Analysis of Cellular Kinases and Aging in PBMCs and Colorectal Tissue

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Version 3.0
21 January 2025

Clinical Trials.gov ID: NCT6132386

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List of Abbreviations

ALT	Alanine transaminase
AK2	Adenylate kinase 2
AST	Aspartate aminotransferase
BUN	Blood urea nitrogen
CBC	Complete Blood count
CKM	Creatine kinase
EMR	Electronic medical record
FTA-ABS	Fluorescent treponemal antibody test absorption
FTC	Emtricitabine
GC/CT	Gonorrhea/Chlamydia
HIV	Human immunodeficiency virus
INR	International normalized ratio
NRTIs	Nucleotide reverse transcriptase inhibitors
PBMC	Peripheral blood mononuclear cells
PLWH	People living with HIV
PrEP	Pre-exposure prophylaxis
PTID	Participant ID
RPR	Rapid plasma reagin
STI	Sexually transmitted infection
TDF	Tenofovir disoproxil fumerate
TFV	Tenofovir
TFV-DP	Tenofovir diphosphate
TFV-MP	Tenofovir monophosphate

I. PROJECT OVERVIEW

This project involves obtaining peripheral blood mononuclear cells (PBMCs) and colorectal tissue samples from study participants to measure adenylate kinase 2 (AK2) and muscle-type creatine kinase (CKM) enzyme levels in an "older adult" group (65-80) when compared to a "younger adult" group (18-50). Both AK2 and CKM has been demonstrated to be vital enzymes in converting the prodrug tenofovir (TFV) into its active form, tenofovir diphosphate (TFV-DP).[1-4] However, no study has yet investigated the effect of aging on AK2 or CKM in colorectal tissues relevant to HIV infection and prevention. This study will investigate the AK2 and CKM variability in individuals from the younger and older age groups in preparation for a follow-up study investigating how the pharmacokinetics (PK) of tenofovir (TFV) vary with altering levels of cellular enzymes in different age populations. A finding of clinically significant differences in the activity of AK2 or CKM, may indicate the need for dose adjustment of TFV for HIV treatment or prevention depending upon age to either reduce toxicity or enhance efficacy.

In this study, individuals who are interested in participating will be screened for eligibility. If eligible, participants will return for a single study visit. At the study visit, blood will be drawn and colorectal tissue will be obtained via flexible sigmoidoscopy. Participants can participate in an optional PK sub-study if they are already taking a tenofovir (TFV)-based regimen for prevention or are willing to take a daily TFV-based regimen for 7 days prior to the flexible sigmoidoscopy. Subsequently, AK2 and CKM will be analyzed in PBMCs and colorectal tissue using mass spectrometry.

II. INTRODUCTION

The prevalence of HIV in older adults (≥50 years of age) living with HIV has increased, in part to the success of antiretroviral therapy extending life expectancy, with nearly half of the people living with HIV (PLWH) in the United States over the age of 50, and 16% ≥65 years of age (data from the Centers for Disease Control and Prevention). However, each year, there are nearly 40,000 new HIV diagnosis infections in the United States and 1.7 million infections globally.[5,6] Individuals over the age of 50 account for 17% of new HIV diagnoses annually. In 2020, men accounted for 80% of new HIV diagnosis in the United States, with nearly 70% in gay, bisexual, or other men who reported male-to-male sexual contact. With this in mind, there is a need to develop and optimize efficacious strategies for prevention of HIV infection in this population[5].

The recent development of pre-exposure prophylaxis (PrEP) for the prevention of HIV can significantly decrease the risk of HIV infection with proper adherence.[7] Tenofovir is a prodrug that is the main antiviral component of current PrEP regimens.[8-10] TFV is from the drug class known as nucleotide reverse transcriptase inhibitors (NRTIs) in which chain termination occurs during DNA synthesis by HIV reverse transcriptase, therefore, suppressing HIV replication. However, only the diphosphate form of tenofovir (TFV-DP), generated by phosphorylation of TFV by intracellular kinases, is capable of inhibiting HIV replication.

