IL-6 Predicts \( p16^{\text{INK4a}} \) Expression, Intracellular Tenofovir/Emtricitabine Exposure, and Endogenous Nucleotide Pools in Adults with HIV

Honors Candidate:
Cynthia Lee
PharmD Candidate 2016
UNC Eshelman School of Pharmacy
University of North Carolina at Chapel Hill

Advisor:
Julie B. Dumond, PharmD, MS, BCPS, AAHIVP
Assistant Professor
Division of Pharmacotherapy and Experimental Therapeutics
UNC Eshelman School of Pharmacy
University of North Carolina at Chapel Hill
**Preface**

I worked with Dr. Julie Dumond and colleagues to study the function of cytokines to aging, antiretroviral drugs, and endogenous nucleotide exposures in HIV-infected subjects. My involvements in the study include participating in subject recruitment and visit, assisting in AUC calculations, conducting literature searches, and contributing to manuscript write-up. The manuscript fully describes the study and is prepared for publication in HIV Medicine journal.

**Abstract**

**Objectives:** It was recently found that p16\(^{INK4a}\), a marker for aging and cellular senescence, is associated with lower intracellular concentrations of endogenous nucleotides and nucleos(t)ide reverse transcriptase inhibitors (NRTIs). This study expands on these findings by determining whether markers of inflammation are predictive of p16\(^{INK4a}\) expression, intracellular metabolite exposure, or endogenous nucleotide concentrations.

**Methods:** Samples from HIV-infected adults receiving daily tenofovir/emtricitabine (TFV/FTC) with either efavirenz (EFV) or atazanavir/ritonavir (ATV/r) were tested for p16\(^{INK4a}\) expression, plasma cytokine concentrations, and intracellular drug concentrations. Elastic net regression was used to identify cytokines that were predictive of p16\(^{INK4a}\) expression and intracellular metabolite/endogenous nucleotide exposures. These outcomes were each compared between groups with detectable versus undetectable levels of predictive cytokines using a Wilcoxon rank-sum test.
**Results:** Enrolled participants had a median age of 48 years (range 23 - 73). Results of the elastic net regression determined that individuals with detectable interleukin-6 (IL-6) concentrations were predicted to have elevated p16\(^{\text{INK4a}}\) expression and lower exposure to tenofovir diphosphate (TFV-dp), emtricitabine triphosphate (FTC-tp), and their respective endogenous nucleotides, deoxyadenosine triphosphate (dATP) and deoxycytidine triphosphate (dCTP).

**Conclusions:** Elevated concentrations of IL-6 have been associated with frailty, morbidity, and mortality in individuals with HIV. Our findings suggest that IL-6 affects cellular senescence, NRTI pharmacokinetics, and endogenous nucleotide pools in an aging HIV population.

**Introduction**

Despite recent advances in antiretroviral (ARV) therapy, HIV-infected patients are at heightened risk for developing complications typically associated with increased age (1). Markers of inflammation are chronically elevated in HIV-infected patients and have been associated with increases in non-AIDS defining morbidity and all-cause mortality (2,3). Given these consequences, understanding the role inflammation plays in the pharmacokinetics of ARVs and the progression of HIV is critical to improving outcomes in aging patients.

Tenofovir and emtricitabine are nucleos(t)ide reverse transcriptase inhibitors (NRTIs) and are recommended in first-line combination ARV therapies. In order to exert their antiviral effect, they must cross the cellular membrane and undergo phosphorylation to
their respective metabolites, tenofovir diphosphate (TFV-dp) and emtricitabine triphosphate (FTC-tp). They work by competing with endogenous nucleotides (deoxyadenosine triphosphate [dATP] for TFV-dp; deoxycytidine triphosphate [dCTP] for FTC-tp) for incorporation into the DNA of infected cells, leading to chain termination. By virtue of this mechanism, it is thought that the ratio of intracellular metabolite to endogenous nucleotide has a greater influence on antiviral efficacy and toxicity than intracellular drug concentrations alone (4).

