Glucose Metabolism Disorders among People Living with HIV/AIDS on Efavirenz and Atazanavir/Ritonavir-Based Combination Antiretroviral Therapy: A Pharmacogenetic and Pharmacokinetic Evaluation

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Identification and recruitment of study participants on HIV patients on EFV or ATV/r-based therapy for Cross sectional study (n=363) Blood sample collection for 1) determination of fasting blood glucose,insulin lipid and other parameters (serum), 2) Pharmacogentic study (whole blood), and 3) determination of plasma concentration of EFV, ATV/r (plasma) Cross-sectional study on predictors and prevlanence of GMD among participants on EFV or ATV/r-based therapy (n=363) based on serum fasting glucose, insulin, HOMA-IR (Paper 1) Whole blood and plasma samples stored at -80 oC and transported to KI lab Blood samples categorized in to Case and Control groups based on GMD status and treatment type (EFV or ATV/r/-based ART) of study paticipants Conduct pharmacokinetic and pharmacogenetic lab analysis and data generation Samples from HIV patients on EFV based ART with random matching of groups Case-control study on PG-GMD association study with GMD on long-term EFV (Paper 2) Case-control study on PG-GMD association study for PK-GMD and PK-PG association on long-term EFV (Paper 3) samples from patients on ATV/r based ART with random matching of groups Samples from patients on ATV/r based ART grouped in to cases & controls PG factors association study (Paper 4) Samples from patients with ATV/r based ART grouped in to cases & controls for PK-PG and PK-GMD association study (Paper 5)

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With this statement, I declare that this dissertation is my original work and I have independently completed this Ph.D. dissertation entitled '*Glucose Metabolism Disorders among People living with HIV/AIDS on Efavirenz and Atazanavir/Ritonavir-Based Combination Antiretroviral Therapy: A Pharmacogenetic and Pharmacokinetic Evaluation'.* The thoughts taken directly or indirectly from external sources are properly marked as such. This dissertation has not been previously presented and submitted to any other academic institution.

Wondmagegn Tamiru Tadesse **Signature:** ____________ **Date** April 13, 2023

Addis Ababa, Ethiopia

Dedicated to my family and mentors, who have always believed in and encouraged my education as a career since I was a child.

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ABSTRACT

Glucose Metabolism Disorders among People living with HIV/AIDS on Efavirenz and Atazanavir/Ritonavir-Based Combination Antiretroviral Therapy: A Pharmacogenetic and Pharmacokinetic Evaluation

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Addis Ababa University, 2022

This dissertation reports the results of cross-sectional and case-control studies investigating the prevalence and the link between pharmacogenetic and pharmacokinetic factors with glucose metabolism disorders (GMDs) among patients on efavirenz (EFV) and ritonavir (RTV)-boosted atazanavir (ATV/r)-based combination.

GMD status was identified based on fasting glucose, fasting insulin, and HOMA-IR values. Cases were defined as the presence of any impaired fasting glucose, insulin resistance (IR), or diabetes mellitus (DM) while controls were those without GMDs. The cross-sectional prevalence study was conducted on EFV- (n=240) and ATV/r -based (n=111) combination antiretroviral therapy (cART). The prevalence and predictors of GMDs were determined by association and regression analysis. Samples from patients on long-term EFV (75 cases and 165 controls) and ATV/r-based cART (22 cases and 89 controls) were then genotyped for _CYP3A4*1B_, _CYP3A5 (*3_ and _*6),_ _CYP2B6*6_, _UGT2B7*2_, _ABCB1 (c.3435C>T, c.4036A>G_), and _SLCO1B1_ (_*1b_, _*5_). The mid-dose (CP12) of EFV, ATV, and RTV plasma concentrations (CP12) was determined using LC-MS/MS. The association of genotypes and CP12 of EFV and ATV/r with the incidence of GMDs were then investigated.

The prevalence of GMDs for all regimens was 27.6% (97/351) [95% CI 23.0-32.6%], with 31.1% (75/240) [95% CI 25.4-37.5%] for EFV-based and 19.8% (22/111) [95% CI 12.9-28.5%)] for ATV/r-based cART group. All genotype frequencies followed the Hardy-Weinberg Equilibrium (p>0.05) between cases and controls. In the EFV group, the *CYP3A5*6* allele ($p = 0.005$) and *CYP3A5*6* genotype ($p = 0.01$) were significantly associated with GMD cases. Similarly, multivariate analysis indicated *CYP3A* haplotype as a significant predictor of GMDs ($p = 0.02$) and IFG ($p = 0.004$), while *CYP2B6*6* significantly predicted DM ($p = 0.03$) in EFV-based cART group. Furthermore, EFV Log CP12 $\geq$ 3.7 (5000) ng/ml was an independent predictor of GMDs.

In ATV-based cART-receiving participants, the C allele carriers of SL*CO1B1*5 c.521 T>C* demonstrated a 2.9 times higher risk of GMDs [AOR=2.9; 95% CI 1.03-8.1, p=0.04] than the wildtype allele carriers. Haplotypes containing any **6* and only **3* of *CYP3A* conferred 80.0% (p=0.03) and 90.0% (p=0.01) protection, respectively, from GMDs than the wildtype combination haplotypes. In contrast, a 90% protection from IR was recorded for both haplotype combinations containing any **6* (p=0.03) and only **3* (p=0.01) types than the wild-type haplotype combinations. According to the plasma concentration analysis, the mean (SEM) logCP12 of ATV was 3.24 ng/ml (0.04) in controls and 3.52 ng/ml (0.06) in cases. But, the logCP12 of both ATV and RTV failed to show significant association with the GMDs.

In conclusion, GMDs are highly prevalent among adults on EFV- than ATV/r-based cARTs. *CYP3A* haplotype and *CYP2B6*6* genotype positively predicted GMDs and DM, respectively, among patients on long-term EFV-based cART. On the other hand, the *CYP3A* haplotypes decreased and the *SLCO1B*5* allele increased the risk of GMDs among PLWH on ATV/r-based regimens. Higher EFV plasma concentration level independently predicted GMDs while

the ATV plasma concentration did not. Our findings warrant further research with a larger sample size.

Key Words: antiretroviral therapy; efavirenz; atazanavir/ritonavir, glucose metabolic disorder; HIV; pharmacogenetic variation, *CYP3A5, CYP3A4, CYP2B6*, genotype, drug transporters, single nucleotide polymorphisms, plasma concentration, Ethiopia.

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LIST OF ACRONYMS/ABBREVIATIONS

3TC = Lamivudine

ABCB1= ATP Binding Cassette Transporter B1

ADRs = Adverse Drug Reactions

AIDS = Acquired Immuno-deficiency syndrome

ARV= Antiretroviral

ATV/r = Ritonavir boosted atazanavir

ATV= Atazanavir

cART = combination ART

cART= Combination antiretroviral therapy

CCR5 = Chemokine receptor-5

C_p = Plasma drug concentration

CP12 = Mid-dose plasma concentration

CYP450 = Cytochrome-P 450

DM = Diabetes mellitus

DTG = Drug Transporter Genes

DTP = Drug Transporter Proteins

EFV= Efavirenz

FBG = Fasting Blood Glucose

GLUT-4 = Glucose Transporter 4

GMDs = Glucose Metabolism Disorders

HDL-c = High-Density Lipoprotein Cholesterol

HIV = Human Immunodeficiency Virus

HOMA = Homeostasis Model Assessment

HOMA-IR = Homeostasis Model Assessment for Insulin Resistance

HWE = Hardy–Weinberg equilibrium

IFG = Impaired Fasting Glucose

IGT = Impaired Glucose Tolerance

IL = Interleukins

ISTIs = integrase strand transfer inhibitors

LDL = Low-Density Lipoprotein Cholesterol

LPV/r = lopinavir/ritonavir

MDR1 = Multidrug resistance 1

mtDNA = mitochondrial DNA

NNRTIs = Non-Nucleoside Reverse Transcriptase Inhibitors

NRTIs = Nucleoside Reverse Transcriptase Inhibitors

OATP = Organic anion transporting polypeptides

PIs = Protease Inhibitors

PLWH = People Living with HIV/AIDS

PMPs = Polymorphisms

PPAR-γ = peroxisomal proliferator-activated receptor-γ

RTV = ritonavir

SLCO1B1 = Solute carrier Organic transporter-1B1

SNPs = Single Nucleotide Polymorphisms

SOPs = Standard Operating Procedures

SSA = Sub-Saharan Africa

T1DM = Type 1 diabetes mellitus

T2DM = Type 2 diabetes mellitus

TASH = Tikur Anbessa Specialized Hospital

TDF = tenofovir

TNF = tumor necrotic factor

UGT = UDP-glucuronosyltransferase

WHO = World Health Organization

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CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 Background

1.1.1 Overview of the natural course of HIV

Acquired immune deficiency syndrome (AIDS) was recognized as a new syndrome of the human immune system in 1981. Later, the causative virus was identified as a retrovirus and named the human immunodeficiency virus (HIV). The virus targets activated CD4 T lymphocytes and its entry relies on the interaction with CD4 and chemokine receptors (CCR5 or CXCR4). Other cells bearing CD4 and chemokine receptors are also infected, which include the resting CD4 T cells, monocytes, macrophages, and dendritic cells. Notably, astrocytes and renal epithelial cells can be infected in a CD4-independent manner (1).

Following acute infection, the virus expands its replication in the absence of an immune response. During this period, the majority of patients develop mononucleosis-like symptoms with increasing viremia. As the immune response develops, the viral load decreases and reaches a set point in which the viral load is stabilized, and the chronic phase is reached. During this phase, the infection is clinically latent. Viral replication continues during latency, particularly in lymphatic tissue, and there is a progressive loss of CD4+ T lymphocytes. As the CD4 cell count drops, the patient develops constitutional symptoms. Due to the loss of cell-mediated immunity, patients are susceptible to a variety of opportunistic infections and cancers with the development of AIDS, which eventually lead to death (2).

1.1.2 HIV Epidemiology

About 38.4 million people were estimated to be living with HIV across the globe in 2021 (3). Sub-Saharan Africa (SSA) was the highest-hit region with about 70% of the global burden (2,4). Particularly, eastern and southern Africa harbors about 20.6 million HIV-infected people compared to 5.0 million in western and central Africa in 2021 (3). In the same year, about 51% of the total new HIV infection, out of 1.5 million in the globe, was in SSA indicating the magnitude of the burden (3). The prevalence of HIV among Ethiopian adults aged 15 to 49 reached 1.1% in 2016. AIDS-related deaths among the stated age group were estimated to account for 18,000 and about 59% of HIV-infected individuals had been on antiretroviral therapy (ART) by the same year (5).

Since its introduction in 1996, ART has been the cornerstone for the management and control of HIV/AIDS disease progression. Over the past few decades, its access has been massively scaled up across the world. Subsequently, ART has changed the complexion of HIV infection from a deadly disease to a chronic manageable disorder and brought about a significant change in its global epidemiology in terms of transmission, incidence, and mortality. In 2021, about 28.7 million people living with HIV were accessing ART and it reached 78% in SSA (3,4).

1.1.3 Antiretroviral Therapy

There are now six classes of antiretrovirals (ARVs) available for combination ART (cART) regimens. These include nucleoside/nucleotide reverse-transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors (FIs), chemokine receptor-5 (CCR5) antagonists, and integrase strand transfer inhibitors (ISTIs). These classes of agents have different modes/mechanisms of action,

pharmacologically (6,7). In most developing health settings, the most popular combination treatment regimens currently involve three or more ARVs, often two NRTIs and either an NNRTI or PI with a ritonavir boost.

Viral load and CD4 cell counts are used as surrogate markers of HIV progression and treatment outcome. In most cases, the CD4 level is considered a marker to start ART in naïve patients, though when to start is controversial and varies from country to country. Successful treatment is marked by the achievement of sustained viral suppression, *i.e.*, persistent viral load <50 copies/mL (8–10). In 2013, WHO increased the CD4 count threshold for starting ART to ≤500 cells/µL from the previous cut-points of <200 and <350 cells/ml, respectively (11). However, the current recommendations warrant the initiation of ART for PLWH as diagnosed, irrespective of CD4 count and viral load based on shreds of evidence that early initiation resulted in reduced morbidity and mortality (12).

In a resource-limited setting, Efavirenz (EFV) and Atazanavir/Ritonavir (ATV/r) serve as core components of combination ARV regimens (13). In Ethiopia, during the study period, the preferred first-line regimen for adults was EFV-based cART, specifically a combination of tenofovir (TDF), lamivudine (3TC), and EFV (14). ATV/r-based regimens were the mainstay of second-line cART replacing lopinavir/ritonavir (LPV/r) (14). Currently, dolutegravir (DTG)-based cART has replaced EFV as a first line for eligible patients. Yet, EFV is used as an alternative to DTG in situations where DTG use is not recommended, such as 1st trimester of pregnancy, associated toxicities, and in settings where DTG is inaccessible (15).

1.1.4 HIV/ART and Glucose Metabolism Disorders

Treatment of HIV is lifelong, embracing frequent clinical evaluation and follow-up (16). During long-term exposure to ART, individual patients may experience treatment-associated adverse events or drug toxicities (16). Long-term exposure to ART may increase the risk of metabolic abnormalities such as lactic acidosis, osteopenia, dyslipidemia, and glucose metabolism disorders (GMDs) (17). GMDs are glucose homeostasis dysregulations that include diabetes mellitus (DM), impaired glucose tolerance (IGT), impaired fasting glycemia (IFG), or insulin resistance (IR) (18).

The literature indicates that the prevalence of IR, IGT, and DM has significantly increased and become a notable clinical concern, as long-term ART (19) and aging-related factors contribute to a higher risk of glucose metabolism abnormalities. DM is now emerging as one of the non-infectious comorbid conditions among People Living with HIV (PLWH) on cART (19). Previous studies reported that patients on ART were found to be four-fold more prone to DM and associated conditions compared to ART naïve individuals (17,18,20–22). Among cART classes, PIs use has been commonly reported to have an association with GMDs. PIs may cause abnormal glucose metabolism, ranging from IR through IGT & IFG to type 2 DM (T2DM) (19,23). The PIs, particularly lopinavir and ritonavir, are linked to an increase in IR and affect lipid and glucose metabolism (24,25). A cross-sectional study reported a five-to-nine-fold elevated prevalence of type 2 DM among PLWH on PIs (23). Recently, a high prevalence of cART-associated dyslipidemia, particularly low High-Density Lipoprotein Cholesterol (HDL-c) and hypertriglyceridemia, has been reported among treatment-experienced HIV-infected children from Ethiopia (26).

1.2 Statement of the Problem and Justification

Based on the above-stated evidence from the literature, it can be concluded that long-term ART may cause an alteration in glucose metabolism among PLWH. Glucose metabolism disorders such as IR, IGT, and overt DM are risk factors for cardiovascular diseases and other chronic complications. Several researchers confirmed that GMDs are prevalent in HIV-infected patients on cART together with other metabolic disorders. Besides managing HIV with ART, adverse drug effects related to glucose handling abnormalities affect treatment outcomes and the quality of life of patients. In such a scenario, managing HIV and metabolic abnormalities would be quite complex among patients on ART. Therefore, the link between ART use and the occurrence of glucose metabolism among PLWH should be investigated, which ultimately may help identify and set directions to mitigate glucose-related metabolic disorders.

The occurrence of GMDs is expectedly affected by the plasma ARVs concentration and genetics of the individual patient. Thus, determining the relationship between such adverse effects and plasma drug levels as well as pharmacogenetic factors may help design evidence-based clinical interventions. In-depth studies related to the effect of ART on glucose metabolism are scarce, especially focusing on pharmacokinetic and pharmacogenetic aspects. The pharmacokinetic aspect covers those factors specifically linked to plasma concentrations, metabolism, and clearance of antiretrovirals while the pharmacogenetic aspects consider polymorphisms of genes encoding proteins important in the metabolism and transport of antiretrovirals with the risk of glucose metabolism disorders. Such studies need to be carried out to identify the effect of pharmacokinetic and pharmacogenetic factors on fasting blood glucose (FBG) levels and other glucose-handling

parameters. Using the generated information, treatment optimization and appropriate clinical precautions may be initiated for HIV-infected patients during and after treatment regimen selection and follow-up.

In response to the emerging burden of glucose and other metabolic abnormalities among PLWH treated with ART, several observational studies were devoted to characterizing risk factors and determining the prevalence of these metabolic disorders. However, such studies, including those conducted in Ethiopia, barely assessed pharmacokinetic and pharmacogenetic factors in relation to ARVs use and the occurrence of GMDs. Hence, this study sought to fill the perceived gap in the existing literature and examine the relationship between PI or NNRTI-based ART and the occurrence of GMDs. To our knowledge, this study is the first in Ethiopia to focus on the pharmacokinetic and pharmacogenetic aspects of these adverse effects.

1.3 Literature Review

1.3.1 Risk Factors for Glucose Metabolism Disorders in HIV Infection

Following the introduction of ARTs in the mid-1990s, abnormalities related to glucose homeostasis have been reported with increasing frequency among HIV patients. T2DM is now emerging as one of the non-infectious comorbid conditions among HIV-infected patients and may pose a perceived threat to ART outcomes and patients' quality of life. Literature indicates that DM-HIV comorbidity is becoming a major public health concern as long-term ART and aging-related factors contribute to the occurrence of GMDs and DM. A study reported that patients on ART were found to have a four-fold higher incidence of DM relative to their counterparts, HIV-uninfected individuals (27).

Risk factors for the development of GMDs include obesity, lipoatrophy, HIV-related inflammation (increased proinflammatory cytokines and/or free fatty acids), use of most PIs, NRTI exposure (particularly stavudine), older age, family history of DM, nonwhite race, and possibly, hepatitis C virus coinfection. GMDs among HIV-infected patients may be a result of traditional risk factors unrelated to HIV infection and are more important than treatment-related factors. The use of niacin, growth hormone, corticosteroids, and antipsychotics may also contribute to hyperglycemia (28–30).

Independent of ART, HIV infection may be associated with metabolic disorders. Listed as one of the risk factors, HIV-related infection has been linked with altered levels of adipokines and decreased leptin. Increased adiponectin and soluble-tumor necrosis factor-receptor 1 (sTNFR1), demonstrating altered adipokines level, were linked with impaired glucose metabolism in a state of HIV infection (31,32). Besides, alterations in CD4+ and CD8+ T-cell function were linked with an impact on glucose metabolism particularly during active HIV infection (33,34). In the Multicenter AIDS Cohort Study (MACS), participants who were living with HIV had a higher prevalence of insulin resistance compared to those HIV-uninfected (35).