In key tissues related to HIV acquisition, it has been found that adenylate kinases and creatine kinases play a significant role in TFV activation.[1] Specifically, in colorectal tissue, it has been found that adenylate kinase 2 (AK2) is the primary kinase in the initial phosphorylation of TFV to tenofovir monophosphate (TFV-MP). Moreover, muscle-type creatine kinase (CKM) has been demonstrated to be the enzyme that is vital for the second phosphorylation, transforming TFV-MP to TFV-DP.

Previous studies have found that age has an effect on CKM expression, post-translational modifications, and activity in multiple tissues.[11-16] Thus, it is hypothesized that expression levels and post translational modifications of AK2 and CKM may differ depending upon age, therefore, altering the amount of active TFV-DP and increasing variability of protection. Therefore, it is imperative to investigate expression and post translational modifications of AK2 and CKM in colorectal tissue and PBMCs from various age populations to see if theses cellular enzymes change with increasing age.

III. RATIONALE

While PrEP has been shown to be highly effective in reducing the risk of HIV infection, despite adherence to PrEP, some individuals become infected with HIV. To gain an understanding of CKM phosphorylation activity and examine whether CKM might also contribute to variability of TFV-DP activation in colonic CD4+ cells in the presence of HIV infection, we will also measure levels of CKM and TFV-DP in colonic CD4+ T cells of younger and older individuals infected with HIV taking tenofovir-based HIV treatment, as well as those taking tenofovir-based PrEP who are not infected with HIV.

This study is designed to investigate the intracellular AK2 and CKM enzymes in both colorectal tissue and PBMCs in a younger and older adult population. It is hypothesized that age alters expression and post translational modifications and expression of AK2 and CKM in colorectal tissue and PBMCs causing variability in TFV-DP levels in individuals taking tenofovir-based medications.

To test this hypothesis, colorectal biopsies and PBMCs will be collected to assess for changes in AK2 and CKM levels using mass spectrometry and changes in enzymatic activity to phosphorylate TFV from the following populations:

Cohort A: 10 younger (age 18-50) and 10 older (age 65-80) adults who are not infected with HIV and not taking tenofovir-based PrEP (“Control Cohort”).

Cohort B: 10 younger (age 18-50) and 10 older (age 65-80) adults who are not infected with HIV, but are taking a TFV-based regimen (either on daily TFV- based PrEP or agree to take a daily TFV-based regimen 7 days prior to procedure). (“PrEP Cohort”)

Cohort C: 10 younger (age 18-50) and 10 older (age 65-80) adults who are infected with HIV, taking a TFV-based regimen. (“ARV Cohort”)

Study Objectives:

Objective	**Endpoint**
1. Determine if levels and activity of AK2 and CKM in colorectal tissue differ between (a) younger adults (age range18-50 years), and older adults (age range 65-80) or	• Proteomics will be used to measure intracellular protein levels and modification status of kinases that phosphorylate TFV. • In individuals taking a TFV-containing regimen, colorectal tissue TFV-DP will be measured at steady-state.

(b) between HIV negative and HIV positive persons.	• In individuals not taking a TFV-based regimen, in vitro incubation of TFV with colorectal tissue biopsies will be performed to measure TFV activation as a function of age.
2. Determine if levels and activity of AK2 and CKM in PBMCs differ between: (a) younger adults, defined as 18-50 years of age, and older adults, defined as 65-80 years of age or (b) HIV negative and HIV positive persons..	• Proteomics will be used to measure intracellular protein levels and modification status of kinases that phosphorylate TFV. • In individuals not taking a TFV-based regimen, in vitro incubations of TFV with PBMCs will be performed to measure TFV activation as a function of age. • In individuals taking a TFV-containing regimen, colorectal tissue TFV-DP will be measured at steady-state.