We previously demonstrated that increased expression of p16\textsuperscript{INK4a}, a biomarker for aging, is associated with lower concentrations of TFV/FTC intracellular metabolites and endogenous nucleotides (5). The work herein extends upon that study and aims to determine whether markers of inflammation previously associated with aging and HIV morbidity / mortality are predictive of p16\textsuperscript{INK4a} expression, intracellular metabolite exposure, or endogenous nucleotide concentrations.

**Methods**

**Clinical Trial Design**

A detailed description of the trial design and eligibility criteria has been previously published (5). In short, HIV-infected adults were recruited from UNC HealthCare Infectious Diseases Clinic (Chapel Hill, NC) and the Cone Health Regional Center for Infectious Diseases (Greensboro, NC). All participants received daily TFV/FTC 300/200 mg with either efavirenz 600mg or atazanavir/ritonavir 300/200mg for at least 2 weeks. Eligible participants had four blood samples taken (predose, 2, 4-6, 10-14 hours).
study protocol was approved by the Institutional Review Boards of both institutions  
(Clinicaltrials.gov NCT01180075).

**Cytokines**

At one of the above pharmacokinetic time points, an additional EDTA tube was 
collected, centrifuged at 3000 RPMs for 10 minutes, and stored at -80ºC for biomarker 
profiling. Analysis was performed in the Duke Regional Biocontainment Laboratory  
(RBL) Immunology Unit (Durham, NC) under the direction of Dr. Gregory D. Sempowski 
using MILLIPLEX® MAP Human Cytokine/Chemokine Premixed 39 Plex bead-based 
assay kit (EMD Millipore Corporation, Billerica, Massachusetts). Due to the large 
number of potential predictor variables, a subset of cytokines that have been associated 
with HIV and aging was included in the analysis: TNFα, IFNγ, IL-1ra, IL-6, IL-12P40, IL- 
12P70, IL-17α, MCP-1, MIP-1α, MIP-1β, MCP-3, MDC, GRO, sCD40L, fractalkine, and 
eotaxin (6–8).

**Pharmacokinetics & p16<sup>INK4a</sup> Expression**

TFV-dp, FTC-tp, dATP, and dCTP concentrations in peripheral blood mononuclear cells 
(PBMCs) were measured using LC-MS/MS in the UNC Center for AIDS Research  
Clinical Pharmacology and Analytical Chemistry Laboratory (5). Drug exposure was 
measured as area under the curve (AUC) using non-compartmental analysis in Phoenix  
Win Nonlin 6.3 (Pharsight, A Certara Company, St. Louis, MO); the linear up/log down 
trapezoidal method was used to calculate AUC over the dosing interval. Expression of 
p16<sup>INK4a</sup> was determined using validated PCR-based methods (9) and final values were 
log<sub>2</sub>-transformed.
**Statistical Analysis**

The primary goal for this analysis was to identify cytokines that are predictive of p16\(^{\text{INK4a}}\) expression and intracellular metabolite/endogenous nucleotide AUCs. Predictor variables were selected using a general linear model elastic net algorithm, a penalized regression technique (10) capable of selecting variables that are most predictive amongst a large number of correlated potential predictors. Tuning parameters for the elastic net algorithm were chosen via 5-fold cross-validation with the optimal tuning parameter values chosen to minimize predicted residual sum of squares (CVPRESS).

The cytokine levels were dichotomized as detectable (1) or undetectable (0) for analysis. Chronological age in years was included as a potential predictor variable. An exact Wilcoxon rank-sum test was used to assess whether p16\(^{\text{INK4a}}\) expression and intracellular metabolite/endogenous nucleotide exposures were different between groups with detectable and undetectable concentrations of predictive cytokines. Analyses were conducted in SAS version 9.4 (SAS Institute, Cary, NC) using the GLMSELECT procedure or R 3.1.2 (r-project.org). A p-value of < 0.05 was considered statistically significant. These analyses were exploratory in nature, so no adjustment was made for multiple comparisons.
Results

Study Participants

The study enrolled 79 participants receiving TFV/FTC. Of those, 54 were receiving EFV and 25 were receiving ATV/r. The median age was 48 years (range 23 - 73) and the median duration of HIV infection was 10 years. Sixty-one percent of participants were African-American. Detailed demographics of the study population have been published (5). One enrolled participant did not have a sample available for cytokine profiling. Of the remaining 78, five did not provide a sample for p16INK4a measurement and six did not provide adequate samples for accurate calculation of intracellular metabolite/endogenous nucleotide exposures. These individuals were excluded from analysis.

