studies showed that *ABCC2* variants were associated with TFV-induced renal side effects in HIV-infected patients.[44,45] One patient had very high TFV-DP CL_{in} in our studied cohort (408% of the median), potentially driving the significance of this relationship; however, the effects of our significant SNPs on this parameter were independent. FTC-TP has higher CL_{out} from the cells given an *ABCC2*_c.1249G>A variant, which increases MRP2 function.[44,45] The $p16^{INK4a}$ expression remains a significant predictor, potentially related to effects of cellular aging on the transport of drugs and/or the catabolic and anabolic processes for the metabolites.[9]

In the interaction analyses using linear regression models, we saw a significant effect of interaction between aging (both chronological age and $p16^{INK4a}$ expression) and gene polymorphism on ATV CL_u/F and TFV-DP CL_{in}. However, we had two individuals with the *ABCC4* variant in the ATV arm and

Table 5 Aging-PGx interaction-focused linear regression analysis

PK parameters	Interaction model with	Factor	*P* value	Q value	Effect size
ATV CL_u/F	Age	*ABCC4*_c.*879T>C	3.60E-02	4.02E-02	−1.33E+03
		Age:*ABCC4*_c.*879T>C	2.07E-02	2.77E-02	3.29E+01
	$p16^{INK4a}$ expression	$p16^{INK4a}$ expression:*ABCC4*_c.*879T>C	1.44E-02	2.28E-02	1.18E+02
RTV CL_u/F	Age	*ABCB1*_c.3435C>T	3.47E-03	7.32E-03	−6.29E+02
		*CYP2B6*1C*_14593C>G	5.75E-03	1.03E-02	6.83E+02
	$p16^{INK4a}$ expression	*ABCB1*_c.3435C>T	1.63E-03	6.18E-03	−6.99E+02
		*CYP2B6*1C*_14593C>G	2.07E-02	2.62E-02	5.71E+02
EFV CL_u/F	Age	*CYP2B6*6*_15631G>T	3.77E-04	1.19E-03	−6.92E-01
		*ABCC4*_c.*38T>G	4.77E-04	1.29E-03	−8.73E-01
		*ABCC4*_c.3348A>G/Del	2.58E-02	3.06E-02	−4.94E-01
	$p16^{INK4a}$ expression	$p16^{INK4a}$ expression	6.60E-03	1.27E-02	−2.41E-01
		*CYP2B6*6*_15631G>T	3.04E-04	1.44E-03	−6.86E-01
		*ABCC4*_c.*38T>G	2.48E-04	1.44E-03	−8.66E-01
		*ABCC4*_c.3348A>G/Del	2.79E-03	6.74E-03	−8.16E-01
TFV CL/F	Age	Age	2.55E-07	1.62E-06	−1.11E+00
		*SLC28A2*_c.531T>C	5.95E-03	1.03E-02	1.52E+01
	$p16^{INK4a}$ expression	*SLC28A2*_c.531T>C	3.94E-02	4.68E-02	1.34E+01
		*SLC28A1*_c.1561G>A	1.63E-02	2.38E-02	−1.47E+01
TFV-DP CL_{in}	Age	*SLC28A2*_c.734G>C	8.28E-10	1.57E-08	3.58E-02
		Age: *SLC28A2*_c.734G>C	8.45E-09	8.03E-08	−6.20E-04
		*ABCB1*_c.1725+38G>A	2.19E-02	2.77E-02	−3.89E-04
	$p16^{INK4a}$ expression	*SLC28A2*_c.734G>C	3.29E-11	6.26E-10	1.68E-02
		$p16^{INK4a}$ expression: *SLC28A2*_c.734G>C	4.99E-09	4.74E-08	−6.14E-03
FTC CL/F	Age	Age	2.08E-04	7.91E-04	−1.65E-01
		*ABCC2*_c.4488C>T	1.45E-04	6.90E-04	6.29E+00
		*SLC22A2*_c.1506A>G	2.45E-03	5.83E-03	3.72E+00
		*SLC22A3*_c.360C>T/G	1.07E-02	1.70E-02	3.11E+00
	$p16^{INK4a}$ expression	$p16^{INK4a}$ expression	1.86E-02	2.52E-02	−1.81E+00
		*ABCC2*_c.4488C>T	2.17E-03	6.74E-03	6.32E+00
		*SLC22A2*_c.1506A>G	2.84E-03	6.74E-03	4.00E+00
		*SLC22A3*_c.360C>T/G	9.33E-03	1.61E-02	3.65E+00
FTC-TP CL_{out}	Age	*ABCC2*_c.1249G>A	1.63E-02	2.38E-02	1.13E-02
	$p16^{INK4a}$ expression	$p16^{INK4a}$ expression	6.69E-03	1.27E-02	9.23E-03

ATV, atazanavir; c., chromosome number; CL/F, apparent clearance; CL_{in}, clearance into the cell; CL_{out}, clearance out of the cell; CL_u/F, apparent unbound drug clearance; EFV, efavirenz; F, undetermined bioavailability; FTC, emtricitabine; FTC-TP, emtricitabine triphosphate; PGx, pharmacogenetics; RTV, ritonavir; TFV, tenofovir; TFV-DP, tenofovir diphosphate.