1.3.2 Epidemiology and the Risk of Glucose Metabolism Disorders in ART

Studies indicate that the prevalence of IR, IGT, and DM has increased significantly and has become a prominent clinical concern following the introduction of ARVs (36,37). Among glucose metabolic abnormalities, DM has been a focus of most literature coverage. According to different cohort studies, despite wide variation in reported proportions, clinically defined T2DM and IR account for 8 to 10% among patients receiving ART, while hyperglycemia with or without DM accounted for 3 to 17%. A recent meta-analysis

reported that the mean FBG level and the risk of DM and metabolic syndrome were significantly higher among ART-exposed patients relative to their naïve counterparts (38).

An observational cohort study by Calza *et al.* (39) reported that DM, IFG, and hyperinsulinemia accounted for 4.5%, 9.4%, and 11.9%, respectively, out of 755 HIV-infected patients on ART in an Italian clinic. Similarly, Galli *et al.* (40) described a higher proportion of DM in HIV patients (4%) than in non-HIV infected controls. Several studies, including the Swiss Cohort Study (26), the Women's Interagency HIV Study (WIHS) (35), the Multicenter AIDS Cohort Study (MACS) (36), and the D:A:D study (37) reported a higher incidence of DM in HIV patients.

In SSA, the prevalence of GMDs among HIV-infected adult patients on ART may range between 20 to 40%. A recent study in Tanzania reported that HIV-infected adults on long-term ART had 5-fold greater odds of GMDs than HIV-negative controls. This study reported that CD4+ T-Cell counts were significantly associated with GMDs even after adjusting for other variables like sex, age, obesity, and socioeconomic status (18). In another cross-sectional study, the HIV-infected patients on ART are reported to have a significantly elevated occurrence of GMDs (32.7% vs. 7.2%) and frank DM (18% vs. 5.2%) than HIV-negative adults (43).

In Ethiopia, little evidence is found regarding the prevalence and incidence of GMDs among HIV patients on ART. A cross-sectional study that involved 134 patients on ART in Addis Ababa reported a 22.4% prevalence of prediabetes and 6.7% (44) to 8.8% (20) of DM (44), which implies that the overall glucose metabolism abnormality would be as high as 31.1%. Although not directly related to glucose metabolism abnormalities, another cross-sectional study in Tikur Anbessa Specialized Hospital (TASH) in Addis Ababa

reported 10.9% and 6.9% prevalence of IFG level and overt DM, respectively, with an overall prevalence of fasting hyperglycemia of 17.8% (45). As high as 8% prevalence of DM has been reported among PLWH taking ART in Gondar, Ethiopia (46). Other community-based cross-sectional studies reported, based on the WHO criteria, that the prevalence of DM and IFG were 3.3% and 3.4%, respectively. The prevalence of GMDs, by adding the two prevalence figures, may at least reach 6.7% across the country (47).

Considering prevalence rates and the risk of glucose metabolism disorders, studies imply that the different classes of antiretrovirals exhibit differences in such incidence and risk of metabolic abnormalities. The sections that follow are devoted to exploring what the literature captured primarily about specific classes of antiretrovirals on which our study focused.

i) NNRTIs

There are sparse and inconsistent data regarding the involvement of NNRTIs in the development of glucose homeostasis alterations and IR. However, recent evidence implicated NNRTIs in disturbed glucose metabolism. These studies reported increased FBG and insulin levels as well as decreased insulin sensitivity with NNRTI-based regimens, particularly with EFV (48,49). A cross-sectional observational study in South Africa also found a significantly higher prevalence of DM or pre-diabetes status among patients receiving EFV compared to nevirapine (73% vs 27%, $p<0.001$), with adjusted covariates including age, CD4 count, and body composition (50). Interestingly, a study conducted in Addis Ababa (Ethiopia) reported elevated FBG levels in EFV-treated groups compared to NVP and LPV/r-treated patients (51).

ii) Protease Inhibitors

Various reports confirmed the association of GMDs with PI use (41,42). PIs may cause abnormal glucose metabolism ranging from IR, IGT, and IFG to T2DM. The PIs increase IR through the hindrance of glucose transport by inhibiting the major glucose transporter, GLUT4 (52). Sequences within the common peptidomimetic core of PIs bind to this transporter (53). Although the newer PIs (amprenavir and atazanavir) are less associated with metabolic and DM-associated adverse effects, indinavir and ritonavir are highly associated with these effects (54). A cross-sectional study reported a five to nine-fold elevated prevalence of T2DM among HIV-infected patients with PIs (23). Similarly, another study that evaluated glucose handling and other factors in Thai adolescents on PI-based ART reported about 22.1% and 3.8% prediabetes and overt T2DM states, respectively.

1.3.3 Mechanism of ART-induced Glucose Metabolism Disorders

In PLWH on ART, the cause of glucose-related abnormalities appears to be multifactorial. Potential mechanisms suggested could be either by direct effects of ARV drugs impairing cellular glucose uptake, or indirectly by altering body fat in the form of induced central obesity and/or peripheral lipoatrophy (55). Interference with glucose uptake, IR, and beta-cell dysfunction are commonly cited glucose-handling disorders during ART. Indeed, IR is described to be the most important pathway relative to beta-cell dysfunction (56,57).

Studies also link the occurrence of DM with an increased level of inflammatory markers typically after the initiation of ART. High-sensitivity C-reactive protein and soluble receptors of tumor necrotic factor (TNF)-α 1 and 2 are common inflammatory markers. Moreover, increased adiponectin and soluble-tumor necrotic factor-receptor-1 (sTNFR1)

and decreased levels of leptin have also been linked with glucose metabolic abnormalities among PLWH. The degree of HIV progression may also play a prominent role since interferon-α levels have been reported to be higher in patients with AIDS compared to healthy controls and HIV-infected controls (58).

In HIV-treated patients, the innate immune system and insulin signaling are integrated and toll-like receptors (TLRs), inducible nitric oxide synthase (iNOS), protein kinase R (PKR), c-Jun N-terminal kinases (JNK), and NF-κB are connected to the insulin receptor and its downstream signaling pathway (IRS/PI3K/Akt). Upon activation of the innate immune system, proteins involved in insulin signaling pathways become post-transcriptionally modified, resulting in reduced insulin action.

In addition, ARVs have been reported to induce IR and other metabolic disorders by altering muscle and adipose tissue, specifically via induced endoplasmic reticulum (ER) stress. Inhibition of mitochondrial synthesis, mitochondrial DNA release, and increased production of reactive oxygen species (ROS) are described as potential mechanisms of ARVs for IR (59). Genetic predisposition to mitochondrial dysfunction may also have contributed to IR and DM pathogenesis, placing people on ART at increased risk. Further, multiple polymorphisms are likely responsible for induced IR and GMDs among PLWH on ART (60).

i) Mechanism of Protease Inhibitors

PIs are linked with the incidence of IR and a few studies also report that they interfere with insulin secretion, specifically by interacting with GLUT-4-mediated glucose transport. The interaction of PIs with the cellular retinoic acid-binding protein type-1 (CRABP1) leads to the inhibition of the peroxisomal proliferator-activated receptor (PPAR)-γ and retinoid-X-

receptor (RXR) heterodimer. Such inhibition enhances adipocyte inflammation and apoptosis ultimately impairing triglyceride storage, the release of free fatty acids, and IR. PIs also interfere with the insulin signaling cascade at the insulin-receptor substrate (IRS)-1 phosphorylation level (55,57).

Mechanistically, PI-induced effects may depend on the duration of exposure. Short-term exposures appear to affect the glucose transport system while long-term is linked with the level of altered insulin signaling along with impaired glucose-stimulated insulin secretion (55). GLUT-4 is another target of ARVs, leading to glucose transport alteration (55,61). PIs like indinavir has been demonstrated to selectively inhibit GLUT-4 in vitro, and this is believed to be the principal mechanism involved in IR. Similar effects have been demonstrated in other PIs, such as saquinavir. Partial reversal of glucose metabolism abnormalities has been documented after the withdrawal of PIs, which leads to the assumption that PIs play a determinant role in IR (62,63).

However, these and other metabolic effects appear to be different among PIs. For instance, indinavir affects IR with no effect on lipid metabolism. Contrary to this, lopinavir and ritonavir increase fasting triglycerides and free fatty acids without a significant effect on insulin sensitivity. In addition, indinavir and ritonavir both interfere with GLUT-4, but no such effect is noted with amprenavir and atazanavir. A reduced beta-cell function is also implicated, as evidenced by about a 25% reduction in insulin release principally in the first phase, especially with nelfinavir, indinavir, lopinavir, or saquinavir therapy. All these differences imply that there is no class-specific mechanism of PIs on glucose and lipid abnormalities (57).

ii) *Mechanism of NNRTIs*

Relative to PIs, NNRTIs appear to induce fewer metabolic adverse effects. Their potential glucose-associated adverse effects and potential mechanisms responsible for GMDs have been less studied. Yet, available studies suggest that EFV has been reported to induce metabolic adverse effects among PLWH by different mechanisms, particularly by acting synergistically with PIs (61) and also NRTIs.

Several lines of evidence indicate that EFV is associated with increased apoptosis, mitochondrial toxicity, and oxidative stress in hepatic cells. Disrupted mitochondrial membrane potential was also reported, which promotes the release of cytochrome C and proapoptotic pathways (64). Moreover, EFV inhibits the electron transport chain (ETC) by altering complex I. It also amplifies the production of ROS and ultimately reduces ATP generation (65). Besides, it causes mitophagy (clearance of damaged mitochondria) due to decreased mitochondrial depolarization and altered mitochondrial morphology in neural cells. In general, mitochondrial toxicity and dysfunction are considered to cause inflammation and oxidative stress (64).

NNRTIs can decrease insulin sensitivity through their pro-inflammatory actions, contributing to IR. According to earlier research, NNRTIs stimulate the production of TNF-α, IL-6, and IL-1β, which results in lower adiponectin levels. The decrease in the insulin sensitivity modulator causes IR to progress. Adiponectin is linked with anti-diabetic, anti-atherosclerotic, and anti-inflammatory effects that slow the advancement of metabolic derangements. Therefore, inhibition causes increased lipid abnormalities and IR, which further culminate into T2DM and other metabolic disorders (65).

1.3.4 Role of pharmacokinetic factors in Glucose Metabolism Disorders

Plasma concentrations of ARVs are directly linked to drug response, efficacy, and adverse drug effects/toxicities. Specifically, treatment failure or side effects of ARVs are often dependent on plasma drug concentration and drug exposure. Pieces of evidence indicate that high drug levels of ARVs are reported as a determinant factor in the occurrence of adverse drug reactions (ADRs). Exclusively, inappropriately high drug concentrations of ARVs may lead to the advent of adverse events ultimately affecting treatment outcomes and quality of life (66).

The relationship between adverse events and plasma concentration of ARVs has been a subject of research interest. Several studies indicated that EFV plasma concentrations have been related to the occurrence of central nervous system (CNS) toxicities, including dizziness, abnormal dreams, and insomnia. It is also suggested that individual differences in drug disposition may be responsible for variability in drug concentrations that in turn results in varied drug responses or adverse effects. ART-associated toxicities, like other pharmacologic agents, may also be induced by augmented plasma drug concentration because of genetic susceptibility. Therefore, altered pharmacokinetics of ART and the resultant adverse drug effects or toxicities may complicate patients' quality of life, and treatment efficacy (67).

Glucose or carbohydrate metabolism disorders may have a relationship with pharmacokinetic factors that cause elevated ARV plasma concentrations among HIV-infected patients. Drug-metabolizing enzymes such as CYP2B6 has received much attention because of their ubiquitous role in the metabolism of ARVs and higher polymorphic genotypes. The most relevant drug in this respect in SSA is EFV and, yet, a

lot is to be done to translate the potential of EFV pharmacogenetics into clinical practice. Unfortunately, there is little or no study that measured and associated the plasma drug concentrations of ARVs with the occurrence of GMDs (68). Only one study from South Africa, which used a multivariate linear regression analysis with adjusted age, body mass index, and total duration on ART, reported a significant association between the log10 transformed EFV mid-dose concentrations and fasting glucose and 2-hour glucose concentration (69).

1.3.5 Role of Pharmacogenetic Factors in Glucose Metabolism Disorders

There are several risk factors associated with GMDs, particularly T2DM, including obesity, physical inactivity, family history, hypertension, and age. A family history of diabetes is associated with a 2 to 4 times greater chance of developing T2DM than unrelated people (70). This may imply that genetic variables have a significant role as risk factors. Indeed, the advent of pharmacogenomics has shown that a growing number of genetic variations and their interactions have a significant impact on the incidence, susceptibility, and management of GMDs, notably T2DM. One of the frequent types of genetic polymorphisms, known as single nucleotide polymorphisms (SNPs), affects the expression of genes associated with glucose metabolism, which has an impact on the incidence of GMDs.

Several genes have been identified as potential risk factors for GMDs (T2DM). Some of the discovered genes of significance in the development of T2DM include AMP-activated protein kinase subunit alpha 2 (PRKAA2) and ABCB1. It has been observed that GCK (Glucokinase) variant mutations and SNPs have been linked to T2DM susceptibility, particularly in populations from China, India, the Netherlands, France, and Japan.

Moreover, it has been demonstrated that T2DM is significantly associated with the glucokinase regulator (GCKR) gene, which codes for a protein from the sugar isomerase family proteins', GCKR subfamily (71).

Some SNPs in the adiponectin gene are also directly linked to Type 1 DM (T1DM), T2DM, and gestational diabetes in pregnant women. T1DM is associated with two major SNPs, rs2241766 and rs1501299, which have a strong connection to gestational diabetes (72). Resistin, a unique signaling molecule secreted from adipocytes, has been linked with IR and the incidence of DM. Published reports implicated SNPs of the gene encoding *resistin*, specifically *RETG +299(G>A)*, in T2DM (73–75).

Genetic changes in a critical gene encoding PPAR-γ that causes IR and lipodystrophy have been related to diabetogenesis (76). As a risk factor for T1DM, genes that affect β-cell function have also been discovered (77). Insulin secretion is dependent on the glucose-induced blockage of an ATP-sensitive potassium channel (KATP). The KATP has two subunits, the sulfonylurea receptor and the ATP-sensitive potassium channel subunits (Kir 6.2), encoded by ABCC8 and KCNJ11 genes, respectively. Due to decreased insulin production, E23K (rs5219) polymorphisms in the ATP-sensitive potassium channel Kir 6.2 (KCNJ11) are invariably linked to an increased risk of T2DM. In addition, insulin and KCNJ11 gene alterations are linked to persistent children/adolescents T2DM (78). Taken together, it appears that many SNPs rather than a single mutation may be a risk factor for diabetes (79).

Pharmacogenetic factors related to EFV and ATV/r

It is suggested that host genetic predisposition may be associated with IR and DM among HIV-infected patients on cART. Modified by genetic susceptibility, the prevalence of DM in PLWH expectedly could vary from one population to the other. This could be explained by the probable contribution of ethnicity-associated genetic susceptibility to the development of diabetes (80). Concerning DM, some common SNPs have been reported. However, studies measuring hyperglycemia and IR as primary end-point are quite scarce, as most of them focus primarily on lipodystrophy and hyperlipidemia (81). Several studies evaluated genes encoding for proteins involved in adipocyte and lipid metabolism such as PPARγ (82,83) and mitochondrial DNA (mtDNA) (84). However, none of these studies found significant associations between genetic variants and GMDs attributed to cART. Thus, only a few studies reported a significant association between the specific polymorphism and the type of GMDs. For example, *resistin* gene polymorphism has been linked with an altered metabolic profile on cART, which included elevated glucose and increased IR (85,86). Another study also revealed that the A allele of Retinol-binding protein 4 (RBP4) −803GA polymorphism is associated with IR in HIV-infected patients receiving HAART (82). Concerning the association between GMDs and polymorphisms of genes important in EFV or ATV/r metabolism, there exists little coverage in the literature. Only one study assessed the effect of *CYP2B6*6* variants on fasting glucose and 2-h glucose concentration among patients on EFV-based regimens. Despite elevated plasma EFV concentration, no significant association was found between *CYP2B6*6* variants and fasting glucose and 2-h glucose concentration (69). Unlike other PIs, ATV is characterized by a relatively lesser metabolic adverse effect. However, data regarding the

role of ATV pharmacogenetics in the incidence of metabolic adverse effects, specifically GMDs, are unavailable to date.

ARVs may be associated with GMDs, probably as a result of adverse metabolic effects, especially in individuals with genetic polymorphisms, affecting their disposition. There is a paucity of data concerning susceptibility to GMDs linked to adverse effects or toxicities, particularly aggravated by genetic factors, in PLWH on long-term cART. The impact of such SNPs on the incidence of GMDs is not fully understood. However, the role of pharmacogenomics in ART is increasingly recognized and considered to be important. Several lines of evidence have been generated showing that genetic variations affect ART and treatment outcomes.

Due to certain genetic variations that impair the activity of CYP2B6, an enzyme involved in the metabolism of EFV, patients in SSA receiving ARV regimens including EFV were more likely to have EFV-induced neuropsychiatric adverse effects (87–90). Indeed, there is a body of evidence linking increased plasma EFV exposure and the CYP2B6 deficient variant allele to abnormalities in liver enzymes (89,91,92) and neuropsychological manifestations (90). But, when the EFV doses in ARV regimens were further adjusted and decreased, there was an enhanced EFV metabolism, which resulted in much lesser neuropsychiatric side effects (93,94).

SNPs of *CYP*, *UGT*, and *ABCB1* genes, which are essential in ATV disposition (95), are associated with variable plasma concentrations, thereby altering the incidence of ADRs of ATV-based regimens. The best example indeed is the association of hyperbilirubinemia to polymorphism of *ABCB1* (*3435 C> T*) among patients on ATV-based therapies (96). The fact that drug-specific metabolic disturbances, including GMDs, or adverse effects do not

arise in all of the individuals on specific ARV, including NNRTI or PI, may imply the involvement of genetic or other factors (97). Thus, pharmacogenetic variation in relevant EFV and ATV/r metabolizing enzymes and transporter proteins may be linked with metabolic derangements and result in clinical conditions, including treatment failure and/or treatment-associated adverse events.

1.4 Significance of the study

Pharmacogenetic research is vital to improve patient care by implementing optimized pharmacotherapy, which enables improved patient care, reduced adverse drug events, and increased treatment outcomes through personalized medicine. Such outcome is dependent on patients' genetic backgrounds and meaningful therapeutic recommendations on time. Therefore, to achieve such goals in precision medicine, pharmacogenetic research and evidence generation is of paramount importance. Since there is a growing body of evidence of increased adverse reactions in patient populations of African ethnicity taking ARVs, pharmacogenetic and precision medicine researches are highly important in guiding ARV therapy.