IV. STUDY DESIGN

Overview

The design of the study will be a two-visit study, including a screening visit (Visit 1) and a sampling visit (Visit 2). During the screening visit, the individual will be assessed for eligibility. For participants scheduled for sigmoidoscopy or colonoscopy for a medical indication, the screening visit may be conducted by phone prior to the procedure. If eligible, the participant will return for the study visit ≤ 42 days following the screening visit. During the study visit, approximately 100 mL of blood will be drawn for PBMC isolation. Following the blood draw, participants will undergo endoscopic evaluation in which 10 colorectal biopsy samples will be obtained between 10-20 cm from the anal verge. Thirty adults from each age population (younger and older) will be enrolled for this study. Three cohorts (each including young and old groups) will be defined by TFV administration and HIV status.

Study Population

Three cohorts of participants will be recruited, with 10 younger and 10 older in each cohort.

Cohort A: Healthy Volunteers, not taking TFV based PrEP (Control Cohort)

Cohort B: Healthy volunteers, steady state TFV-based regimen (either on daily TFV- based PrEP or taking daily TFV-based regimen 7 days prior to procedure) (PrEP Cohort)

Cohort C: Persons infected with HIV taking TFV/FTC-based HIV treatment (ARV Cohort)

Inclusion Criteria
Participants who meet the following criteria are eligible for inclusion in this research:

1. English speaking male or female volunteers between the ages of 18-50 for younger adults or 65-80 for older adults
2. Willing to provide written informed consent
3. Willing to abstain from insertion of anything in rectum for 72 hours before and 72 hours after the endoscopic procedure for colorectal tissue collection.
4. Not currently participating in other research studies involving drugs and/or medical devices.

For participants not infected with HIV (Control Cohort A)

1. No known risk for HIV exposure or a documented negative HIV-1/HIV-2 Ag/Ab test in the past 3 months (HIV risk, but not on PrEP)
2. Documented negative HBsAg in those taking study TFV (i.e. cohort B)

For participants not infected with HIV, but taking or willing to take TFV (PrEP Cohort B)

1. Currently taking TFV-based oral PrEP daily or willing to take oral TFV-containing PrEP for one week
2. Documented negative HIV-1/HIV-2 Ag/Ab test in the past 3 months
3. Documented negative HBsAg

For participants infected with HIV (ARV Cohort C)

1. Virologically suppressed HIV for at least 6 months prior to screening.
2. Taking tenofovir disoproxil fumarate or tenofovir alafenamide containing regimen to treat HIV

Exclusion Criteria
Participants who meet any of the following criteria at screening will be excluded from the study:

1. History of inflammatory bowel disease or active inflammatory condition of the GI tract
2. History of significant gastrointestinal bleeding
3. Current medically-indicated use of warfarin or heparin or other anticoagulant medications associated with increased risk for bleeding following mucosal biopsy (e.g., daily high dose aspirin [>81 mg], non-steroidal anti-inflammatory drugs [NSAIDs], or Pradaxa®)
4. Use of systemic immunomodulatory medications within 4 weeks of enrollment
5. Use of rectally administered medications within 4 weeks of enrollment
6. Use of product containing nonoxynol-9 within 4 weeks of enrollment
7. Use of any investigational products within 4 weeks of enrollment
8. Any other clinical condition or prior therapy that, in the opinion of the investigator, would make the participant unsuitable for the study or unable to comply with the study requirements. Such conditions may include, but are not limited to, current or recent history of

severe, progressive, or uncontrolled renal, hepatic, hematological, gastrointestinal, endocrine, pulmonary, neurological, or cerebral disease.

9. Active rectal infection (GC, Chlamydia, HSV). Participants screening positive for GC/CT at the time of endoscopy will be excluded from analysis (and replaced).

<u>For participants not undergoing a concurrent endoscopic procedure for indication unrelated to this study:</u>

10. Hct <36%
11. Platelet count <150/mm^3
12. INR > 1.2

Study Procedures

Note: This protocol requires colorectal biopsies to support the primary objectives. These are collected using an endoscope (e.g., flexible sigmoidoscope or colonoscope). We plan to use a flexible sigmoidoscope as the least invasive method to achieve our objectives. If, however, the research participant already has a colonoscopy scheduled for a medically indicated purpose, e.g., a preventive colon cancer screening, then we will coordinate with the clinical endoscopy team to obtain the biopsies by this method, in order to reduce the number of procedures related to our study. The protocol indicates some minor differences in procedures to accommodate this option.