Elastic Net Analysis

P16INK4a expression and AUC measurements were available for 73 and 72 participants, respectively. Table 1 presents the elastic net results for p16INK4a expression and the four AUC measures using dichotomized cytokine predictor variables. Detectable concentrations of interleukin-6 (IL-6) were found to be associated with higher p16INK4a expression and lower AUC of FTC-tp, TFV-dp, dATP, and dCTP. Macrophage inflammatory protein-1β (MIP-1β) was negatively associated with p16INK4a expression and positively associated with dCTP exposure. Monocyte chemoattractant protein 3 (MCP-3) was associated with lower TFV-dp exposure. Age positively predicted p16INK4a expression, which has been previously observed (11).
Cytokines were dichotomized based on detectability because a sizeable proportion of raw concentration values were left censored. The $R^2$ values for the optimal model are presented for each outcome and represent the proportion of variation explained by predictor variables in the model. The penalized parameter estimates for the selected variables describe the direction of prediction. The magnitude does not provide for meaningful comparisons between models and significance testing cannot be applied to the model parameters as in ordinary least squares (10).

Outcomes of interest were then compared between groups with detectable and undetectable concentrations of IL-6. Figure 1 shows that participants with detectable concentrations of IL-6 had significantly higher $p16^{\text{INK4a}}$ expression and lower TFV-dp, FTC-tp, dATP, and dCTP exposures in PBMCs. Means and standard deviations are provided in Table 2. Comparisons between groups with detectable and undetectable concentrations of MIP-1$\beta$ and MCP-3 were not statistically significant (Wilcoxon $p > 0.05$).

**Discussion**

Results from the elastic net regression showed detectable concentrations of IL-6 predicted increased expression of $p16^{\text{INK4a}}$. PIM-1 is a proto-oncogene expressed in the hematopoietic and lymphoid system. It encodes for a serine/threonine protein kinase that regulates cell cycle progression and apoptosis (12). IL-6 mediates PIM-1 expression through signal transducer and activator of transcription-3 (STAT3), an important transcription factor in cytokine signaling pathways (13). Overexpression on
PIM-1 increases markers of aging and cellular senescence, including p16\textsuperscript{INK4a}. The relationship between IL-6 and p16\textsuperscript{INK4a} seen in our HIV positive cohort could be explained by through this mechanism.

It was also observed that participants with detectable IL-6 had decreased exposure to TFV-dp and FTC-tp. This finding could be explained by membrane transporter modulation. TFV is a known substrate for efflux transporters P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), and FTC is transported by multidrug resistance-associated protein-1 (MRP-1) (14). IL-6 has been shown to directly increase P-gp and BCRP cell-surface expression through the PIM-1 pathway (15) and MRP-1 expression through unknown mechanisms in human cells lines (16). Modulation of membrane transporters is already known to affect intracellular accumulation of protease inhibitors (17). Increased expression of these efflux transporters on PBMCs could result in decreased intracellular NRTI concentrations.

Detectable concentrations of IL-6 were also predictive of lower amounts of dATP and dCTP. Reduced exposure to these nucleotides may be related to cell cycle arrest in senescent cells. Demand for nucleotide biosynthesis varies during the cell cycle and is highest during S-phase (Bayes, et al. 2014). To meet this heightened demand, enzymes involved in the synthesis of nucleotides and deoxynucleotides, such as phosphoribosyl pyrophosphate (PRPP) and ribonucleotide reductase (RNR), are upregulated (18). IL-6 has been described to induce premature senescence in human fibroblasts (19). Since senescent cells are arrested in the G-1 phase and are no longer dividing, demand for endogenous nucleotides, such as dATP and dCTP, remains low and their synthesis is attenuated.
MIP-1β was selected as a predictor of p16\(^{\text{INK4a}}\) expression and dCTP AUC. MCP-3 was predictive of TFV-dp exposure. However, it was decided to focus on IL-6, as it was consistently predictive of all outcomes of interest.