The *P* values, Q values, and effect sizes shown from interaction models containing gene polymorphisms (single-nucleotide polymorphisms [SNPs]) and age as factors or SNPs and $p16^{INK4a}$ expression as factors. Only factors that were significant after a multiple testing correction at the false discovery rate of $q < 0.1$ are shown. Drug clearance was modeled on the log scale for drug EFV. The expression of $p16^{INK4a}$ is presented as logarithm (base 2) of gene expression.

A colon between two variables indicates interaction between the two.

two individuals with the *SLC28A2* variant in the cohort, limiting the power of the current study. Other demographic variables were not included in the linear regression models, due to the small sample size. Hence, we performed Random Forest analysis that included all the available variables. Random Forest has been shown to have improved power over regression approaches to detect interactions in genetic association studies.[46] However, due to the large number of predictors as compared with the limited sample size, the interaction effects of aging and polymorphisms may have been suppressed by other, stronger predictors in the Random Forest analysis.

Our study has some limitations. First, the small sample size, especially the ATV/RTV treatment arm, has limited our conclusions. We might have ruled out some important SNPs because of the low allele frequency. The low numbers of participants also compromised the distribution of some studied factors and the statistical power of our analysis; particularly, we lack participants with the frailty phenotype, limiting our ability to investigate frailty. Second, due to the large number of variables, we conducted the interaction analysis only involving aging (both chronological and cellular) and genetic factors, potentially excluding the interaction of other demographic factors with genetic factors. The interaction analyses were limited by low numbers of the minor alleles for the SNPs studied. The estimated effect sizes should be used to motivate well-powered follow-up

Table 6 Top three predictors for drug PK in the Random Forest analysis

PK parameters	Top three predictors
ATV CL_u/F	*ABCC4*_c.*879T>C (MRP4)
	*SLCO1B1*_c.571T>C (OATP1B1)
	Sex
RTV CL_u/F	*ABCB1*_c.3435C>T (P-gp)
	*CYP1A2*1C*_-3860G>A (CYP1A2)
	BMI
EFV CL_u/F	*CYP2B6*6*_15631G>T (CYP2B6)
	*ABCC4*_c.3348A>G/Del (MRP4)
	*ABCC4*_c. 311G>A (MRP4)
TFV CL/F	*SLC28A1*_c.1561G>A (CNT1)
	Age
	CrCL
TFV-DP CL_{in}	*ABCB1*_c.1725+38G>A (P-gp)
	Sex
	BMI
FTC CL/F	Age
	CrCL
	$p16^{INK4a}$ expression
FTC-TP CL_{out}	*ABCC2*_c.1249G>A (MRP2)
	*SLC28A1*_c.1149A>G (CNT1)
	$p16^{INK4a}$ expression

ATV, atazanavir; BMI, body mass index; c., chromosome number; CL/F, apparent clearance; CL_{in}, clearance into the cell; CL_{out}, clearance out of the cell; CL_u/F, apparent unbound drug clearance; CNT, concentrative nucleoside transporter; CrCL, creatinine clearance; CYP, cytochrome P450; EFV, efavirenz; F, undetermined bioavailability; FTC, emtricitabine; FTC-TP, emtricitabine triphosphate; MRP, multidrug resistance protein; OATP, organic anion-transporting polypeptide; P-gp, P-glycoprotein; PK, pharmacokinetic; RTV, ritonavir; TFV, tenofovir; TFV-DP, tenofovir diphosphate.
Drug clearance was modeled on the log scale for drug EFV. The expression of $p16^{INK4a}$ is presented as logarithm (base 2) of gene expression. Protein names related to the genes are presented in the parentheses.

studies to work to establish genetic factors as potential PK predictors.

Nevertheless, our study has several advantages. Using a novel statistical methodology, a population PK model is able to describe the PK processes more physiologically and explain the variability associated with those processes more appropriately than traditional PK analysis. Therefore, our analysis is based on more precise PK parameters, partially compensating for the small sample size. Additionally, we explored the SNPs at a gene-wide level with the DMET array platform, maximizing the chance to identify relevant SNPs/genes. We found the associations of several SNPs with ARV PK that have not been reported before, generating interesting hypotheses for future study. Moreover, we evaluated the interaction between aging and SNPs in predicting ARV PK, using both the chronological and cellular aging. The significant interactions between aging and the genetic factors were seen for both chronological age and $p16^{INK4a}$ expression.

Our study provides data on the effect of aging on ARVs PK in the context of PGx, highlighting the importance of the cellular aging biomarker, $p16^{INK4a}$, in predicting the ARV drug disposition and the potential alterations in aging-PK relations by PGx. The investigations add knowledge to the current PGx of ARVs, and facilitate further studies with focus on precision treatments, especially those containing drugs with similar disposition profiles with our studied ARVs, for the growing older HIV-infected population.

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