This section includes information on visit specific study procedures. This study includes two visits, a screening visit and a sampling visit. The breakdown of the procedures on each day are as follows:

Visit 1: Screening Visit

Prescreening via email or a phone call will take place with potential participants. Those interested in the study can request that the informed consent documents be emailed or mailed to them prior to the Screening Visit.

Written informed consent will be obtained from study participants prior to any study procedures taking place. To obtain informed consent, an interactive question and answer process will occur in which the potential subject will be asked specific questions to determine their understanding of the purpose, procedures, risks and benefits of participating in the study. If, in the judgment of the investigator, the subject cannot demonstrate sufficient understanding, then that subject will be excluded from participation in the protocol. If participants do not meet the eligibility criteria, screening will be discontinued once ineligibility is determined.

The following list of activities will be completed during the screening visit:

Table 1: Visit 1 - Screening

Component	Procedure/Analysis

Administrative	• Obtain written informed consent
	• Assign participant ID (PTID)
	• Assess eligibility
	• Obtain demographic and locator information
	• Provide counseling:
	  • HIV/STI risk reduction counseling
	  • HIV pre- and post- test counseling, if indicated
	  • Provide a copy of pre- and post- visit reminders
	• Participants not undergoing screening colonoscopy will be provided with a Enema and directions for use prior to Sampling Visit.

Clinical	• Obtain complete medical history, documenting pre-existing conditions • Review and record concomitant medications • Obtain vital signs – blood pressure (BP), heart rate (HR), height, and weight (height may be obtained from EMR, and weight captured in the EMR within 6 months of screening may be obtained from EMR Note: Medical history and physical examination data may be extracted from electronic medical record, or performed at the time the participant presents for an endoscopic procedure scheduled for clinical indication.

Safety Labs and STI Screening (for participants not undergoing a concurrent endoscopy procedure only):	• Perform general physical exam
	• Collect rectal swab for GC/CT
	• Collect blood specimens for:
	  ○ Complete blood count (CBC) with differential[1]
	  ○ AST, ALT, BUN, Creatinine[1]
	  ○ HIV testing[2]
	  ○ Syphilis testing[3]

PrEP cohort only for those not already on TFV-based PrEP prior to screening	• HBsAg (result from prior test within 12 months acceptable) • Dispense F/TDF or F/TAF for daily dosing to begin 7 days prior to study procedure (PrEP Cohort B only, not already on PrEP) • Commence daily virtual DOT for 7 days prior to study procedure

[1] Screening laboratory values are valid up to 42 days.

[2]See Appendix II: HIV Testing Algorithm; HIV testing not to be done in participants known to be infected with HIV

[3]Syphilis Chemiluminescence assay (with confirmatory RPR; FTA-ABS performed on RPR negative samples)

Visit 2: Sampling Visit (≤ 42 days since **Screening Visit**)

Note: Where applicable, Visit 2 may be combined with Visit 1

After eligibility is confirmed, participants will be contacted to schedule the study visit (Visit 2). Where possible, Study Visit 2 may be coordinated with a scheduled clinical procedure, such as a screening colonoscopy.