The results from this work are consistent with our earlier findings. P16\(^{\text{INK4a}}\) was found to be negatively associated with drug metabolite and endogenous nucleotide exposures. Our analysis demonstrated that detectable concentrations of IL-6 are positively predictive of p16\(^{\text{INK4a}}\) expression, while negatively predictive of intracellular drug metabolite and nucleotide exposures. This relationship suggests that cytokines, such as IL-6, could play a role in the inverse association between p16\(^{\text{INK4a}}\) expression and drug/nucleotide exposure previously seen.

Also, although a relationship between IL-6 and intracellular metabolite/endogenous nucleotide exposures is evident, our study lacks the ability to identity a clear causal mechanism for the relationships being seen. Future \textit{in vitro} and \textit{in vivo} studies are planned to confirm these relationships and explore potential mechanisms.

HIV-infected patients exhibit significantly higher plasma concentrations of IL-6 compared to uninfected controls (20) and IL-6 has been associated with increased all-cause mortality in this population (2). The results of this study suggest that IL-6 affects cellular senescence, NRTI pharmacokinetics, and endogenous nucleotide pools. These findings emphasize the importance that markers of inflammation play in the treatment of HIV in an aging population, and further support the idea of cytokines as potential drug targets.
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Disclosures

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References


Figure 1: Boxplots showing the distributions of a) p16\textsuperscript{\textit{INK4a}} expression, b) TFV-dp AUC, c) FTC-tp AUC, d) dATP AUC, and e) dCTP AUC between participants with undetectable (0) and detectable (1) levels of IL-6.

Each box corresponds to the inter-quartile range (middle 50% of observations) for the group. The median is indicated by the horizontal line within the box. The distance between the upper and lower whiskers represents the range of the data that are not considered outliers. Outliers are denoted by a closed circle (•). Participants with detectable concentrations of IL-6 exhibited significantly higher expression of p16\textsuperscript{\textit{INK4a}} than those with undetectable concentrations (a). Conversely, participants with detectable IL-6 concentrations had a lower TFV-dp AUC (b), FTC-tp AUC (c), dATP AUC (d), and dCTP AUC (e). ** p < 0.01, *** p < 0.001

AUC, area under the curve; TFV-dp, tenofovir diphosphate; FTC-tp, emtricitabine triphosphate; dATP, deoxyadenosine triphosphate; dCTP, deoxycytidine triphosphate, IL-6, interleukin-6.
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Table 1: Elastic net results using dichotomized cytokine values as predictor variables for p16<sup>INK4a</sup> expression, TFV-dp AUC, FTC-tp AUC, dATP AUC, and dCTP AUC.

The R² value is the proportion of variation explained by predictor variables in the model. Variables chosen by the elastic net algorithm that best predict each outcome are shown with their corresponding penalized parameter estimates. The penalized parameter estimates show whether a variable is positively or negatively predictive of the outcome. Detectable concentrations of IL-6 are positively predictive of p16<sup>INK4a</sup> expression and negatively predictive of TFV-dp AUC, FTC-tp AUC, dATP AUC, and dCTP AUC. Detectable concentrations of MIP-1β predict p16<sup>INK4a</sup> expression and dCTP AUC. Detectable MCP-3 is a predictor for TFV-dp AUC.

AUC, area under curve; TFV-dp tenofovir diphosphate; FTC-tp, emtricitabine triphosphate; dATP, deoxyadenosine triphosphate; dCTP, deoxycytidine triphosphate.
### Table 2: Comparison of p16\textsuperscript{INK4a} expression, TFV-dp AUC, FTC-tp AUC, dATP AUC, and dCTP AUC measurements between participants with undetectable and detectable concentrations of IL-6.

Values are reported as mean ± standard deviation.

AUC, area under the curve; TFV-dp, tenofovir diphosphate; FTC-tp, emtricitabine triphosphate; dATP, deoxyadenosine triphosphate; dCTP, deoxycytidine triphosphate, IL-6, interleukin-6.