This study could generate evidence regarding the long-term cART and the incidence of GMDs. Thus, it will help identify pharmacokinetic and pharmacogenetic factors related to GMDs in the long-term use of EFV and ATV/r-based cARTs. The evidence generated from this study may also have use in setting directions to minimize ARV-related GMDs through evidence-based drug selection and follow-up. Moreover, it can indicate interventions to minimize the risk of co-morbid non-communicable disease among PLWH on ART that may reduce health care costs in the long run. Expectedly, the findings of this research will

provide further pharmacokinetic and pharmacogenetic evidence to the existing body of knowledge and may also encourage other researchers to do further investigations in the area.

1.5 Hypothesis and Research Questions

We hypothesized that long-term EFV and ATV/r-based cART are associated with a higher incidence of GMDs, and pharmacokinetic (higher plasma concentrations) and pharmacogenetic factors (SNPs important in the disposition of EFV and ATV/r) could contribute to the increased incidence.

Based on the hypothesis, the following research questions were formulated:

- Are long-term EFV and ARV/r-based cART associated with the incidence of GMDs?
- What are the predictors of GMDs among PLWH on long-term EFV and ATV/r-based cART as a whole and specific to the individual regimen category?
- Can pharmacogenetic variations in genes involved in the disposition of EFV and ATV/r explain the increased incidence of GMDs among PLWH on long-term cART?
- Do the pharmacokinetic factors, such as mid-dose plasma concentrations or concentration categories (supratherapeutic, therapeutic, or subtherapeutic plasma levels) of EFV or ATV/r affect the incidence of GMDs among PLWH on long-term cART?

CHAPTER 2. OBJECTIVES

2.1. General Objective

To investigate the association of polymorphisms of drug-metabolizing enzymes and transporters important in the disposition of EFV and ATV/r (pharmacogenetic) and the influence of mid-dose plasma concentrations with the occurrence of glucose metabolism disorders among people living with HIV who were receiving EFV and ATV/r-based cART.

2.2. Specific Objectives

- To determine the prevalence and predictors of Glucose Metabolism Disorders among HIV patients on EFV- and ATV/r-based cART.
- To determine the association of polymorphisms of *CYP3A* (*4*1B*, *5*3*, and *5*6*), *CYP2B6*6*, *UGT2B7*, *ABCB1* (rs 3435 and rs 4036), and *SLCO1B1* (**1* and **5*) with the occurrence of GMDs among people living with HIV patients who were on EFV- and ATV/r-based cART
- To determine the association of mid-dose plasma concentrations of EFV, ATV, and RTV with the occurrence of Glucose Metabolism Disorders among people living with HIV who were receiving EFV- and ATV/r-based cART
- To evaluate the association of polymorphisms of *CYP3A* (*4*1B*, *5*3*, and *5*6*), *CYP2B6*6*, *UGT2B7*, *ABCB1* (rs 3435 and rs 4036), and *SLCO1B1* (**1* and **5*) and plasma mid-dose concentrations of EFV, ATV, and RTV with the occurrence of glucose metabolism disorder among people living with HIV who were receiving EFV- and ATV/r-based cART.

CHAPTER 3. METHODS

3.1. Study Design and Period

The study was a facility-based prospective cross-sectional study, which was carried out from August 2019 to March 2020, (Paper I) and followed by a comparative case-control sub-group analysis (Paper II-V) (Figure 1). For the case-control sub-group comparative study, case and controls were defined as cases were those individuals with at least one diagnosis of IR, IFG, or DM based on the cut-off values of fasting blood glucose (FBG), Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), or serum insulin level as stated elsewhere (98). Whereas, controls were those individuals with normal FBG, HOMA-IR, and fasting serum insulin levels as stated in Paper I. There was no significant difference in variables such as age, cART duration of therapy (previous and current length of exposure), weight, waist circumference, and body mass index (BMI) between the case and the control sub-groups.

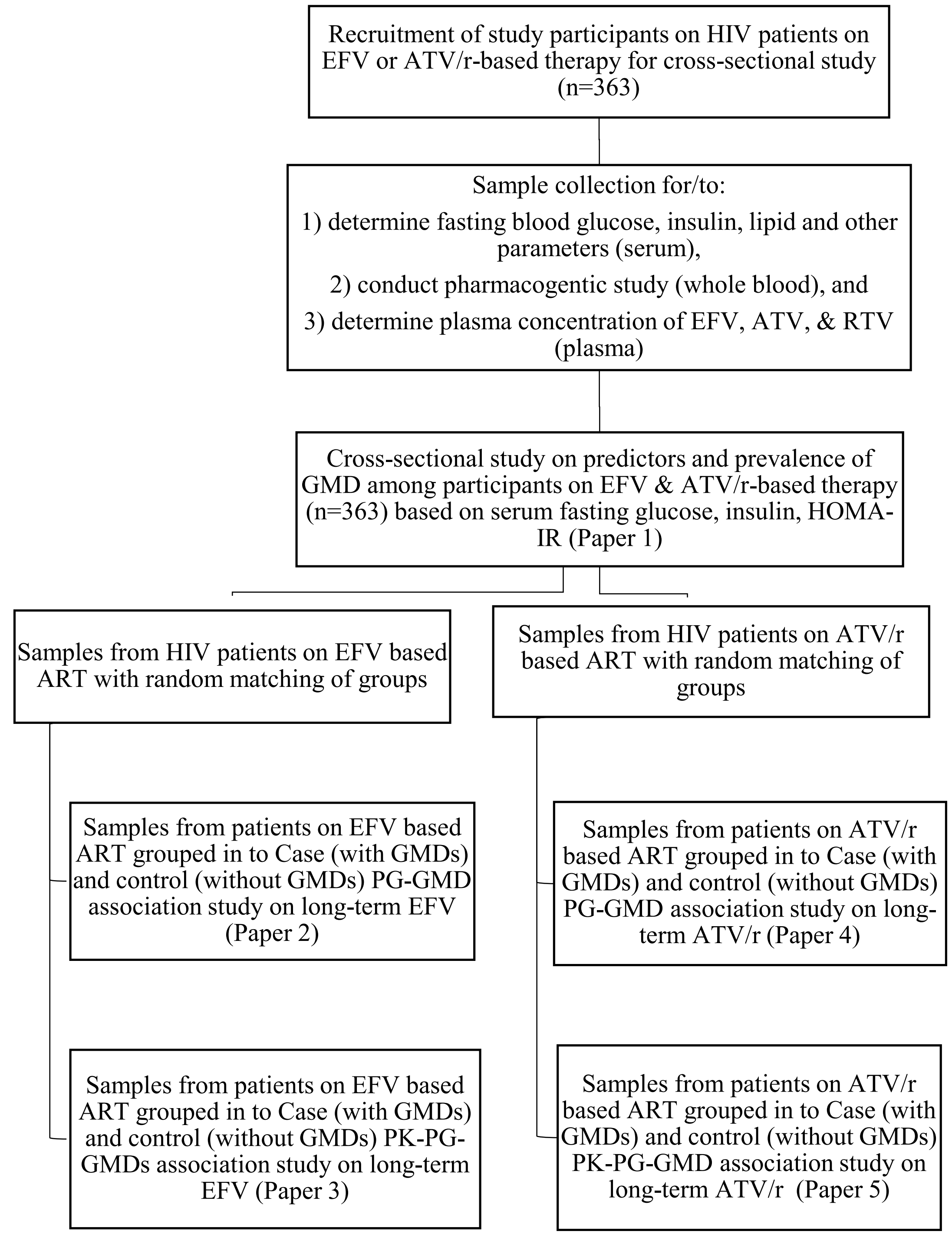

Figure 1 Flowchart describing the study design, grouping, and respective specific objectives with publication outputs of the study.

3.2. Study Setting

The study was conducted at the ART clinic of Tikur Anbessa Specialized Hospital (TASH), College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia. TASH is the largest tertiary-level teaching and referral hospital in Ethiopia. This setting regularly receives referred patients from different parts of the country. With more than 800 beds, it provides various specialized clinical services for more than 500,000 patients each year (99). The ART clinic at TASH provides HIV/AIDS prevention, patient care, and ART services. During the study period, approximately 4,000 registered follow-up patients were attending ART service at the clinic, comprising a mix of professionals ranging from senior clinical specialists, residents, general practitioners, and registered nurses to pharmacists.

3.3. Source and Study Population

All adult PLWH on EFV- or ATV/r- based ART attending the ART clinic of TASH formed the study population. Study participants were confirmed PLWH recruited based on the inclusion and exclusion criteria.

3.4. Inclusion and Exclusion Criteria

PLWH aged 18 years and above and on EFV or ATV/r-based cART at least for one year were included. Besides, the participant's willingness to participate in the study was also considered as an inclusion criterion. Patients known to have recorded DM prior to ART initiation, pregnancy, cancer, renal disease, liver disease, uncontrolled hypertension, and heart failure were excluded from the study. Moreover, patients on certain co-administered

medications such as antipsychotics, cancer chemotherapy, anti-TB, corticosteroids, hormonal agents, or antidiabetics were excluded.

3.5. Sample Size Determination and Sampling Techniques

The sample size was determined using single proportion formula for cross-sectional studies, with a qualitative variable (100). To estimate the specific prevalence for sample size calculation, the sum of the prevalence of IFG (pre-diabetes) and DM in PLWH on ART was considered from two local cross-sectional studies (44,101). Accordingly, a prevalence of 31.2% was calculated. In addition, 90% power, 95% confidence interval, and 0.05 level of significance was considered for sample calculation.

$$Sample\ size = \frac{{Z_{1-\alpha/2}}^{2} p(1-p)}{d^2}$$

Where: $Z_{(1-\alpha/2)}$ =standard normal variate at 5% type I error ($p<0.05$) =1.96,

d = absolute error values of 5%= 0.05, p = proportion of GMDs among HIV patients on ART =31.2%, form (44,101).

The calculated sample size needed for the cross-sectional study was therefore about 330 patients. Adding a 10% contingency for the probability of missing data, the total sample size reached 363.

The overall proportion of patients receiving EFV- and ATV/r-based cART during the study period was 69% and 31%, respectively. Accordingly, 251 participants were recruited from those on EFV-based cART and 112 from those on ATV/r-based cART. A convenient sampling technique was used to recruit study participants based on their consent and inclusion/exclusion criteria. The overall flow of the study was depicted in Figure 1.

For the case-control studies, all participants from the prevalence study were considered and grouped according to their GMDs status into case and control groups.

3.6. Interview and Medical Record Data Abstraction

Relevant data including socio-demographic and clinical characteristics; adherence based on a self-reported 3 days recall test, in which study participants were asked to report the number of doses they missed over the last three consecutive days before the sampling date (102–104); and anthropometric measurement was collected using a semi-structured interview questionnaire, patient medical charts, and prospective laboratory sample analysis. Waist circumference and body weight were measured with participants wearing light clothing and barefooted. Waist circumference was measured at the umbilical level to the nearest 0.1 cm using a tape measure. BMI was computed as weight divided by height squared (kg/m^2). Waist circumference and BMI were defined according to WHO recommendations (105).

Data were collected by two trained nurses and three pharmacists. Collected data were checked daily for accuracy and consistency by a team of experts. Moreover, the instrument was pre-tested in subjects that were not included in the actual study.

3.7. Blood Sample Collection

Blood samples were collected after overnight fasting (8 to 12 h) in 2 EDTA (2 ml each) and 1 SST tube (3 ml) 12 h after the last dose. The EDTA tubes were inverted 8-10 times for gentle mixing. Plasma was extracted from one of the EDTA tubes and immediately separated by centrifugation at 3,000 x g for 10 min. The other EDTA (whole blood sample)

was saved for DNA extraction for a pharmacogenetic study. Both plasma and whole blood samples were stored at -80 ºC before being transported in dry ice to the laboratory at Karolinska Institute, Stockholm, Sweden, until analysis.

Similarly, serum was separated from the SST tube immediately by centrifugation at 3,000 x g for 10 min, and FBG, insulin, lipid profiles, and renal and liver function tests were determined. HOMA-IR was calculated by using the following equation (106).

$$HOMA - IR = \frac{\left[Fasting\ insulin\ \left(\frac{\mu U}{mL}\right) X\ Fasting\ glucose\ \left(\frac{mmol}{L}\right)\right]}{22.5}$$

3.8. Genotyping

Whole blood samples were collected for genotype analysis in EDTA coated vacutainer tube (2 ml) and inverted 8–10 times for thorough mixing with EDTA. The samples were then transferred to cryotubes and stored at −80 °C until genotyping analysis. Genomic DNA was isolated from whole-blood samples using QIAamp DNA MidiKit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The purity and quantity of DNA were assessed using NanoDrop 2000 (Thermo Scientific, Saveen Warner, Sweden). Allelic discrimination assay was performed using TaqMan drug metabolism genotyping assay (Applied Biosystems, Foster City, CA, USA).

Functional variant alleles in enzymes and transport proteins relevant to the disposition of EFV and ATV/r-based cART were selected for genotyping (107,108). The variant alleles were *CYP3A4*1B*, *CYP3A5*3*, *CYP3A5*6*, *CYP2B6*6*, *UGT2B7*2*, *ABCB1* (*c.3435C>T*

and c.4036A>G), and SLCO1B1 (*1B and *5). Genotyping was performed using TaqMan® allele-specific PCR (Applied Biosystems Genotyping Assays) with the following ID numbers for the respective SNPs: C__7586657_20 for ABCB1c.3435C>T, C_11711730_20 for ABCB1c.4036A>G, C__7817765_60 for CYP2B6 c.516G>T (CYP2B6*6), C__30720663_20 for UGT2B7 −327G>A (UGT2B7*2b, *2c, *2d, *2f), C_9440184_20 for UGT2B15*4, C__26201809_30 for CYP3A5*3 (6986A>G), C__30203950_10 for CYP3A5*6 (14690G>A), C_1837671_50 for CYP3A4*1B, C_1901697_20 for SLCO1B1*1B, C_30633906_10 for SLCO1B1*5. The 7500 Real-Time PCR system (Applied Biosystems) was used for genotyping. The final volume was 10 µL for each reaction, consisting of 9 µL of TaqMan Universal PCR Master Mix® (Applied Biosystems, Waltham, MA, USA), DNA/RNA free water, TaqMan 20× drug-metabolism genotyping assay mix (Applied Biosystems), and 1 µL of genomic DNA. The PCR conditions were as follows: an initial step at 60 °C for 30 s, hold-stage at 95 °C for 10 min, and PCR stage for 40 cycles step 1 at 95 °C for 15 and step 2 at 60 °C for 1 min, and after read-stage with 60 °C for 30 s.

3.9.Quantification of Mid-dose Plasma Concentration

Plasma EFV, ATV, and RTV concentrations were determined by an LC-MS/MS system consisting of an ACQUITY Ultra Performance LC-system coupled to a Xevo TQ-S Micro (Waters, Milford, MA, USA). The positive ion mode for MS/MS analysis was selected, and data were processed by MassLynx 4.2 software (Waters). Fragment transitions of EFV and efavirenz-d4 (Toronto Research Chemicals, ON, Canada) were detected at m/z 316.15→168.15 and 320.05 →172.10, respectively. Similarly, ATV, atazanavir-d_5

(Toronto Research Chemicals, ON, Canada), RTV, and ritonavir-d_6 (Toronto Research Chemicals, ON, Canada) fragment transitions were detected at m/z 705.32 $\rightarrow$ 335.27, 710.45 $\rightarrow$ 340.25, 721.28 $\rightarrow$ 296.17, and 727.32 $\rightarrow$ 302.25, respectively. The chromatographic column consisted of ACQUITY UPLC BEH C18, 1.8 μ, 2.1 × 50 mm, reversed-phase column (Waters). LC-MS analytical grade acetone, acetonitrile, ammonium acetate, isopropanol, methanol, formic acid, and acetic acid were obtained from Merck (Darmstadt, Germany).

The mobile phase A and B were 0.1% aqueous formic acid and methanol, respectively. The initial composition of the mobile phase was 50% B, followed by a linear gradient to 99% in 1.5 min, with a flow rate of 0.4 mL/min. The chromatography run time was approximately 3 min for each sample. The lower limit of quantification (LLQ) was 15.8 ng/ml and the upper limit of quantification (ULQ) was 15.8 μg/ml for the three drugs.

Calibration samples were prepared by spiking blank plasma samples (Blood Bank of Karolinska University Hospital, Huddinge, Stockholm, Sweden) with reference standards of EFV (Merck, Darmstadt, Germany), ATV, and RTV (Sigma-Aldrich, Saint Louis, MO, USA) included in each analytical run. The lower, middle, and higher concentration quality control samples were also prepared by spiking in blank plasma. Each plasma sample (50 μL) was precipitated in acetonitrile solution (200 μL), which contains the internal standard [500 ng/mL of efavirenz-d4, atazanavir-d_5, and ritonavir-d_6 in methanol]. Then, 5 μL of the supernatant was injected into the LC-MS system, after 30 s vortexing and centrifugation (2100x g for 5 min), to determine plasma EFV, ATV, and RTV concentration.

The method was calibrated at each analysis. The calibration curve was calculated with linear regression based on the analyte/internal standard area ratios, weighted as 1/x. The method validation fulfilled the European Medicines Agency Guideline on bioanalytical criteria. The accuracy range was ±10% throughout the quantification range, and precision was <6% of the coefficient of variation (CV) except for LLQ (below 10 CV%). Log10 transformed EFV, ATV, and RTV plasma concentration values were used for statistical analysis.

3.10. Data Analysis and Interpretation

Data were sorted and entered as codes suitable for Statistical Package for Social Sciences (SPSS) statistical software version 25. Socio-demographic, anthropometric, and clinical as well as laboratory results were presented using descriptive statistics (frequency, mean, median, interquartile range). Continuous variables were reported as mean ± standard error of the mean (mean ± SEM), while categorical variables were presented as percent proportions. HOMA-IR was calculated, as stated in section 3.7, to determine IR using FBG level and insulin concentrations. The following definitions were used for data interpretation and analysis:

- DM was defined as a fasting glucose level of 126 mg/dL or higher (109).
- IFG was defined as a fasting glucose level between 110 and 125 mg/dL (109).
- Normoglycemia was defined as a fasting serum glucose level between 70 and 109 mg/dL (109,110).

- IR was diagnosed by either a Homeostasis model assessment insulin resistance (HOMA-IR) value of ≥3.8, fasting plasma insulin of ≥20 μU/ml, or fasting glucose/insulin ratio of ≥4.5 (111,112).
- GMDs were defined as the presence of IFG, IR, or DM (18,113).

For genetic variants, Hardy–Weinberg equilibrium was assessed by the chi-square test for each SNP between cases and controls. Haploview version 4.2 was employed to determine the association and case-control analysis of genotypes and haplotypes. The effect of EFV, ATV, and RTV CP12 was analyzed between cases and controls using chi-square association by considering their respective sub-therapeutic (<1000 ng/ml for EFV CP12 and <150 ng/ml for ATV CP12), therapeutic (1000-4000 ng/ml EFV CP12 and ATV CP12 150-850 ng/ml) and supratherapeutic plasma levels (≥ 4000 ng/ml for EFV CP12 and >850 ng/ml for ATV CP12). The mid-dose plasma (CP12) level of EFV, ATV, and RTV was analyzed between cases and controls among alleles of the different genotypes.