The following activities will take place at the Study Visit:

Table 2: Visit 2 – Sampling Visit

Component	Procedure/Analysis
Administrative	• Confirm participant understood and signed informed consent • Review/update locator information • Confirm eligibility • Provide counseling: • HIV/STI risk reduction counseling • Provide a copy of pre- and post- visit reminders
Clinical	• Review/update medical history • Review/update concomitant medications • Perform symptom-directed physical exam as needed • Obtain vital signs – blood pressure (BP), heart rate (HR), • Obtain 10 colorectal biopsies via flexible sigmoidoscopy or during clinically-indicated study procedure
STI Screening (For participants undergoing a concurrent endoscopy for clinical indications):	• Collect rectal swab for CT/GC testing
Blood for PBMC Collection	• Pre-endoscopic Procedure
TFV PK (only in HIV uninfected participants taking TFV)	• Blood plasma

V. RECRUITMENT AND OUTREACH

Sources of recruitment will be as follows:

- Referral from treating clinicians at any regional clinic
- Prior Hopkins/Affiliates study participants who have given informed consent to be reached for future studies
- Individuals who learn about the study through advertisements or peer/network recruiting

Approved recruitment flyers will be distributed throughout the community. In addition, approved messages for social media platforms, internet recruitment and department website will be used. Staff will also follow-up with all persons who express an interest in the study in a timely manner.

VI. COMPENSATION TO SUBJECTS

Participants will be compensated for their time and inconvenience related to participation in this study. Compensation will be $425 for completion of the study and is based upon the schedule established for DDU studies.

VII. COSTS TO SUBJECTS

There will be no costs to the subjects outside of costs to travel to the study site.

VIII. PROTECTION OF HUMAN RESEARCH PARTICIPANTS

Risks

Flexible Sigmoidoscopy: Endoscopy is uncomfortable for some persons and poses only a very small risk of perforation and bleeding when biopsies are taken. The most common adverse events associated with endoscopy are gas, bloating, and cramping which usually resolve within hours of the procedure. Taking the biopsies causes no discomfort due to the insensate epithelial lining of the colon. Pinch biopsies may cause minor bleeding which stops on its own. More significant and rare adverse events can include prolonged bleeding and/or perforation. Participants will be screened and excluded if taking any blood thinners or have conditions that may prolong bleeding. Risk of perforation, which is a medical emergency that may require surgery, will be minimized by enrolling participants who do not have significant bowel disease and having the procedure performed by a credentialed endoscopist. Where possible, the endoscopic procedure will be paired with a procedure that may have a clinical indication, such as screening colonoscopy for colon cancer. In this case, the only additional risk to the participant are the risks of collecting pinch biopsies, which are lower in risk than the endoscopy itself..

Phlebotomy is performed by drawing blood from a vein in the forearm or antecubital fossa. This may cause pain during the insertion of the needle or slight bruising afterward.

TFV (administered as part of this research protocol): TFV disoproxil fumarate (TDF) and TFV alafenamide (TAF) are both TFV prodrugs licensed by FDA for the prevention and treatment of HIV. TFV-based PrEP regimens currently approved include emtricitabine / tenofovir alafenamide (F/TAF) or emtricitabine / tenofovir disoproxil fumarate (F/TDF). Both combination regimens are well tolerated. Risks of TFV include acute exacerbation of hepatitis B virus Immune Reconstitution Syndrome; New or worsening renal impairment; lactic acidosis; hepatomegaly with steatosis; lower bone mineral density; diarrhea; nausea; headache; fatigue; abdominal pain; rash. Risks will be minimized by confirming participants do not have hepatitis B or HIV and baseline safety labs will be monitored prior to receiving study medication. TFV-naïve participants who are not already prescribed TFV-containing regimens for PrEP or treatment will take only 7 doses, which will minimize the risks associated with chronic administration.

STI testing: Participants may become embarrassed, worried, or anxious when completing their HIV-related interviews and/or receiving STI counseling. Certain positive results (Chlamydia, gonorrhea) may need to be reported to appropriate Department of Public Health Agencies. The reporting only happens if the test result is positive and is always done confidentially.

Incidental Findings: Significant clinical findings may be discovered during study procedures, which may warrant referral for medical care and further testing. If significant clinical findings are discovered during the procedure, study subjects will be referred to the proper Johns Hopkins Division for additional evaluation and treatment as necessary.

Social Risk: Although the study site will make every effort to protect participant's privacy and confidentiality, it is possible that involvement in the study could become known to others, and that social harms may result (i.e., subjects could become known as HIV-infected or at "high risk" for HIV infection) and, consequently, be treated unfairly or discriminated against by families, friends and/or communities.

Loss of confidentiality: Although every effort will be made to protect the privacy and confidentiality of all participants, it is possible that their involvement in the study could become known to others. All guidelines for the protection of Personal Health Identifiers will be followed.

Benefits

There are no direct benefits to subjects who participate in this study. However, there may be personal satisfaction in the knowledge that they are aiding in important medical research, and that the knowledge obtained from this study will advance the field of HIV prevention. Participants will be reimbursed for their time and inconvenience.

The risks to individual participants are outweighed by potential benefits to the scientific community and to society – namely, contribution to our microbicide knowledge-bank.

IX. STATISTICAL ANALYSIS

To compare protein levels between the younger adult and older adult groups, protein abundances will be averaged within age groups. Differences in protein levels will be determined by unpaired two-tailed t test using the Benjamini-Hochberg correction for multiple comparisons, with significant differences being defined as having an adjusted p-value of <0.05. Based on our previous studies of TFV phosphorylation we estimate ~80% power to detect a 1.5-fold difference between study groups using our measured outcomes and >95% power to detect a 1.75-fold difference between study groups at our sample size.

X. CONFIDENTIALITY

Given the sensitive nature of the proposed studies, the investigational team will keep the study information private to the extent possible by law. Where possible, clinical information will be identified by a unique number assigned to each individual research participant. The research team will make every effort to register research subjects in the hospital information systems for clinical testing using this identifier. Access to study records will be limited to the study team, the Office of Human Research Protections, and Johns Hopkins University officials, where applicable and required by law. Enrolled patients will be assigned study numbers for data collection and

evaluation. Publications in medical journals arising from this study will delete the names of the subjects involved.

The key linking study to numbers will be kept as an electronic file. Electronic files will be maintained on the DDU's computer server, which is password protected for access solely by study investigators at the level of the individual protocol.

This study will not require a Certificate of Confidentiality. However, a Certificate of Confidentiality has been issued as a term and condition of this NIH-Funded research. All records are stored in a locked area and only research staff will have access to them. Paper files are maintained in a locked cabinet located in the Division of Clinical Pharmacology, again, with access limited to study investigators.

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APPENDIX I: SCHEDULE OF EVENTS

	Screening Visit	Sampling Visit
Visit Day	≤ -42	1
Visit #	1	2
ADMINISTRATIVE		
Informed consent	x	
Assessment of Eligibility	x	
HIV/STI Risk Reduction Counseling	x	
Dispense and provide directions for enema[2]	x	
Dispense TFV to TFV-naïve participants[3]		
CLINICAL		
History/Concomitant meds	x	
Physical Exam, Vital signs, height, weight [1]	x	
Oral TFV-based regimen dosing and virtual DOT[6]	Day -7 to -1	
SAFETY LABS[6]		
Blood CBC w/ diff, AST, ALT, BUN, Creatinine[2]	x	
Blood HIV testing[4]	x	
Blood syphilis screening[5]	x	
Rectal swab GC/CT NAT	x	x[6]
Blood HBsAg[6]	x	
SPECIMEN COLLECTION		
Blood PBMC		x
Rectal biopsy		x
Blood for TFV[6]		x

[1]After the screening visit, physical exam is symptom-directed; vital signs consist of heart rate and blood pressure only.
[2]For participants not undergoing a concurrent endoscopy procedure.
[3] For PrEP cohort only in participants not already taking PrEP.
[4]See Appendix II: HIV Testing Algorithm. Will not be completed for those with known HIV infection.
[5]Syphilis Chemiluminescence assay (with confirmatory RPR; FTA-ABS performed on RPR negative samples)
[6]For participants in PrEP or ARV Cohort taking TFV-based regimen for 7 days prior to procedure.
[7]With the exception of rectal GC/CT screening, safety labs will not be obtained in participants undergoing screening colonoscopy. In those individuals, rectal GC/CT screening can be performed concurrently with Visit #2.