A stepwise multivariate logistic regression analysis was performed to determine independent predictors of the primary outcomes. Variables with univariate analysis of $p<0.2$ were entered in the stepwise multivariate analysis. Clinical, organ function, and genetic markers were also considered in the linear regression analysis against the log-transformed EFV, ATV, and RTV plasma concentration. In all analyses, p-values were two-sided, and p-values of < 0.05 were considered statistically significant. The regression coefficient (β), Crude odds ratio (COR), and adjusted odds ratio (AOR) were recorded from univariate and multivariate logistic regression.

3.11. Ethical Consideration

Ethical clearance was obtained from the Institutional Review Board of the College of Health Sciences, Addis Ababa University (Protocol No. 019/19/SoP) and National Ethical Review Committee, Ministry of Science and Higher Education, Addis Ababa, Ethiopia (Ref. No. MoSHE/RD14.1/9324/20). Written informed consent was obtained from each study participant after a full explanation of the purpose and the procedures. All participants were informed about their right to withdraw from the study. Study participants were also briefed that collected data and results would be kept confidential, coded, and no identifiers would be used in any form or publication. All data were kept safely in a locked cabinet and analyzed anonymously. In cases of severe abnormal values, participants were contacted through the HIV clinic and referred for a timely clinical evaluation and follow-up at the same hospital.

CHAPTER 4. RESULTS

4.1. Prevalence and Predictors of Glucose Metabolism Disorders

The baseline characteristics of study participants (n=351) are summarized in Table 1 (Paper I). A large proportion of the participants (68.4%) were on EFV-based 1st line cART. The EFV group had a significantly longer cumulative time on cART since initiation (123.9±2.9 months) compared with the ATV/r-group (112.0±4.3 months). No apparent difference was observed between the two groups for serum insulin and HOMA-IR. In contrast, the EFV-based group showed significantly higher FBG (p=0.018) than the ATV/r-based group.

The overall prevalence of detected GMDs was 27.6%, with IFG, DM, and IR accounting for 12.8%, 5.7%, and 14.8 %, respectively (Paper I). The incidence of GMDs was significantly higher ($p<0.05$) in the EFV-based (31.3%) than in ATV/r based (19.8%) group. IFG contributed to this difference (15.4% vs. 7.2%, $p<0.05$), as there were no detectable differences in DM and IR between the two groups.

Multivariate logistic regression analysis revealed that age ≥46 years old, male gender, history of comorbid conditions, and serum triglycerides level were found to be independent predictors of GMDs (Paper I). Participants with these characteristics had a 2 to 5 times more likelihood to develop GMDs. On the other hand, a significantly lower risk of developing GMDs was observed in participants with a higher cumulative duration on ATV/r-based second-line cART and waist circumference.

4.2. Pharmacogenetic Factors and Glucose Metabolism Disorders

4.2.1. EFV-based cART

A total of 240 participants on long-term EFV-based ART regimens with complete genotyping and GMD data were included in the case-control study. Cases and controls were comparable in most of the sociodemographic, clinical, and biochemical variables (Table 1, Paper II). All genotype frequencies were in accord with Hardy–Weinberg equilibrium (HWE) ($p > 0.05$). A comparison of genotype and allele frequencies revealed that the *CYP3A5*6* variant was significantly higher among cases than controls (0.41 versus 0.24, $p = 0.005$) (Paper II). Linkage among the three *CYP3A* valiant alleles was found with a relatively stronger linkage disequilibrium observed between *CYP3A4*1B* and *CYP3A5*3* (Paper II). The major *CYP3A* haplotype was *CYP3A5*3* alone (53.8%), followed by *CYP3A4*1B* alone (17.3%), and *CYP3A5*1B* linkage with *CYP3A5*6* (10.9%).

The Haploview case-control analysis also demonstrated that whilst the *CYP3A5*6* allele was significantly associated with IFG, IR, and overall GMDs; the *CYP2B6*6* allele was associated only with DM (Paper II). Multivariate analysis revealed that the carriers of *3A4*1B* + *3A5*3* + *3A5*6 (*1B/*3/*6)* haplotype combination had about a two-fold increased risk of experiencing GMDs than those carrying the wildtype or *CYP3A5*3* alone haplotype combinations (overall $p = 0.04$). With regards to IFG and IR, the risk was 2.8- and 2.6-fold higher, respectively, among *3A4*1B* + *3A5*3* + *3A5*6* haplotype carriers than the wildtype or *CYP3A5*3*-alone haplotypes carriers ($p = 0.04$ and 0.05). DM, on the other hand, appeared to be influenced by *CYP2B6*.

Carriers of *CYP2B6*6* alleles were at a significantly higher risk of DM by 4-fold than the wild-type carriers (*p* = 0.03).

4.2.2. ATV/r-based cART

A total of 111 participants were recruited for genotype analysis, of which about 73.9% (82/111) were females (Paper IV). With regards to the GMDs status, a significant mean difference (p<0.001) between biochemical markers of GMDs between controls and cases was observed. All genotype frequencies were as per HWE (p>0.05) in cases and controls. The **3* alleles of *CYP3A5*3* c.6986A>G showed a significantly higher frequency among controls (0.91) than cases (0.73) (p=0.02). The major *CYP3A* haplotype was *CYP3A5*3* alone (49.1%), followed by *CYP3A4*1B* alone (19.2%), and then by **1B* linkage with **3* (9.8%) (Paper IV).

The main finding of this study was that, firstly, the SL*CO1B1*5 c.521 T >C* genotype significantly predicted GMDs, and the C allele carriers demonstrated a 2.9-fold higher risk of GMDs [AOR=2.9; 95% CI 1.03-8.1, p=0.04] than the wildtype allele carriers. Secondly, the case-control and multivariate analyses showed that carriers of the wild type were at higher risk of IR than any **6* or *3-only haplotype combinations of *CYP3A*. Participants with any **6* and only **3* containing haplotypes of *CYP3A* had a significantly reduced risk for GMDs by 80.0% (p=0.03) and 90.0% (p=0.01), respectively than the wildtype combination haplotypes. Moreover, the Haploview analysis indicated that the **1B*-alone haplotype was significantly associated with IR (p=0.03).

4.3. Mid-dose Plasma Concentration and Glucose Metabolism Disorders

4.3.1. EFV-based cART

Plasma samples (n=240) were analyzed for CP12 EFV concentration in cases (n=75) and controls (n=165). Of these, 6 samples (2 from cases and 4 from controls) were not analyzable. Thus, a total of 234 PLWH on EFV-based regimens consisting of 73 cases and 161 controls (163 females and 71 males) were included in the statistical analysis. The detailed sociodemographic and clinical characteristics of the study participants are described in Table 1 (Paper III).

The Log CP12 EFV concentrations for controls and cases were 3.5±0.02 and 3.6±0.04, respectively, and no significant difference (p=0.14) was observed between the two groups (Paper III). However, when the geometric means were analyzed by an independent t-test, a significant difference (p=0.03) was observed between controls (3829.8±230.5 ng/ml) and cases (4934.8±536 ng/ml). Based on concentration ranges, 3.0%, 65.4%, and 31.6% of subjects had sub-therapeutic, therapeutic, and supratherapeutic plasma EFV concentrations, respectively.

The proportion of participants with CP12 above the recommended level (log EFV CP12 ≥3.6 or geometric mean >4000 ng/ml) was higher in cases (38.4%) than in controls (28.6%), though not statistically significant (p=0.14). However, looking at the specific traits and mean log EFV CP12, IFG cases demonstrated a significantly higher (p=0.005) log CP12 mean difference (3.62±0.05, geometric mean=4168.9 ng/ml) than controls (3.50±0.03, geometric mean=3162.3 ng/ml), (Figure 1, Paper

III). Cases were also compared using the proportion of cases with slightly increased CP12 level above the recommended level. Accordingly, no apparent difference was observed in the proportion of participants having CP12 < log 3.6 (4000) ng/ml and ≥ log 3.6 (4000) ng/ml in both overall GMDs as well as specific GMDs. Increasing the recommended level to log 3.69 (5000) ng/ml, however, produced a significant difference in the proportion of almost all cases but IR (Figure 2, Paper III). This indicates that a slightly higher concentration achieved above the recommended level could increase the risk of developing GMDs.

Multivariate analysis of pharmacokinetic factors (Paper III) of EFV revealed that gender, triglyceride level, and EFV log CP12≥3.69 ng/ml were independent predictors of GMDs among patients on EFV-based cART (Table 2, Paper III). Patients with EFV Log CP12≥3.7 ng/ml showed a 2.2 times higher risk of GMDs than EFV CP12 below 3.7 ng/ml (AOR=2.2, 95% CI 1.1-4.6, p=0.03). Increasing the cut-off to CP12≥4.0 (geometric ≥10,000) ng/ml doubled the risk of GMDs (β=1.4, AOR=4.2, 95%CI 1.4-13.0, p=0.01).

The EFV log CP12 was significantly higher in both cases (p=0.002) and controls (p=0.000) carrying *CYP2B6*6* compared to their corresponding wildtype allele (Paper III). Among the overall study participants, the multivariate analysis identified that age (β=0.005, 95%CI 0.001-0.008, p<0.01) and *CYP2B6*6* (β=0.215, 95%CI 0.143-0.288, p<0.001) were positively associated, while participants' weight (β=-0.003, 95%CI -0.006-0.000, p=0.03) was negatively associated with CP12 of EFV.

4.3.2. ATV/r-based cART

A total of 111 subjects, 89 controls, and 22 cases, were recruited and plasma samples were collected for determination of mid-dose plasma concentration (CP12) of ATV and RTV. However, 90 study subjects (74 controls and 16 cases) were included (66 females and 24 males) in the statistical analysis after meeting concentration requirements according to our method (Paper V). The sociodemographic characteristics, clinical characteristics, and allele frequencies of the study subjects are shown in Table 1 (Paper V).

The mean log CP12 of ATV was 3.24±0.04 ng/ml in controls and 3.52±0.06 ng/ml in cases (Table 1, Paper V). On the other hand, the mean log RTV CP12 was 2.26±0.07 ng/ml in controls and 2.54 ±0.1 ng/ml in cases. Only about 10% (9/90) of the subjects achieved the desired CP12 of ATV (150-850 ng/ml). About 1.1 % (1/90) of the study participants had CP12 ATV concentrations less than 150 ng/ml (the minimum goal), while 88.9% (80/90) had levels >850 ng/ml. No apparent difference (p=0.84) was observed in the proportion of cases (87.5%) and controls (89.2%) with ATV CP12 >850 ng/ml. The log CP12 of both ATV and RTV did not show a statistically significant relationship with any of the GMDs. Furthermore, regression analysis (Table 3, Paper V) revealed that CP12 of both ATV and RTV did not predict the occurrence of any of the GMDs.

On the other hand, the *CYP3A5*3* allele lowered the incidence of overall GMDs by 20% (AOR=0.2, 95%CI 0.03-0.9, p=0.04) and IR by 10% (AOR=0.1, 95%CI 0.02-0.9, p=0.04) than the wildtype allele in subjects on ATV/r-based cART. Similar to

findings of the genotype analysis, the C allele of *SLCO1B1*5* increased the incidence of overall GMDs by nearly nine-fold than the wildtype allele (AOR= 8.8, 95%CI 1.8-41.7, p=0.006) (Paper V).

Assessing factors, based on linear regression prediction model, BMI (p=0.036), creatinine serum level (p=0.014), direct bilirubin (p=0.022), HOMA-IR (p=0.026), *CYP3A5*3* (p=0.047), and *ABCB1 c.4036A>G* (p=0.019) alleles were positive predictors of ATV CP12 in the overall study participants (Paper V). In the case of CP12 of RTV, pharmacogenetic factors such as *CYP3A5*3* allele (p=0.047), *SLCO1B1*5* (p=0.013), and **1B* (p=0.045), and *ABCB1 c.4036A>G* (p=0.05) showed a positive association (Table 4, Paper V).

CHAPTER 5. DISCUSSION

This study is the first and unique in that it attempted to evaluate the pharmacogenetic and pharmacokinetic factors in the study of ART-induced GMDs. The prevalence study showed a relatively higher GMD incidence among participants on EFV-based cART than ATV/r-based cART. The prevalence obtained in the present study (27.6%) for the overall GMDs is similar to that reported for dysglycemia (26.0%) among PLWH on first-line ART by Levitt *et al.* (19). A Tanzanian study also reported a prevalence of GMDs (32.7%) among HIV-infected patients on ART (18), disregarding the methodological difference that this study did not consider a specific type of cART, unlike our study. However, our study reported a lower IFG (12.8% vs. 24%) but a higher DM prevalence (5.7% vs. 2%) than the South African study (69) among EFV-based cART-treated participants. These discrepancies could be due to the variations in methodology, study design, or inclusion of IR parameters, apart from IFG and DM, in the stated studies.

The pharmacogenetics of EFV and ATV/r might have a role in the incidence of GMDs in long-term cART, although it is difficult to suggest a direct possible mechanism. Supporting this notion, our study found that SNPs of EFV and ATV/r metabolizing enzyme and transporter genes demonstrated a significant association with the risk of GMDs. The association of the *CYP3A* haplotype combination, especially the *3A5*6* and *CYP3A4*1B* variants, and their co-occurrence as well as the strong tendency to predict the risk of IR, IFG, and GMDs among patients on EFV-based cART could be cited as evidence for the notion. A different pattern, however, emerged with ATV/r-

based cART. Here, *CYP3A4*1B* and haplotype combinations of *CYP3A* with any **6* and **3* significantly reduced the risk for IR or GMDs, unlike in the EFV-based cART group. Our study demonstrated that *CYP3A5*3* brought about a 20% risk reduction than the wildtype genotype among ATV/r receiving group.

Moreover, the *CYP2B6*6* genotype significantly predicted the incidence of DM only with EFV-based cART in both pharmacogenetic and pharmacokinetic studies. However, no such positive association was observed between *CYP3A* haplotypes, which are important in the metabolism of ATV/r, and GMDs or DM in ATV/r-based therapies. The fact that there is a consistent association between the risk of GMDs and variants of genes coding for drug-metabolizing enzymes in EFV-based but not in ATV/r-based therapy reinforces the notion that EFV is the likely culprit in inducing GMDs in PLWH. Indeed, several studies showed that the *CYP2B6*6* genotype determines interindividual variability of plasma EFV exposure, which could explain the difference in the incidence of metabolic adverse effects.

Data implicating pharmacogenetic factors as a risk factor for GMDs are sparse in the literature and those available are derived from non-ART-treated patients. A Japanese case-control study reported the implication of *CYP3A4* (*13989A>G*) polymorphism in the prevalence of T2DM (114). Another study also reported the association of *CYP3A4*18B* with the incidence of tacrolimus-induced new-onset diabetes while reporting no association of *CYP3A5* variants with new-onset T2DM in renal transplant recipients (115). Nevertheless, our findings, particularly related to EFV, showed a statistically significant association, which could stimulate active research

in the area. It is plausible to assume that the association of GMDs with these variants could be due to a defective enzyme function which might result in higher plasma drug exposure linked with the risk of GMDs. However, the exact molecular mechanism that links *CYP3A* and *CYP2B*6* variants to GMDs remains to be explored.

Only among ATV/r-based cART-receiving participants, the C allele (*SLCO1B1*5 c.521 T >C*) of *SLCO1B1,* a gene encoding for the uptake (influx) transporter OATP1B1, increased the risk of GMDs among cases in both the pharmacogenetics and plasma concentration of drug analysis. SNPs of both c.521 and c.388, particularly c.521, were reported to have a major effect on OATP1B1 activity by reducing the uptake transport of substrates to the liver (116). In addition, studies indicated that the C allele carriers of *SLCO1B1*5 c.521* had a significantly higher RTV intracellular concentration than the T allele carriers (117). Therefore, it is conceivable to assume that the increased risk of GMDs observed among the C allele carriers could be due to reduced activity of the *SLCO1B1*5 c.521* variant.

GMDs were observed in a higher proportion of individuals with supratherapeutic plasma levels among EFV- than ATV/r -based cART group. This finding is consistent with other findings showing that the incidence of adverse effects, especially the CNS side effects, are associated with supratherapeutic levels of EFV (118–120). In contrast, neither the log CP12 of ATV/RTV nor the ATV supratherapeutic concentration range was associated with the occurrence of GMDs. This finding is discordant with the belief that elevated ATV concentration may predict IR and GMDs. It also contradicts the notion that associates the incidence of

hyperbilirubinemia, as one of the major adverse effects, with elevated ATV concentrations. Published reports in the literature indicate that ATV is considered to have a safe metabolic profile with fewer effects on lipids and glucose (121), reinforcing our finding that ATV had no clinically significant effect on glucose metabolism and GMDs incidence.

Importantly, we demonstrated that PLWH with EFV log CP12 above 3.7 ng/ml (or above 5000 ng/ml) had a significant risk for GMDs. This suggests that EFV concentration higher than the classical supratherapeutic concentration (≥4000 ng/ml) might significantly contribute to the incidence of GMDs. The risk of GMDs also increased when the EFV log CP12 increased above 3.7 (5000) ng/ml. This might indicate that increased exposure and high plasma concentration of EFV could have a role in GMD incidence among PLWH on long-term EFV.

Growing evidence implicates EFV in energy metabolism, mitochondrial function, and other cellular processes involved in oxidative stress (122,123). EFV exposure lowers glucose uptake and inhibits SLC2A1, which mediates cellular glucose uptake (124). Several studies suggested that EFV-containing cART is linked with elevated blood glucose levels due to mitochondrial toxicity or IR (49,125,126). Several lines of recent evidence also implicated NNRTIs in disturbed glucose metabolism. For example, studies reported that increased fasting plasma glucose, insulin levels, and decreased insulin sensitivity were observed in NNRTI-based regimens, particularly with EFV (125–127). A study also showed that 8-Hydroxy-efavirenz, the primary metabolite of EFV, stimulates the glycolytic flux in cultured rat astrocytes (128). The

association of high plasma EFV concentrations with high plasma fasting lipid and glucose concentrations in South African HIV patients treated with EFV-based ART has been reported (129). Thus, long-term EFV-based cART-associated metabolic derangements, including GMDs, are likely due to mitochondrial toxicity (130) and could be aggravated by high plasma exposure.

CHAPTER 6. LIMITATIONS OF THE STUDY

Due to budget and resource constraints, we used a one-time sampling and follow-up for the diagnosis and measurement of lab parameters. Yet, the average value of two to three repeated test results was considered for analysis, which might offset the stated limitation, especially in the prevalence study. Despite the exclusion of participants with documented DM, our study design lacks mechanisms to exclude study participants who may have other GMDs because of lack of documented diagnosis; for instance, insulin measurements were quite uncommon to be conducted as a routine clinical parameter that prior insulin resistance indicators were never been recorded.

Moreover, our study did not assess the causal association between long term-cART and GMDs, as the study had no control groups and prior baseline data. One of the reasons for the lack of non-ART control groups was connected to the current ART initiation guideline that we could not find any ART naïve control groups. As to the lack of baseline data, the main problem was linked to medical records and data repositories due to an inefficient record system. As the best option, we could have followed our study participants prospectively taking the first findings as baseline data, however, such a way of design would require a large sum of budget and a long follow-up duration.

The study also shares the limitations emanating from the effect of the study format, as we used a single institution (due to budgetary constraints), a cross-sectional study, and consecutive sampling during recruitment (to accommodate a smooth and fast

recruitment process) for the prevalence study. Hence, the prevalence findings might not be extrapolated to the general PLWH receiving treatment in Ethiopia. Nevertheless, one should note that the study site is the largest referral hospital in Ethiopia, where patients from different parts of the country are referred to receive care.

Our study also focused only on genes important in the pharmacokinetics of EFV and ATV/r and did not consider phenotypic parameters such as detecting the level of EFV and ATV/r metabolites as these tests may incur more costs. Because of similar constraints, our study did not also consider other SNPs of genes directly involved in carbohydrate metabolism and insulin release. The time considered for the last dose sampling was based on individual participants' reports. However, most participants reported that they took their medications in the evening (20:00-22:00), which allowed a suitable sampling time the next morning. The case-control comparative studies involved a relatively small number of study subjects. However, the studies meet the minimum recommendation set for case-control studies i.e., at least an approximate case: control ratio of 1:4. Despite the limitations, our study generated multifaceted data correlating genetic and kinetic factors with clinical and sociodemographic variables. Therefore, it can be used as a baseline to conduct further studies with a larger sample size and more extended and repeated follow-up using comparative case-control, and prospective study design.

CHAPTER 7. CONCLUSIONS AND RECOMMENDATIONS

The prevalence of GMDs was higher among EFV than ATV/r-based cART-receiving participants. Pharmacogenetic and pharmacokinetic factors differentially predicted the risk for GMDs in the two groups. Genes linked to metabolic enzymes (*CYP3A* haplotype and the *CYP2B6*6*) positively predicted, while the *CYP3A* haplotype combination consisting of **6* and **3*-only of *CYP3A 4*1B/5*3/5*6* negatively predicted among HIV patients on long-term EFV- and ATV/r-based cART, respectively. On the other hand, variant alleles linked to transporter proteins (the C allele of *SLCO1B*5*) were identified as a positive predictor of GMDs among cases with ATV/r-based. Whereas, the mid-dose plasma concentration of EFV was found to be a positive predictor of GMDs in EFV.

Based on our findings, close monitoring for IFG during long-term EFV-based cART is recommended for early diagnosis of T2DM and its management. Further, plasma EFV concentration measurements and identifying carriers of *CYP3A* haplotypes and *CYP2B*6/*6* would significantly improve the early detection of GMDs and improve clinical decision and regimen switch for patients on long-term EFV-based cART. Our findings warrant the need for further investigation of the pharmacogenetic-pharmacokinetic risk factors of GMDs in both EFV and ATV/r-based cARTs prospectively and consisting of a larger sample size (case-control) to confirm our findings.

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APPENDEX

PAPER I

PLOS ONE

RESEARCH ARTICLE

Prevalence and predictors of glucose metabolism disorders among People Living with HIV on combination antiretroviral therapy

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Abstract

Objective

We investigated prevalence and predictors of glucose metabolism disorders (GMDs) among People Living with HIV (PLWH) on efavirenz- and atazanavir/ritonavir-based combination antiretroviral therapy (cART).

Methods

This cross-sectional study involved adult PLWH on efavirenz- (n = 240) and atazanavir/ritonavir-based (n = 111) cART. The prevalence of GMDs was determined by fasting serum glucose, insulin, and homeostasis model assessment. A logistic regression model was used to determine predictors.

Results

The overall prevalence of GMDs for all regimens was 27.6% (97/351) [95% CI 23.0–32.6%] s, with 31.1% (75/240) [95% CI 25.4–37.5%] for efavirenz-based and 19.8% (22/111) [95% CI 12.9–28.5%)] for atazanavir/ritonavir-based cART group. The prevalence of impaired fasting glycemia was significantly higher (p = 0.026) in the efavirenz- [(15.4%) (37/240); 95%CI (11.1–20.6%)] than atazanavir/ritonavir-based [(7.2%) (8/111), (95%CI (3.2–13.7%)] cART. However, no significant difference was observed in the prevalence of diabetes mellitus and insulin resistance between the two regimens. Age ≥46 years old and specific type of ARV contained in cART, such as TDF, were independent predictors of GMD in both groups. Whereas the male gender and BMI category were predictors of GMDs among EFV-based cART group, AZT- and ABC- containing regimens and triglyceride levels were predictors in the ATV/r-based group.

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Conclusions

GMDs were highly prevalent among adults on EFV- than ATV/r-based cARTs. Age ≥46 years and TDF-containing cARTs are common predictors in both regimens. Close monitoring for impaired fasting glucose during long-term EFV-based cART is recommended for early diagnosis of type-2 diabetes and management.

Introduction

HIV/AIDS has remained a public health problem in sub-Saharan Africa [1]. Over the last three decades, HIV-associated mortality and disease transmission rate has progressively declined, mainly because of the rapid expansion and availability of combination Antiretroviral Therapy (cART) [2]. The introduction of cART has changed the complexion of HIV infection from a deadly disease to a chronic manageable disorder that notably changed patients' quality of life and longevity [3]. It also changed the global epidemiology of transmission, morbidity, and mortality of HIV [2, 4].

Treatment of HIV is lifelong, embracing frequent clinical evaluation and follow-up [5]. During long-term exposure to antiretroviral therapy (ART), individual patients may experience treatment-associated adverse events or drug toxicities [5]. Long-term exposure to ART may increase the risk of metabolic abnormalities such as lactic acidosis, osteopenia, dyslipidemia, and glucose metabolism disorders (GMDs) [6]. GMDs are glucose homeostasis dysregulations that include diabetes mellitus (DM), impaired glucose tolerance (IGT), impaired fasting glycemia (IFG), or insulin resistance (IR) [7].

The literature indicates that the prevalence of IR, IGT, and DM has significantly increased and became a notable clinical concern, as long-term ART [8] and aging-related factors contribute to a higher risk of glucose metabolism abnormalities. DM is now emerging as one of the non-infectious comorbid conditions among People Living with HIV (PLWH) on cART [8]. Previous studies reported that patients on ART were found to be four-fold more prone to DM and associated conditions compared to HIV uninfected individuals [6, 7, 9–11]. Among cART classes, protease inhibitors (PI) use has been commonly reported to have association with GMDs. PIs may cause abnormal glucose metabolism, ranging from IR through IGT & IFG to type 2 diabetes [8, 12]. The PIs, particularly lopinavir and ritonavir, are linked to an increase in IR and to have effect on lipid and glucose metabolism [13, 14]. A cross-sectional study reported a five-to-nine-fold elevated prevalence of type 2 DM among PLWH on PIs [12]. Recently, a high prevalence of cART-associated dyslipidemia, particularly low High-Density Lipoprotein Cholesterol (HDL-c) and hypertriglyceridemia, has also been reported among treatment-experienced HIV-infected children from Ethiopia [15].

In a resource-limited setting, Efavirenz (EFV) and Atazanavir/Ritonavir (ATV/r) serve as a backbone of combination antiretroviral regimens [16]. In Ethiopia, during the study period, the preferred first-line regimen for adults was EFV-based cART, specifically a combination of tenofovir (TDF), lamivudine (3TC), and EFV [17]. ATV/r-based regimens were the mainstay of second-line cART replacing lopinavir/ritonavir (LPV/r) [17].

The magnitude of and risk factors for GMDs are well investigated in developed countries. Several studies from sub-Saharan African countries also reported a high prevalence of glucose-related abnormalities and risk factors. However, regimen-specific prevalence and predicting factors, particularly for EFV- and ATV/r-based regimens, are limited in Ethiopia. Principally, data are almost unavailable concerning glucose metabolism-related alterations of ATV/r-

based regimens, at least in Ethiopia. Moreover, data comparing the incidence of GMDs and the respective risk factors of Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs), including EFV, and new PIs, such as atazanavir (ATV) are sparse. Thus, this study would provide data and generate evidence for interested researchers and clinicians in sub-Saharan Africa in general and in Ethiopia in particular. Therefore, this study aimed to determine the prevalence and predicting factors of GMDs among PLWH on EFV- and ATV/ritonavir (ATV/r)-based cARTs.

Methods

Study design, population, and setting

This is an institution-based cross-sectional study conducted among treatment-experienced PLWH on EFV- or ATV/r-based cART. The study was conducted from August 2019 to March 2020 at the ART clinic of Tikur Anbessa Specialized Hospital (TASH), College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia. TASH is the largest tertiary level teaching and referral hospital in Ethiopia. This setting regularly receives referred patients from different parts of the country. With more than 800 beds, it provides various specialized clinical services for more than 500,000 patients each year [18]. The ART clinic at TASH provides HIV/AIDS prevention, patient care, and ART services.

All adult PLWH on EFV- or ATV/r- based ART attending at ART-clinic of TASH formed the study population. Study participants were confirmed PLWH recruited based on the inclusion and exclusion criteria.

Sample size determination and sampling techniques

The sample size was determined using single proportion formula for cross-sectional studies, with a qualitative variable [19]. To estimate the specific prevalence for sample size calculation, the sum of the prevalence of IFG (pre-diabetes) and DM in PLWH on ART were considered from two local cross-sectional studies [20, 21]. Accordingly, a prevalence of 31.2% was calculated. In addition, 90% power to detect a prevalence difference of 10% between the two groups, 95% confidence interval, and 0.05 level of significance was considered for sample size calculation using the single proportion formula.

The calculated sample size needed for the cross-sectional study was therefore about 330 patients. Adding a 10% contingency for the probability of missing data, the total sample size reached to 363.

The overall proportion of patients receiving EFV- and ATV/r-based cART during the study period was 69% and 31%, respectively. Accordingly, 251 participants were recruited from those on EFV-based cART and 112 from those on ATV/r-based cART. A convenient sampling technique was used to recruit study participants based on their consent and inclusion/exclusion criteria.

PLWH aged 18 years and above and on EFV or ATV/r-based cART at least for one year were included. Patients known to have DM, pregnancy, cancer, renal disease, liver disease, uncontrolled hypertension, and heart failure were excluded from the study. Moreover, patients on certain co-administered medications such as antipsychotics, cancer chemotherapy, anti-TB, corticosteroids, hormonal agents, or antidiabetics were excluded.

Data collection

Relevant data including socio-demographic, clinical characteristics, adherence based on self-reported 3 days recall test, in which study participants were asked to report the number of

doses they missed over the last three consecutive days prior to sampling date [22–24], and anthropometric measurement were collected using a semi-structured interview questionnaire, patient medical charts, and prospective laboratory sample analysis. Waist circumference and body weight were measured with participants wearing light clothing and barefooted. Waist circumference was measured at the umbilical level to the nearest 0.1 cm using a tape measure. BMI was computed as weight divided by height square (kg/m^2). Waist circumference and BMI were defined according to WHO recommendations [25].

Blood tests were performed after overnight fasting (8 to 12 h). About 5 ml blood was collected from the brachial artery in serum separator tubes and fasting blood glucose (FBG), insulin, and lipid profiles were determined.

Operational definitions

- DM was defined as a fasting glucose level of 126 mg/dL or higher [26].
- IFG was defined as a fasting glucose level between 110 and 125 mg/dL [26].
- Normoglycemia was defined as a fasting serum glucose level between 70 and 109 mg/dL [26, 27].
- Hyperglycemia was defined as a fasting glucose level of 110 mg/dL or higher [26, 27].
- IR was diagnosed by either Homeostasis model assessment insulin resistance (HOMA-IR) value of $\geq$3.8, fasting plasma insulin of $\geq$20 μU/ml, or fasting glucose/insulin ratio of $\geq$4.5 [28, 29].
- GMDs were defined as the presence of IFG, IR, or DM [7, 30].

Data management and analysis

Data were sorted and entered as codes suitable for Statistical Package for Social Science (SPSS) statistical software version 25. Socio-demographic, anthropometric, and clinical as well as laboratory results were presented using descriptive statistics (frequency, mean, median, interquartile range). Continuous variables were reported as mean ± standard error of the mean (SEM), while categorical variables were presented as percent proportions. HOMA-IR was calculated to determine IR using FBG level and insulin concentrations. HOMA-IR is given by the product of fasting insulin concentration (μU/ml) and fasting glucose (mmol/L) level divided by the constant normalizing factor, 22.5 [31].

To determine associations of variables against GMDs, univariate logistic regression analysis was performed for each socio-demographic, anthropometric, and clinical lab variables. Multivariate analysis was performed using backward-stepwise logistic regression analysis and independent predictors of the primary outcome were identified. Variables with a p-value less than 0.05 were considered statistically significant, while variables with p values less than 0.2 in univariate analysis were candidates for multivariate logistic regression analysis.

Ethical consideration

Ethical clearance was obtained from the Institutional Review Board of College of Health Sciences, Addis Ababa University (Protocol No. 019/19/SoP) and National Ethical Review Committee, Ministry of Science and Higher Education, Addis Ababa, Ethiopia (Ref. No. MoSHE/RD14.1/9324/20). Written informed consent was obtained from each study participant after a full explanation of the purpose and nature of all procedures used. In cases of severe abnormal

values, participants were contacted through the HIV clinic and referred for a timely clinical evaluation and follow-up at the same hospital.

Results

Baseline characteristics of study participants

Out of the 363 recruited study participants, 351 had complete clinical laboratory data for FBG fasting serum insulin, and HOMA-IR value, and thus considered for statistical analysis. As depicted in Table 1, there was a female preponderance in the study participants (70.4%), EFV- (68.8%), and ATV/r-based (73.9%) regimens. Majority of the participants (58.4%) belong to the age group of 18–45 years, with 54.6% and 66.7% in EFV- and ATV/r-based cART group, respectively. A large proportion of participants were non-smokers (99.1%), non-khat chewers (98.6%), or non-alcohol users (96.6%). Related to anthropometric characteristics, the overall mean (SEM) lean weight was 62.4 (0.71) kg, waist circumference was 34.11 (0.26) cm, and BMI was 24.0 (0.26) kg/m^2.

About 240 (68.4%) of the participants were on EFV-based 1st line cART (Table 1). The overall mean (±SEM) of baseline and latest CD4 counts were 206 ±18.3 and 464.6 ±13.6, respectively. Unlike the baseline (225.5±25.6 vs. 163.3±16.2), a significantly elevated latest (523.5 ±16.4 vs. 336.8±19.3) CD4 counts were observed in the EFV- than ATV/r-group. Based on the latest medical records, only 31 (8.8%) of the overall study participants experienced virologic failure, with viral loads of >1000 copies/ml, out of which a significant majority were on ATV/r-based cART (p = 0.000). The EFV group had a significantly longer cumulative time on cART since initiation (123.9±2.9 months) than the ATV/r-group (112.0±4.3 months) (Table 1). Whilst the cumulative time on EFV-based regimen was 101.4±2.8, it was 34.3±3.0 months for ATV/r-based cART. Participants on ATV/r-based cART were on 1st-line cART for 90.5±11.9 months, mainly on Nevirapine (NVP)-based for 33.4±4.3 months and later, on EFV-based regimens for 40.8±4.2 months.

Large majority of the participants (339, 96.6%) were adherent to their respective cART based on the self-reported three-day adherence test. Treatment adherence was higher among participants in ATV/r- (97.3%) than EFV-based groups (96.3%), though no statistically significant difference was found (p = 0.616). Based on the overall clinical lab analysis, the mean (SEM) FBG was 99.2 (1.5) mg/dL and serum insulin ranged from 0.46 to 160.5 μU/mL, with a mean (SEM) of 9.6 (0.6) (Table 1). Moreover, the mean (SEM) HOMA-IR value was found to be 2.5 (0.2), ranging from 0.14 to 57.39. Unlike fasting serum insulin and HOMA-IR values, the EFV-based group showed a significantly elevated FBG than ATV/r-based (p = 0.018). In general, the clinical lab values were relatively elevated in the EFV- than the ATV/r-based group, except for the triglyceride level. However, only LDL level showed a statistically significant elevation among EFV- than ATV/r-based cART receiving group (Table 1).

Prevalence of GMDs

The prevalence of GMDs is shown in Table 2. The overall prevalence of GMDs was found to be 27.6% (n = 97/351). Among the overall study participants, about 12.8% (45/351) were with impaired glycemia and 5.7% (20/351) with a diabetic range of fasting serum glucose. IR was detected in about 14.8% (n = 52/351) of the study participants. A significantly higher ($p<0.05$) prevalence of GMDs was found in patients taking EFV-based first-line therapy (31.3%) than ATV/r therapy (19.8%). Disaggregating the data revealed that only the prevalence of IFG was found to be significantly higher ($p<0.05$) in the EFV- (15.4%) than the ATV/r-based group (7.2%). Although the prevalence of DM and IR tended to be higher in the EFV group than the ATV/r group, it did not reach statistical significance (Table 2).

Table 1. Baseline characteristics of study participants on efavirenz-based or ritonavir-boosted combination antiretrovirals.

Variables	Categories	Overall	EFV-based	ATV/r-based	χ^2/F or t	p
		n (%)	n (%)	n (%)		
Age (years)	Median (IQR)	43.0 (37.0–50.0)	45.0 (38.0–52.0)	40.0 (33.0–48.0)	3.5	0.000
Age category	18≤45	205(58.4)	131 (54.6)	74 (66.7)	4.6	0.033
	≥46 years	146 (41.6)	109 (45.4)	37 (33.3)		
Gender	Female	247 (70.4)	165 (68.8)	82 (73.9)	1.0	0.328
	Male	104 (29.6)	75 (31.3)	29 (26.1)		
Marital status	Single	76 (21.7)	47 (19.6)	29 (26.1)	3.0	0.387
	Married	127 (36.1)	93 (38.8)	34 (30.6)		
	Widowed	86 (24.5)	59 (24.6)	27 (24.3)		
	Divorced	62 (17.7)	41 (17.1)	21 (18.9)		
Educational status	Up to primary	145 (41.3)	96 (40.0)	49 (44.1)	0.9	0.332
	Above primary	194 (55.3)	138 (57.5)	56 (50.5)		
Khat use (self-report)	Never	346 (98.6)	236 (98.3)	110 (99.1)	0.317	0.573
	Current or previous	5 (1.4)	4 (1.7)	1 (0.9)		
Smoking (self-report)	Never	348 (99.1)	238 (99.2)	110 (99.1)	0.004	0.949
	Current or previous	3 (0.9)	2 (0.8)	1 (0.9)		
Alcohol use (Self-report)	Never	339 (96.6)	231 (96.3)	108 (97.3)	0.252	0.616
	Current or previous	12 (3.4)	9 (3.8)	3 (2.7)		
Treatment adherence (3-day test)	Adhered	339 (96.6)	231 (96.3)	108 (97.3)	0.252	0.616
	Non-adhered	12 (3.4)	9 (3.8)	3 (2.7)		
BMI category	<18.5	40 (11.4)	25 (10.4)	15 (13.5)	5.4	0.143
	18.6–24.9	163 (46.4)	105 (43.8)	58 (52.3)		
	25–29.9	104 (29.6)	80 (33.3)	24 (21.6)		
	≥30	34 (9.7)	24 (10.0)	10 (9.0)		
Viral load status(n = 335)	≥1000 copies/ml	31 (8.8)	3 (1.3)	28 (25.2)	61.7	0.000
	<1000 copies/ml	304 (86.6)	225 (93.8)	79 (71.2)		
History of comorbidity (self-reported)	HIV-only	340 (96.9)	233 (97.1)	107 (96.4)	0.118	0.731
	HIV + comorbidity	11 (3.1)	7 (2.9)	4 (3.6)		
cART backbone-type		351 (100)	240 (68.4)	111 (31.6)		
Specific ARVs contained in cART	TDF containing	266 (75.8)	216 (90.0)	50 (45.0)	80.9	0.000
	AZT containing	71 (20.2)	24 (10.0)	47 (42.3)	45.3	0.000
	ABC containing	13 (3.7)	-	13 (11.7)	29.2	0.000
Time since HIV confirmed date (months) [δ]		131.14±2.6	134.5±3.1	123.9±4.9	1.9	0.062
Cumulative time on cART (month) [δ]		120.2±2.4	123.9±2.9	112.0±4.3	2.3	0.021
Cumulative time on EFV-based 1st-line (month) [δ]		82.3±2.7	101.4±2.8	40.8±4.2[#]	12.2	0.000
Cumulative time on ATV/r-based 2nd-line (month) [δ]		11.3±1.3	0.7±0.5[*]	34.3±3.0	115.1	0.000
Time on current cART regimen type (month) [δ]		74.6±2.5	93.7±2.7	33.1±2.6	16.2	0.000
Time on prior cART regimen types (month) [δ]		52.4±5.6	34.8±5.6	90.5±11.9[#]	349	0.000
Time on NVP-based 1st-line (Prior to EFV) (month) [δ]		25.4±2.1	21.7±2.4	33.4±4.3[#]	178.7	0.018
Time on LPV/r-based 2nd-line (prior to ATV/r) (month) [δ]		1.4±0.5	0.2±0.2[*]	4.1±1.6	112.6	0.018
Waist circumference (cm) [δ]		34.1±0.26	34.6±0.3	33.3±0.4	2.4	0.016
CD4+ (cells/ul) (baseline) [δ]		206.0±18.3	225.5±25.6	163.3±16.2	1.6	0.114
CD4+ (cells/ul) (recent) [δ]		464.6±13.6	523.5±16.4	336.8±19.3	7.4	0.000
Fasting glucose (mg/dL) [δ]		99.2±1.5	101.7±1.7	94.2±2.6	2.4	0.018
Fasting insulin (uU/ml) [δ]		9.6±0.6	10.2±0.8	8.4±0.6	1.4	0.150
HOMA-IR (μU/ml) [δ]		2.5±0.2	2.8±0.3	2.0±0.2	1.4	0.154
Total cholesterol(mg/dL) [δ]		200.8±6.9	208.5±9.9	184.2±4.1	1.6	0.103

(*Continued*)

Table 1. (Continued)

Variables	Categories	Overall	EFV-based	ATV/r-based	χ^2/F or t	p
		n (%)	n (%)	n (%)		
Triglyceride(mg/dL) $^{\delta}$		155.9±4.0	152.2±5.0	163.7±6.4	1.3	0.179
HDL-C(mg/dL) $^{\delta}$		45.3±1.7	46.3±1.8	43.1±3.6	0.9	0.370
LDL-C(mg/dL) $^{\delta}$		126.1±6.3	135.6±9.0	105.5±3.4	2.2	0.026

Values are frequencies or $^{\delta}$mean ± SEM (N = 351), TDF = Tenofovir, AZT = Zidovudine, ABC = Abacavir, HDL-C = High density lipoprotein Cholesterol, LDL-C = Low density Lipoprotein Cholesterol.

[#]Prior regimens in subsequent order of NVP-based, EFV-based, and LPV/r-based before switched to the current ATV/r-based 2nd-line cART.

[*]Few study participants had a switch to 2nd-line between initiation and current 1st-line cART. HIV Comorbidities refer to conditions such as dyslipidemia, hepatitis or asthma based on patients' report. χ^2 = chi-square test for categoric variables, F = F-test for categoric variables with 1 cell expected count <5 or t = independent t-test for continuous variables.

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We also tried to calculate the prevalence of these variables based on tenofovir (TDF)-, zidovudine (AZT)-, and abacavir (ABC)-containing combinations. The prevalence of GMDs was higher in participants taking AZT- (29.6%) than TDF- (27.3%) and ABC (15.4%)- containing combinations. Looking at the individual variables, the AZT-containing combination showed a relatively higher rate for IR (18.3%) and IFG (14%) than TDF-(13.9% for IR and 13.1% for IFG) and ABC- (7.7% for IR and 0% for IFG) containing combinations. By contrast, prevalence of DM was higher for ABC- (7.7%) than TDF- (5.6%), and AZT- (2.8%)-containing combinations.

Predictors of glucose metabolism disorders

Predictors for overall study participants. Univariate analysis revealed that BMI, serum level of triglycerides, age, gender, khat use, comorbid conditions, and history of hypertension were significantly associated with GMDs (Table 3). However, in multivariate logistic regression analysis, age ≥46 years old, male gender, history of comorbid conditions, and serum triglycerides level were found to be independent predictors of GMDs.

Patients with age ≥46 years [AOR = 2.1, 95% CI 1.2–3.6, p<0.01] and males had a two-fold risk of GMDs [AOR = 2.6, 95% CI 1.1–3.5, p<0.01]. Likewise, individuals with comorbid conditions had a nearly five-fold risk of GMDs [AOR = 4.7, 95% CI 1.3–18.9, p<0.05] than those without comorbid conditions. Serum level of triglycerides showed a statistically significant association with the incidence of GMDs. For each unit increase in the level of triglycerides, the likelihood to develop GMDs increased by 0.5% [AOR = 1.005, 95% CI 1.001–1.008), p<0.05].

On the other hand, cumulative time on ATV/r-based second-line cART conferred a significant protection from GMDs, *i.e.*, a 2% lower incidence of GMD was observed for each month stay on ATV/r-based cART (Table 3). Paradoxically, waist circumference was negatively

Table 2. Prevalence of glucose metabolism disorders relative to the overall and specific type of combination antiretroviral treatment category among the study participants.

Variables	Overall (N = 351)	EFV-Based cART (n = 240)	ATV/r-Based cART (n = 111)	χ^2	p
Impaired Fasting Glycemia	45 (12.8%)	37 (15.4%)	8 (7.2%)	5.0	0.026
Diabetes Mellitus	20 (5.7%)	17 (7.1%)	3 (2.7%)	1.5	0.318
Insulin Resistance	52 (14.8%)	36 (15%)	16 (14.4%)	0.1	0.748
Glucose Metabolism Disorders	97 (27.6%)	75 (31.3%)	22 (19.8%)	4.6	0.039

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Table 3. Predictors of glucose metabolism disorders determined by logistic regression analysis among all study participants (n-97).

Variables	Categories	Univariate		Multivariate	
		COR	P	AOR	P
Age category	≤45	1	-	1	0.006
	≥46	2.3 (1.4, 3.8)	0.000	2.1(1.2, 3.6)	
Gender	Female	1		1	0.001
	Male	3.2 (2.0, 5.3)	0.000	2.6 (1.4, 4.5)	
Educational status	Up to Primary	1	-		
	Above primary	1.6 (0.9, 2.5)	0.063		
Marital status	Single	1			
	Married	1.0 (0.6,2.0)	0.913		
	Widowed	0.9 (0.4,1.8)	0.768		
	Divorce	1.1 (0.5, 2.3)	0.856		
Ever khat use (self-report)	Never	1	0.034		
	Current or previous	10.9 (1.2, 98.6)			
Ever smoking (self-report)	Never	1	-		
	Current or previous	5.3 (0.5, 59.4)	0.174		
Ever alcohol use (Self-report)	Never	1	-		
	Current or previous	1.9 (0.6, 6.2)	0.276		
Treatment adherence (3-day test)	Non-adhered	1	-		
	Adhered	4.3 (0.5, 34.1)	0.162		
BMI category	≥30	1			
	<18.5	0.13 (0.04, 0.43)	0.001		
	18.6–24.9	0.5 (0.2, 1.0)	0.066		
	25–29.9	0.4 (0.2, 1.1)	0.074		
CD4 (n = 342) (recent)	<350	1			
	≥350	0.9 (0.6, 1.5)	0.762		
Viral load status(n = 335)	≥1000 copies/ml	1	-		
	<1000 copies/ml	1.3 (0.6, 3.2)	0.524		
History of comorbidity (self-reported)	HIV-only	1	-	1	0.027
	HIV + comorbidity	4.9 (1.4, 17.0)	0.013	4.7 (1.2, 18.4)	
cART backbone-type	ATV/r-based	1	-		
	EFV-Based	1.8 (1.1, 3.1)	0.027		
Specific ARVs contained in cART	TDF containing	0.9 (0.5, 1.6)	0.826		
	AZT containing	1.1 (0.6, 2.0)	0.682		
	ABC containing	0.5 (0.1, 2.1)	0.325		
Time since HIV confirmed date (months)		1.003(0.998, 1.008)	0.243		
Cumulative time on cART (month) $^{\delta}$		0.993 (0.992, 0.995)	0.0.00		
Cumulative time on EFV-based 1st-line (month) $^{\delta}$		0.993 (0.990, 0.995)	0.000		
Cumulative time on ATV/r-based 2nd-line (month) $^{\delta}$		0.966(0.952,0.980)	0.000	0.98(0.96,0.99)	0.011
Time on current cART regimen type (month) $^{\delta}$		0.992(0.989,0.995)	0.000		
Time on prior cART regimen types (month) $^{\delta}$		0.989(0.985,0.993)	0.000		
Time on NVP-based 1st-line (Prior to EFV) (month) $^{\delta}$		0.989(0.984,0.995)	0.000		
Time on LPV/r-based 2nd-line (prior to ATV/r) (month) $^{\delta}$		1.0(0.98,1.02)	0.907		
Waist circumference (cm)		1.1 (0.99, 1.11)	0.055	0.96(0.94,0.99)	0.002
Total cholesterol (mg/dL)		1 (0.998, 1.002)	0.972		
Triglyceride (mg/dL)		1.008 (1.004, 1.011)	0.000	1.005 (1.001, 1.008)	0.017
HDL-C (mg/dL)		0.997 (0.987, 1.007)	0.566		
LDL-C (mg/dL)		1.001 (0.999, 1.002)	0.587		

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associated with the incidence of GMD. A 4% lower incidence of GMD was noted with a 1 cm increase in waist circumference.

Predictors for EFV-based cARTs. Being age ≥46 years old, male gender, BMI category, and TDF-containing combinations were independent predictors to GMDs (Table 4). A 2.1-fold high risk of GMDs was observed among participants with age ≥46 years old (AOR = 2.1, 95% CI 1.1–4.0, p = 0.02). The male gender had a 4.3 times higher risk of GMDs (AOR = 4.3, 95% CI 2.2–83, p = 0.000). Underweights (BMI <18.5) had an 80% lower incidence of GMDs (AOR = 0.2, 95% CI 0.04, 0.7, p = 0.014) than obese participants (BMI ≥30). Likewise, TDF consisting regimens demonstrated a lower incidence of GMDs by about 70% (AOR = 0.3, 95% CI 0.1, 0.6, p = 0.001) than AZT consisting EFV-based cARTs. This was consistent with chi-square analysis, which found a significant association of GMDs with AZT-containing EFV-based regimen than AZT-containing ATV/r-based cART (χ^2 = 6.3, p = 0.012).

Predictors for ATV/r-based cARTs. Being age ≥46 years old, type of specific ARVs contained in ATV-based cART, and serum triglycerides level were independent predictors of GMDs incidence (Table 4). Participants with age ≥46 years old exhibited a 5.6 times higher risk of GMDs (AOR = 5.6, 95% CI 1.6, 21.3, p = 0.006) than age ≤45 years. A >90% lower incidence of GMDs was recorded for TDF-, AZT-, and ABC- containing than their corresponding non-containing type of ATV/r-based cARTs (Table 4). Concerning triglycerides level, the likelihood to develop GMDs increased by 0.9% (AOR = 1.009, 95% CI 1.002–1.016, p<0.05) for each unit increase.

Discussion

Our study aimed at determining the prevalence and predictors of GMDs among EFV and ATV/r-based cART receiving patients. The overall prevalence of GMDs among participants on EFV-based cART was relatively high than ATV/r-based cART. Remarkably, it was shown that EFV-based cART was associated with the occurrence of IFG than ATV/r-based regimen. Our study linked TDF-containing cART as an independent predictor of GMDs in both EFV- and ATV/r-based combination regimens. Our study considered IR along with IFG and DM as a measure of glucose abnormalities unlike other similar studies [9, 21, 32–34]. This may have increased the chance of detecting GMDs in our study while addressing the pathological hierarchy of glucose-related abnormalities, in which most ARVs in cART are implicated.

The prevalence of DM among PLWH on EFV-based cART in the present study (5.7%) is even higher than reported by WHO in 2016 (3.8%) and IDF (3.2%) [35, 36] for the general population of Ethiopia. This finding may be suggestive of a higher prevalence of GMDs among PLWH on cART, especially on NNRTIs like EFV. A comparative study by Levitt *et al.* [8] reported a prevalence of dysglycemia (26.0%) among PLWH on first-line ART, which is similar to the overall prevalence of GMDs in the present study (27.6%). A relatively close prevalence of GMDs (32.7%) was also reported in a study conducted in Tanzania among HIV-infected patients on ART, though the specific ARV drugs were not indicated [7]. Our study reported lower IFG (12.8% vs. 24%) but a higher DM prevalence (5.7% vs. 2%) than the South African study [37] among EFV-based cART treated participants. In addition, the DM prevalence in this study was slightly lower than reported from North-east (8.8%) [21] and North-west (8.8%) [9] Ethiopia. But it is concordant with that reported from another North-west Ethiopian (5.1%) [33] and Zambian (5%) [38] studies. On the other hand, a lower prevalence of IR was observed in this study as compared to the 21% prevalence reported in a longitudinal study by Araujo *et al.* [29] and 34.2% by Guillen *et al.* [10].

In general, a varied prevalence has been reported for IR, IFG and DM across the literature. These discrepancies could be due to variations in methodology. For instance, the above-stated

Table 4. Predictors of glucose metabolism disorders among EFV- (n = 75) and ATV/r-based (n = 22) cART receiving groups.

Variables		Categories	EFV-based				ATV/r-based			
			Univariate		Multivariate		Univariate		Multivariate	
			COR	p	AOR	p	COR	p	AOR	p
Age category		≤45	1		1		1		5.6(1.6,19.3)	0.006
		≥46	0.5 (0.3,0.8)	0.006	2.1(1.1, 4.0)	0.02	0.4 (0.2, 1.1)	0.069		
Gender		Female	1		1		1			
		Male	4.6 (2.6,8.4)	0.000	4.3 (2.2, 8.3)	0.000	1.1 (0.4,3.1)	0.891		
Educational status		Up to primary	1				1			
		Above Primary	1.8 (0.99,3.1)	0.055			1.1 (0.4, 2.9)	0.868		
Marital status		Single	1				1			
		Married	0.5 (0.3,0.8)	0.002			0.2 (0.1,0.4)	0.000		
		Widowed	0.3 (0.2,0.9)	0.000			0.4 (0.1,0.8)	0.017		
		Divorce	0.5 (0.2, 0.9)	0.022			0.3 (0.1,0.9)	0.023		
Treatment adherence (3-day test)		Non-adhered	1				1			
		Adhered	0.48 (0.37,0.63)	0.000			0.24 (0.15,0.39)	0.000		
BMI category		≥30	1				1			
		<18.5	0.1 (0.04, 0.46)	0.001	0.2 (0.04,0.7)	0.014	0.1 (0.01,0.54)	0.011		
		18.6–24.9	0.7 (0.5,0.99)	0.042	0.8 (0.3,1.7)	0.499	0.3 (0.1, 0.5)	0.000		
		25–29.9	0.4 (0.23,0.62)	0.000	0.5 (0.2,1.1)	0.096	0.3 (0.1, 0.7)	0.008		
CD4 (n = 342) (recent)		<350	1				1	0.000		
		≥350	0.4 (0.3, 0.6)	0.000			0.2 (0.1,0.4)			
Viral load status(n = 335)		≥1000 copies/ml	1				1	0.000		
		<1000 copies/ml	0.5 (0.3, 0.6)	0.000			0.2 (0.1,0.4)			
History of comorbidity (self-reported)		HIV-only	1				1	1.000		
		HIV +comorbidity	2.5 (0.5,12.9)	0.273			1.0 (0.1, 7.1)			
Specific ARVs contained in cART	TDF containing	No	1		1	-	1		1	0.03
		Yes	0.4 (0.3,0.6)	0.000	0.3 (0.1,0.6)	0.001	0.2 (0.1,0.5)	0.000	0.101 (0.013,0.802)	
	AZT containing	No	1				1		1	0.016
		Yes	0.9 (0.4,2.0)	0.842			0.2 (0.1,0.5)	0.000	0.092 (0.013,0.637)	
	ABC containing	No					1		1	0.012
		Yes					0.2 (0.04,0.8)	0.027	0.034 (0.002,0.474)	
Time since HIV confirmed date (months)			0.995 (0.993,0.997)	0.000			0.989 (0.986,0.997)	0.000	0.99 (0.98,1.0)	0.054
Cumulative time on cART (month) $^{\delta}$			0.995 (0.993,0.997)	0.000			0.989 (0.985,0.993)	0.000		
Cumulative time on EFV-based 1st-line (month) $^{\delta}$			0.994 (0.991,0.996)	0.000			0.984 (0.975,0.992)	0.000		
Cumulative time on ATV/r-based 2nd-line (month) $^{\delta}$			1.0(0.97,1.04)	0.891			0.961 (0.946,0.977)	0.000		
Time on current cART regimen type (month) $^{\delta}$			0.993 (0.991,0.996)	0.000			0.968 (0.954,0.982)	0.000		
Time on prior cART regimen types (month) $^{\delta}$			0.992 (0.986, 997)	0.003			0.987(0.981, 0.992)	0.000		
Time on NVP-based 1st-line (prior to EFV) (month) $^{\delta}$			0.992 (0.986,0.999)	0.017			0.985 (0.977,0.994)	0.001		

(*Continued*)

Table 4. (Continued)

Variables	Categories	EFV-based				ATV/r-based			
		Univariate		Multivariate		Univariate		Multivariate	
		COR	p	AOR	p	COR	p	AOR	p
Time on LPV/r-based 2nd-line (prior to ATV/r) (month) $^{\delta}$		1.7	1.000			1.0(0.98,1.02)	0.728		
Waist circumference (cm)		0.979 (0.972,0.987)	0.000			0.961 (0.948,0.975)	0.000		
Total cholesterol (mg/dL)		0.996 (0.995,0.998)	0.000			0.993 (0.99,0.995)	0.000		
Triglyceride (mg/dL)		0.997 (0.996,0.999)	0.001			0.994 (0.991,0.996)	0.000	1.009 (1.002,1.016)	0.014
HDL-C (mg/dL)		0.984 (0.978,0.99)	0.000			0.967 (0.955,0.978)	0.000		
LDL-C (mg/dL)		0.996 (0.994,0.998)	0.000			0.988 (0.983,0.992)	0.000		

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studies from Northwest Ethiopia, Northeast Ethiopia, and Zambia considered IFG and DM, while our study included IR parameters in addition to IFG and DM to determine the prevalence and risk factors [9, 21, 33, 38]. The sensitivity of kits or equipment used to determine fasting glucose might also differ, accounting for the observed differences (we used the Cobas 6000 (c501) analyzer machine and glucometer was used by the Zambian study). Study designs employed could also contribute to the differences. Araujo *et al.* [29] determined the prevalence using a prospective cohort study, recruiting participants from different types of cART, unlike ours which used a cross-sectional study design using only EFV- and ATV/r-based regimens.

Several studies suggested that EFV containing cART is linked with elevated blood glucose levels due to mitochondrial toxicity or IR [34, 39, 40]. This notion could explain why a high prevalence of GMDs was observed among EFV- than ATV/r-based cART receiving study participants. In contrast, a cross-sectional study from Tanzania indicated that neither EFV nor other ARVs had an association with GMDs among HIV-infected patients on cART [7]. Nonetheless, several lines of recent evidence implicated NNRTIs in disturbed glucose metabolism. For example, studies reported that increased fasting plasma glucose, insulin levels, and decreased insulin sensitivity were observed in NNRTI-based regimens, particularly with EFV [34, 39, 40].

This is one of the few studies that assessed GMDs among HIV-infected patients on ATV/r-based cART, particularly in Ethiopia. As ATV/r-based therapy is relatively new to most resource-limited health settings, our study may provide baseline evidence concerning GMDs during ATV/r-based regimen use. Thus, the findings could help guide ARV drug selection when switching to PI-based second line is considered, particularly for the high-risk group of HIV patients. Based on the finding of this study, those participants on ATV/r-based therapies had a low prevalence of GMDs (3.6%), which is similar to the DM estimates of the Ethiopian population for 2016 (3.8%) [35].

The findings highlighted that the type of specific ARVs contained in cART could influence the occurrence of GMDs, as participants on TDF-containing regimens had a significantly reduced risk for developing GMDs than those on without TDF. Considering IR as a component of GMDs, our study is consistent with the interpretation of "The Women's interagency HIV study" that reported a lack of clear elevation or precise association between cumulative exposure to five NRTIs, including AZT, ABC or TDF, and HOMA [11].

Consistent with previous studies, our study demonstrated that patients above 45 years of age are at higher odds of developing GMDs in both regimen types as well as in the overall

study participants. Aging is a well-recognized traditional risk factor for IFG, diabetes or GMDs in general. A rise in the incidence of GMDs might occur among HIV-infected patients because of increased survival or aging in the face of long-term exposure to cART [7, 21, 41]. Our findings also indicated male patients, specifically among EFV-based cART, were at a higher risk of encountering GMDs than females, which is in line with other studies [34, 38, 41–44]. A meta-analysis also found a significantly higher prevalence of IFG in men among the general population of Eastern, Middle, and Southern African countries [45]. It is thus plausible to assume that the same trend might occur in HIV-infected patients on cART. Although direct evidence is lacking, previous studies suggest that in addition to socio-cultural, lifestyle, or behavioral factors; anthropometric, metabolic, and endocrine differences could contribute to the gender disparity in the prevalence of GMDs [46, 47]. It is also suggested that differences in type and composition of fat might have a role in the risk for IFG, IR, or DM between men and women [48].

Study limitations

The use of a one-time sampling to define glycemic status rather than confirmation on a subsequent day as recommended can be considered as one of the limitations. However, determination of fasting glucose was run three times for each sample and the average was reported, which could probably offset this limitation. Despite the exclusion of participants with DM, our study design lacks excluding study participants who may have other GMDs. Moreover, our study lacks assessing casual association between long term-cART and GMDs as the study did not have control groups and prior baseline data. The study may also share the limitations emanating from the study design effect, as we used a single institution, a cross-sectional study, and a consecutive sampling during recruitment. Hence, the findings might not be extrapolated to the general PLWH receiving treatment in Ethiopia. Nevertheless, one should note that the study site is the largest referral hospital in Ethiopia, where patients from different parts of the country are referred to receive care. Despite the limitations, this study generated findings related to higher prevalence of GMDs among HIV-infected adult patients, particularly those on EFV-based cART. However, future studies with large sample size, comparative case-control, and prospective study design comprising these regimens should be conducted to confirm the predictors and determine if there exist casual relationships between GMDs and long-term cART.

Conclusions

In conclusion, we report a high prevalence of GMDs, such as IR, IFG, and DM, among PLWH on EFV-based cART. Age 46 and above and TDF-containing cART were common predictors of GMDs in both EFV- and ATV/r-based treatment groups. The male gender and BMI are predictors of GMDs in EFV-based cART group. AZT- containing and ABC-containing ATV/r-based cARTs as well as elevated serum triglycerides are predictors of GMDs in ATV/r-based cART receiving group. Close monitoring for impaired fasting glucose during long-term efavirenz-based cART is recommended for early diagnosis of type-2 diabetes and management.

Supporting information

S1 Raw data.
(SAV)

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PAPER II

Article

CYP3A and *CYP2B6* Genotype Predicts Glucose Metabolism Disorder among HIV Patients on Long-Term Efavirenz-Based ART: A Case-Control Study

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Abstract: Long-term antiretroviral treatment (cART) increases the risk of glucose metabolism disorders (GMDs). Genetic variation in drug-metabolizing enzymes and transporters may influence susceptibility to cART-associated GMDs. We conducted a case-control study to investigate the association of pharmacogenetic variations with cART-induced GMDs. A total of 240 HIV patients on long-term efavirenz-based cART (75 GMD cases and 165 controls without GMDs) were genotyped for *CYP3A4*1B*, *CYP3A5 (*3,*6)*, *CYP2B6*6*, *UGT2B7*2*, *ABCB1* (*c.3435C>T*, *c.4036A>G*), and *SLCO1B1* (**1b*, **5*). GMD cases were defined as the presence of impaired fasting glucose, insulin resistance, or diabetes mellitus (DM). Case-control genotype/haplotype association and logistic regression analysis were performed by adjusting for age, sex, and BMI. The major *CYP3A* haplotype were *CYP3A5*3* (53.8%), *CYP3A4*1B* (17.3%), combinations of *CYP3A4*1B*, and *CYP3A5*6* (10.9%), and *CYP3A* wild type (7%). *CYP3A5*6* allele ($p = 0.005$) and *CYP3A5*6* genotype ($p = 0.01$) were significantly associated with GMD cases. Multivariate analysis indicated *CYP3A* haplotype as a significant predictor of GMD ($p = 0.02$) and IFG ($p = 0.004$). *CYP2B6*6* significantly predicted DM ($p = 0.03$). *CYP3A* haplotype and *CYP2B6*6* genotype are independent significant predictors of GMD and DM, respectively, among HIV patients on long-term EFV-based cART.

Keywords: antiretroviral therapy; efavirenz; glucose metabolic disorder; HIV; pharmacogenetic variation; *CYP3A5*; *CYP2B6*; genotype; Ethiopia

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1. Introduction

The introduction of combination antiretroviral therapy (cART) significantly improved survival and health-related quality of life among people living with HIV (PLWH) [1,2]. Efavirenz (EFV)-based cART is among the first-line antiretroviral regimen to treat HIV recommended by the World Health Organization [1]. Before its takeover by dolutegravir, EFV served as the backbone of the preferred first-line antiretroviral regimen due to its clinical, operational, and programmatic benefits.

Various observational and clinical trial studies link EFV use with a range of treatment-associated adverse events manifested depending on the duration of therapy. While liver enzyme abnormalities [3–6] and neuropsychological manifestations [7,8] can occur during early treatment, HIV patients on long-term antiretroviral therapy are at an increased risk of developing metabolic derangements, including lipid dystrophy and glucose metabolism

disorders (GMDs) [9–11]. Indeed, a higher risk of GMDs among HIV patients on EFV-based cART than on atazanavir/ritonavir-based cART has been reported recently [11].

Several factors determine individuals' susceptibility to cART-associated adverse events [12,13], including comorbidities and concomitant medications, host-genetic factors, and population variation [14–16]. Genetic variations in genes coding for drug-metabolizing enzymes and transporter proteins can alter plasma concentration and drug exposure, ultimately affecting treatment outcomes. Efavirenz is metabolized by genetically polymorphic CYP enzymes expressed in the liver. It is primarily metabolized to hydroxylated metabolites by CYP2B6, though other CYPs, including CYP3A4/5, CYP1A2, and CYP2A6, also play a minor role [17]. Genetic variations in drug-metabolizing enzymes affect EFV plasma exposure [15,18,19], thereby altering treatment outcomes [4,8].

Pharmacogenetic variation in relevant EFV metabolizing enzymes and transporter proteins may be linked with metabolic derangements and result in clinical conditions, including treatment-associated adverse events. Indeed, the association of *CYP2B6* defective variant allele and higher plasma efavirenz exposure with liver enzyme abnormalities [3–5,20] and neuropsychological manifestations is reported [7,8,20].

Accumulating evidence in the literature indicates that long-term efavirenz-based cARTs are associated with GMDs in PLWH [11,21,22]. The importance of pharmacogenetic variations for cART-induced liver and CNS toxicities is well explored. Inter-individual variations in drug metabolic capacity due to genetic variations of drug-metabolizing enzyme and transporter genes may play a role in the predisposition to GMDs (insulin resistance (IR) or impaired fasting glycemia (IFG)) in antiretroviral therapy (ART).

No studies have been conducted to assess the association of GMDs with genetic variations in enzymes and transporter proteins involved in the disposition of EFV. Pharmacogenetic association studies are important to identify genetic biomarkers that predict specific ADRs associated with ARVs. Identifying risk factors of EFV-based cART-associated GMD is imperative in Africa, where HIV remains a significant public health problem. Here, we assessed the association of common functional variant alleles in relevant drug-metabolizing enzymes (*CYP3A4*, *CYP3A5*, *CYP2B6*, and *UGT2B7*) and transporters (*ABCB1* and *SLCO1B1*) with cART-associated GMDs.

2. Materials and Methods

2.1. Study Setting Design and Participants

The study setting was the HIV clinic of Tikur Anbessa Specialized Hospital (TASH), College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia. TASH, being the largest tertiary level teaching and referral hospital in Ethiopia, serves about 500,000 patients each year through its various specialized clinical services. The hospital receives referred patients from different corners of the country and the city, providing an opportunity to recruit study participants representing a heterogeneous mix of different ethnic populations and socioeconomic statuses of the country. The HIV clinic at TASH coordinates various activities encompassing HIV/AIDS prevention, patient care, and ART services for patients through scheduled follow-ups.

The study design was a case-control genotype association study, and the study population is described in our previous cross-sectional study [11]. In brief, a total of 240 participants on long-term EFV-based ART regimens with complete genotyping and GMD data participated in this study. The inclusion and exclusion criteria were stated in detail in our previous publication [11]. In brief, PLWH on EFV-based cART at least for one year with age $\geq$ 18 years were included, while PLWH diagnosed with DM, pregnancy, cancer, renal disease, liver disease, uncontrolled hypertension, or heart failure were excluded from this study. Additionally, patients on concomitant treatment with antipsychotics, anti-cancer agents, anti-TB, corticosteroids, hormonal agents, and antidiabetic agents were excluded.

Whole blood was collected after overnight fasting (8 to 12 h). Serum fasting blood glucose (FBG), insulin, and lipid profiles were determined. Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated by using the following equation [23].

$$\text{HOMA} - \text{IR} = \frac{\left[\text{Fasting insulin } \left(\frac{\mu\text{U}}{\text{mL}}\right) \times \text{Fasting glucose}\left(\frac{\text{mmol}}{\text{L}}\right)\right]}{22.5}$$

2.2. GMD Case Definitions

The following operational definitions were used [11].

1. DM was defined as a fasting glucose level of 126 mg/dL or higher.
2. IFG was defined as a fasting glucose level between 110 and 125 mg/dL.
3. IR was diagnosed by either a homeostasis model assessment insulin resistance (HOMA-IR) value of ≥3.8, Fasting plasma insulin of ≥20 µU/mL, or fasting glucose/insulin ratio of ≥4.5′.
4. GMDs were defined as the presence of IFG, IR, or DM.

Controls were those individuals with normal fasting serum glucose, HOMA-IR, or serum insulin level based on the above-stated cut-off values as stated by Tadesse et al. [11]. The cases were those individuals with at least one diagnosis with insulin resistance, impaired fasting glycemia, or diabetes mellitus. The case and control groups were matched to each other in terms of age, duration since cART start, duration on current cART, weight, waist circumference, and BMI. Out of 240 participants, 75 cases and 165 controls were recruited in this study.

2.3. Genotyping

Whole blood samples were collected for genotype analysis in EDTA coated vacutainer tube and inverted 8–10 times for thorough mixing of EDTA. The samples were then transferred to cryotubes and stored at −80 °C until genotyping analysis. Genomic DNA was isolated from whole-blood samples using QIAamp DNA MidiKit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Purity and quantity of DNA were assessed using NanoDrop 2000 (Thermo Scientific, Saveen Warner, Sweden). Allelic discrimination assay was performed using TaqMan drug metabolism genotyping assay (Applied Biosystems, Foster City, CA, USA).

Functional variant alleles in enzymes and transport proteins relevant for disposition of EFV based cART were selected for genotyping [15,17]. The variant alleles were *CYP3A4*1B*, *CYP3A5*3*, *CYP3A5*6*, *CYP2B6*6*, *UGT2B7*2*, *ABCB1* (*c.3435C>T* and *c.4036A>G*), and *SLCO1B1* (*1B, *5). Genotyping was performed using TaqMan® allele-specific PCR (Applied Biosystems Genotyping Assays) with the following ID numbers for respective SNPs: C__7586657_20 for *ABCB1c.3435C>T*, C_11711730_20 for *ABCB1c.4036A>G*, C__7817765_60 for *CYP2B6 c.516G>T* (*CYP2B6*6*), C__30720663_20 for *UGT2B7 −327G>A* (*UGT2B7*2b*, *2c, *2d, *2f), C_9440184_20 for *UGT2B15*4*, C__26201809_30 for *CYP3A5*3* (*6986A>G*), C__30203950_10 for *CYP3A5*6* (*14690G>A*), C_1837671_50 for *CYP3A4*1B*, C_1901697_20 for *SLCO1B1*1B*, C_30633906_10 for *SLCO1B1*5*. The 7500 Real-Time PCR system (Applied Biosystems) was used for genotyping. The final volume was 10 µL for each reaction, consisting of 9 µL of TaqMan Universal PCR Master Mix® (Applied Biosystems, Waltham, MA, USA), DNA/RNA free water, TaqMan 20× drug-metabolism genotyping assay mix (Applied Biosystems), and 1 µL of genomic DNA. The PCR conditions were as follows: an initial step at 60 °C for 30 s, hold-stage at 95 °C for 10 min and PCR stage for 40 cycles step 1 with 95 °C for 15 and step 2 with 60 °C for 1 min and after read-stage with 60 °C for 30 s.

2.4. Statistical Analysis

Hardy–Weinberg equilibrium was assessed by chi-square test for each SNP to determine any differences between observed and expected genotype frequencies. Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 26

(IBM® SPSS® Statistics, Chicago, IL, USA) and Haploview version 4.2 (Broad Institute, Cambridge, MA, USA). Baseline sociodemographic and laboratory parameters were described as means and standard deviation (SD) or medians, and interquartile range (IQR) for continuous variables and proportions for categorical variables.

Haploview version 4.2 was employed to determine the association and case-control analysis of genotypes and haplotypes. The association of GMDs with genetic variants was determined through logistic regression analysis. Univariate followed by a multivariate logistic regression model was used to determine the association of GMD with genotype variants after adjusting for factors such as age, sex, and BMI among the groups. Genotypes with a univariate analysis of $p < 0.2$ were entered in the multivariate analysis. A stepwise conditional-backward analysis was employed when three or more genotypes meet the stated entry criteria for multivariate analysis. When only one or two genotypes meet the entry criteria, a direct enter method (without a stepwise) was used in the multivariate analysis. p-values of <0.05 were considered statistically significant. The regression coefficient (β), crude odds ratio (COR), and adjusted odds ratio (AOR) were recorded from univariate and multivariate logistic regression.

2.5. Ethical Consideration

Ethical clearance was obtained from the Institutional Review Board of College of Health Sciences, Addis Ababa University (Protocol No. 019/19/SoP) and National Ethical Review Committee, Ministry of Science and Higher Education of Ethiopia (Ref. No. MoSHE/RD14.1/9324/20). Written informed consent was obtained from each study participant after fully explaining the purpose and nature of all procedures used.

3. Results

3.1. Characteristics of Study Participants

A comparison of sociodemographic, clinical, and biochemical parameters between HIV patients who developed GMD (cases) versus those who did not develop GMD (controls) is presented in Table 1. The highest mean age (46.6 ± 1.3 years) was recorded in the case group. Unlike the case group, the distribution of males (21.7%) and females (78.3%) was disproportional among the control group. The other characteristics, such as weight, BMI, and duration on cART, did not show a significant difference between the case and the control groups (Table 1). Comparative clinical laboratory data for lipid measurements, fasting serum glucose, fasting serum insulin, and HOMA-IR values are shown in Table 1. A significant mean difference ($p < 0.001$) was observed in the biochemical markers of GMD between the control and the case groups (Table 1).

Table 1. Sociodemographic and biochemical parameters among HIV patients with glucose metabolic disorders (cases) versus without (controls).

Variables		GMD Cases	Control	*p*
Sample (*n*) (case–control ratio)		75	165	(1:2.2)
Gender *n* (%)	Male	40 (52.7)	35 (21.7)	<0.001
	Female	35 (47.3)	130 (78.3)	
Age (years)		46.6 ± 1.3	43.8 ± 0.8	0.06
Duration since HIV confirmed date (month)		141.6 ± 5.9	130.5 ± 3.7	0.10
Cumulative time on cART (month)		129.2 ± 5.3	120.9 ± 3.4	0.19
Time on current cART regimen type (month)		95.3 ± 5.2	92.1 ± 3.1	0.59
Cumulative time on EFV-based 1st-line (month)		104.3 ± 5.0	100.2 ± 3.3	0.49
Time on prior cART regimen types (month)		32.2 ± 4.4	36.2 ± 8.0	0.74

Table 1. *Cont.*

Variables		GMD Cases	Control	*p*
Time on NVP-based 1st-line (Prior to EFV) (month)		24.8 ± 4.0	20.8 ± 3.0	0.43
Weight (Kg)		64.5 ± 1.4	62.7 ± 1.1	0.34
Waist circumference (cm)		34.8 ± 0.6	34.2 ± 0.4	0.37
BMI (Kg/m^2)		24.1 ± 0.5	24.2 ± 0.4	0.86
Total cholesterol (mg/dL)		205.4 ± 5.1	209.5 ± 14.2	0.85
Triglyceride (mg/dL)		185.0 ± 11.8	136.8 ± 4.3	<0.001
HDL-C (mg/dL)		44.9 ± 2.3	46.8 ± 2.4	0.63
LDL-C (mg/dL)		139.7 ± 18.5	133.4 ± 10.0	0.75
Biochemical markers of GMD	Fasting serum glucose (mg/dL)	117.7 ± 4.9	94.0 ± 0.6	<0.001
	Fasting serum insulin (μLU/mL)	16.9 ± 2.3	7.1 ± 0.3	<0.001
	HOMA-IR	5.3 ± 1.0	1.7 ± 0.1	<0.001

3.2. Genotype, Allele, and Haplotype Frequencies

Comparison of *CYP3A4*1B*, *CYP3A5*3*, *CYP3A5*6*, *CYP2B6*6*, *UGT2B7*2*, *ABCB1 c.3435C>T*, *ABCB1c.4036A>G*, *SLCO1B1*1B*, and *SLCO1B1*5* genotype and allele frequencies between control and case groups are depicted in Table 2. All genotype frequencies were in accordance with Hardy–Weinberg equilibrium (HWE) ($p > 0.05$).

The variant allele frequency *CYP3A5*6* was significantly higher among GMD cases than in the control groups (0.41 versus 0.24) ($p = 0.005$). The linkage disequilibrium (LD) analysis demonstrated a linkage among the three *CYP3A* valiant alleles, and the relatively stronger linkage was between *CYP3A4*1B* and *CYP3A5*3* (Figure 1). The major *CYP3A* haplotype was *CYP3A5*3* alone (53.8%), followed by *CYP3A4*1B* alone (17.3%) and then by *CYP3A5*1B* linkage with *CYP3A5*6* (10.9%) (Table 3). Based on Haploview case-control analysis, the *CYP3A5*6* allele was significantly associated with IFG, IR, and overall GMD, while the *CYP2B6*6* allele was significantly associated with DM (not shown in tables).

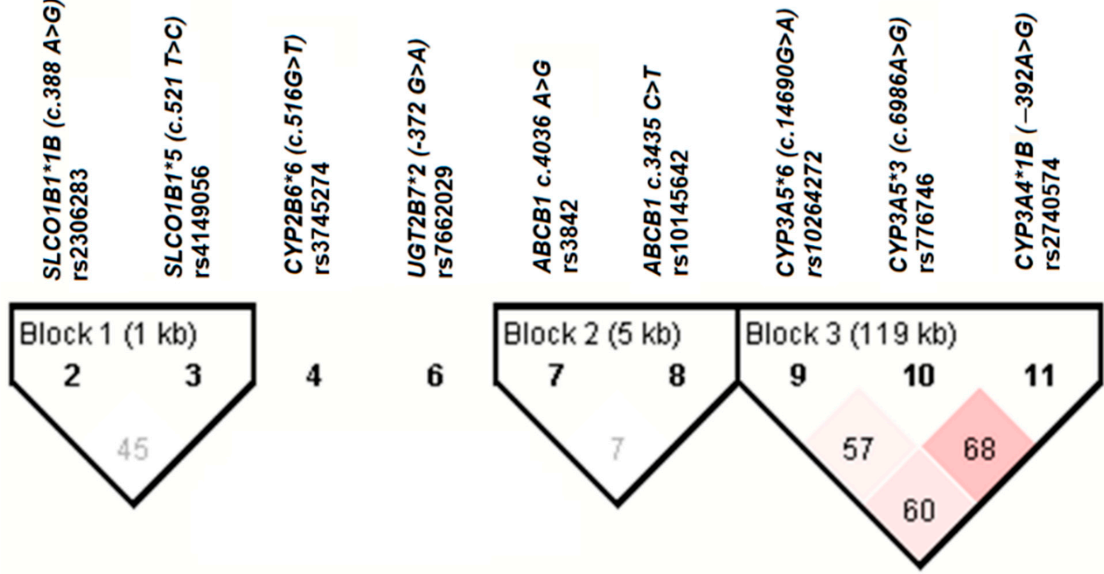

Figure 1. Linkage disequilibrium (LD) plot of the studied genotype variants among Ethiopian PLWH on EFV-based cART. The values in the diagonal square boxes represent the respective observed D′ values of the pair-wise LD relationship. The red color gradient indicates higher LD values while the white represents lower LD values (D′ 1–0).

Table 2. Genotype and variant allele frequency distribution and association with glucose metabolism disorders (GMD) among PLWH (*N* = 351).

Genotypes	Variants	Control, *n* (%)	Case, *n* (%)	*p*
*CYP 3A4*1B (−392A>G)*	*1/*1 *1/*1B *1B/*1B	70 (42.4) 79 (47.9) 16 (9.7)	28 (37.3) 36 (48.0) 11 (14.7)	0.48
*CYP3A5*3 c.6986A>G*	*1/*1 *1/*3 *3/*3	16 (9.7) 82 (49.7) 67 (40.6)	10 (13.3) 38 (50.7) 27 (36.0)	0.63
*CYP3A5*6 c.14690G>A*	*1/*1 *1/*6 *6/*6	126 (76.4) 36 (21.8) 3 (1.8)	44 (58.7) 29 (38.7) 2 (2.7)	0.01
*CYP2B6*6 c.516G>T*	*1/*1 *1/*6 *6/*6	81 (49.1) 70 (42.4) 14 (8.5)	30 (40.0) 38 (50.7) 7 (9.3)	0.42
*UGT2B7*2 (−372G>A)*	GG GA AA	39 (23.6) 86 (52.1) 40 (24.2)	17 (22.7) 36 (48.0) 22 (29.3)	0.70
ABCB1 c.3435C>T	CC CT TT	93 (56.4) 62 (37.6) 10 (6.1)	45 (60.0) 24 (32.0) 6 (8.0)	0.65
ABCB1 c.4036A>G	AA AG GG	107 (64.8) 55 (33.3) 3 (1.8)	53 (70.7) 19 (25.3) 3 (4.0)	0.32
*SLCO1B1*1B c.388A>G*	AA AG GG	22 (13.3) 91 (55.2) 52 (31.5)	8 (10.7) 43 (57.3) 24 (32.0)	0.84
*SLCO1B1*5 c.521T>C*	TT TC CC	108 (65.5) 49 (29.7) 8 (4.8)	49 (65.3) 24 (32.0) 2 (2.7)	0.71
Minor Variant Allele		Allele Frequency		*p*
*CYP3A4*1B (−392A>G)*	*1B	0.58	0.37	0.46
*CYP3A5*3 (c.6986A>G)*	*3	0.9	0.87	0.4
*CYP3A5*6 (c.14690G>A)*	*6	0.24	0.41	0.005
*CYP2B6*6 (c.516G>T)*	*6	0.51	0.6	0.19
*UGT2B7*2 (-372G>A)*	A	0.76	0.77	0.87
ABCB1 c.3435C>T	T	0.44	0.4	0.6
ABCB1 c.4036A>G	G	0.35	0.29	0.38
*SLCO1B1*1B (c.388A>G)*	G	0.87	0.89	0.56
*SLCO1B1*5 (c.521T>C)*	C	0.35	0.35	0.99

Table 3. GMD case-control analysis of *CYP3A* haplotype using Haploview among HIV patients on long-term EFV-based cART.

CYP3A **Haplotypes**	***CYP3A*** **Allele Combinations**			**F (%)**	***p*-Values**			
	CYP3A4*1B −392A>G	***CYP3A5*3 c.6986A>G***	***CYP3A5*6 c.14690G>A***		**GMD**	**IFG**	**IR**	**DM**
AGG	wt	*3	wt	53.8	0.40	0.20	0.41	0.56
GAG	*1B	wt	wt	17.3	0.90	0.81	0.89	0.27
GAA	*1B	wt	*6	10.9	0.04	0.008	0.04	0.35
GGG	*1B	*3	wt	6.3	0.45	0.17	0.57	0.35
AAG	wt	wt	wt	7.0	0.13	0.99	0.15	0.49
AGA	wt	*3	*6	3.4	0.34	0.32	0.60	0.99

F (%) represent haplotype frequency distribution; level of significance (*p*-values) are based on Haploview case-control analysis for *CYP3A* haplotypes; Wt—wildtype; GMD—glucose metabolism disorder; IFG—impaired fasting glycemia; IR—insulin resistance; DM—diabetes mellitus.

3.3. Genotype/Haplotype Association with GMD

Based on Haploview case-control analysis, *3A4*1B + 3A5*6* haplotype combinations of *CYP3A* showed a significant association with GMD ($p = 0.04$) (Table 3). The multivariate analysis revealed that the *3A4*1B + 3A5*3 + 3A5*6 (*1B/*3/*6)* haplotype combination of CYP3A was found to be an independent predictor of GMD when the wildtype and the *1B haplotype carriers used as a reference group (overall $p = 0.02$) (Table 4). Among carriers of *CYP3A4*1B + 3A5*3 + 3A5*6* haplotype, the odds of experiencing GMD were about 2.2-fold higher (AOR = 2.2; 95% CI 1.0–4.8), $p = 0.04$) than carriers of the wildtype (with two functional alleles) or *CYP3A5*3* alone haplotype combinations.

Table 4. GMD regression analysis of drug-metabolizing and transporter genotypes with glucose metabolism disorders (GMD) and impaired fasting glycemia (IFG) among HIV patients on long-term EFV-based cART ($n = 240$).

Genotype		GMD among EFV-Based cART						IFG among EFV-Based cART					
		β	COR (95% CI)	*p*	β	AOR (95% CI)	*p*	β	COR (95% CI)	*p*	β	AOR (95% CI)	*p*
CYP3A haplotypes (*3A4*1B/3A5*3/3A5*6*)	**1/*1/*1* or **1B/*1/*1*		1	0.02		1	0.02		1	0.004		1	0.004
	**1/*3/*1* or **1B/*3/*1*	−0.4	0.7 (0.2, 2.4)	0.56	−0.4	0.6 (0.2, 2.3)	0.5	−0.6	0.6 (0.1, 2.8)	0.47	−0.6	0.6 (0.1, 2.8)	0.47
	**1B/*1/*6* or **1/*3/*6* (any *3A5*6*)	0.5	1.6 (0.4, 5.7)	0.49	0.4	1.5 (0.4, 5.6)	0.52	0.7	1.9 (0.4, 9.7)	0.42	0.7	1.9 (0.4, 9.7)	0.42
*CYP2B6*6* (*c.516G>T*)	**1/*1*		1						1				
	**1/*6 or *6/*6*	0.4	1.4 (0.8, 2.5)	0.19	0.4	1.5 (0.9, 2.7)	0.13	0.01	1.0 (0.5, 2.0)	0.97			
*UGT2B7*2b* (*−327G>A*)	GG		1						1				
	GA or AA	0.1	1.1 (0.6, 2.0)	0.87				0.3	1.4 (0.6, 3.3)	0.49			
ABCB1 c.3435C>T	CC		1						1				
	CT or TT	−0.2	0.9 (0.5, 1.5)	0.6				0.2	1.2 (0.6, 2.4)	0.65			
ABCB1 c.4036A>G	AA		1						1				
	AG or GG	−0.3	0.8 (0.4, 1.4)	0.38				−0.05	1.0 (0.5, 2.2)	0.9			
*SLCO1B1*1B* (*c.388A>G*)	AA		1						1				
	AG or GG	0.3	1.3 (0.5, 3.0)	0.56				−0.1	0.9 (0.3, 2.5)	0.84			
*SLCO1B1*5* (*c.521T>C*)	TT		1						1				
	TC or CC	0.01	1.0 (0.6, 1.8)	0.99				−0.1	0.9 (0.4, 1.9)	0.77			

COR—crude odds ratios; AOR—adjusted odds ratio.

3.4. Genotype/Haplotype Association with IFG

As depicted in Table 3, the Haploview case-control analysis revealed that *3A4*1B + 3A5*6* haplotype combinations of *CYP3A* demonstrated a significant association with IFG ($p < 0.01$), whereas the multivariate analysis (Table 4) showed that the overall *CYP3A* haplotypes were significantly associated with IFG ($p = 0.004$). In alternative multivariate analysis, the odds of developing IFG was 2.8-times higher among *3A4*1B + 3A5*3 + 3A5*6* haplotype carriers compared to the wildtype or *CYP3A5*3*-alone haplotypes carriers (AOR = 2.8, 95% CI 1.1–7.1, $p = 0.04$).

3.5. Association with Insulin Resistance

Based on Haploview case-control analysis, carriers of *CYP3A4*1B + 3A5*6* haplotype combination of *CYP3A* locus showed a significant association with the incidence of IR ($p = 0.04$) (Table 3). *CYP3A* haplotypes showed marginal association compared to the wildtype and the **1B* haplotype carriers ($p = 0.06$) (Table 5). In alternative multivariate analysis (not shown in tables), *3A4*1B + 3A5*3 + 3A5*6* haplotype combination predicted the incidence of IR. The odds of IR risk were 2.6 for *3A4*1B + 3A5*3 + 3A5*6* haplotype carriers (AOR = 2.6; 95% CI 1.0–6.8, $p = 0.05$) compared to the wildtype or *CYP3A5*3*-alone haplotype combinations. In addition, the T allele of *ABCB1 c.3435T* showed a significant 60% protection of IR (AOR = 0.4, 95% CI 0.2–0.9, $p = 0.04$) relative to the wildtype *ABCB1 c.3435* C allele (Table 5).

Table 5. Logistic regression analysis of drug-metabolizing and transporter genotypes with insulin resistance (IR) and diabetes mellitus (DM) among HIV patients on long-term EFV-based cART ($n = 240$).

Genotype		IR among EFV-Based cART						DM among EFV-Based cART					
		β	COR (95% CI)	*p*	β	AOR (95% CI)	*p*	β	COR (95% CI)	*p*	β	AOR (95% CI)	*p*
CYP3A haplotypes (*3A4*1B/3A5*3/3A5*6*)	**1/*1/*1* or **1B/*1/*1*		1	0.06		1	0.06		1	0.68			
	**1/*3/*1* or **1B/*3/*1*	−0.5	0.6 (0.1, 3.1)	0.58	−0.7	0.5 (0.1, 2.6)	0.40	−0.1	0.9 (0.1, 7.5)	0.92			
	**1B/*1/*6* or **1/*3/*6* (any **6*)	0.4	1.5 (0.3, 7.8)	0.60	0.2	1.2 (0.2, 6.5)	0.82	−0.7	0.5 (0.05, 5.3)	0.57			
*CYP2B6*6* (*c.516G>T*)	**1/*1*	0.1	1						1				
	**1/*6* or **6/*6*	−0.1	1.0 (0.5, 1.9)	0.9				1.4	4.0 (1.1, 14.5)	0.03	1.4	4.0 (1.1, 14.5)	0.03
*UGT2B7*2b* (*−327G>A*)	GG		1						1				
	GA or AA	−0.3	0.8 (0.3, 1.7)	0.5				−0.1	0.9 (0.3, 2.9)	0.87			
ABCB1 c.3435C>T	CC		1						1				
	CT or TT	−0.6	0.5 (0.3, 1.2)	0.12	−0.8	0.5 (0.2, 0.98)	0.05	−0.2	0.8 (0.3, 2.3)	0.68			
ABCB1 c.4036A>G	AA		1						1				
	AG or GG	−0.5	0.6 (0.3, 1.4)	0.25				0.5	1.6 (0.6, 4.5)	0.36			
*SLCO1B1*1B* (*c.388A>G*)	AA		1						1				
	AG or GG	1.8	5.8 (0.8, 44.0)	0.09	1.9	6.3 (0.8, 49.1)	0.08	−0.5	0.6 (0.2, 2.2)	0.44			
*SLCO1B1*5* (*c.521T>C*)	TT		1						1				
	TC or CC	0.2	1.2 (0.6, 2.6)	0.56				0.1	1.1 (0.4, 3.3)	0.8			

COR—crude odds ratios; AOR—adjusted odds ratio.

3.6. Association with DM

As depicted in Table 5, *CYP2B6*6 c.516G>T* genotype significantly predicted the incidence of DM among study participants on EFV-based cART. Based on multivariate regression analysis, carriers of *CYP2B6*6* alleles were at a significantly higher risk of DM by 4-fold than the wildtype carriers (AOR = 4.0; CI 95% 1.1–14.5; $p = 0.03$). No significant association of DM with the other genotypes was observed.

4. Discussion

Long-term ART is associated with several metabolic adverse effects, including GMDs (i.e., IR, IFG, or Type 2 diabetes). Long-term efavirenz-based cART-associated metabolic derangements, including GMD, are likely due to mitochondrial toxicity [9]. Growing evidence implicates the role of efavirenz in energy metabolism, mitochondrial function, and other cellular processes involved in oxidative stress [24,25]. Efavirenz exposure lowers glucose uptake and alters bioenergetic cell profiles in human cell lines through inhibition of SLC2A1, which mediates cellular glucose uptake [26]. This result in reduced glucose metabolism in immune cells, suppressing immune cell activation. The primary metabolite of efavirenz, 8-Hydroxy-efavirenz, stimulates the glycolytic flux in cultured rat astrocytes [27]. Association of high plasma efavirenz concentrations with high plasma fasting lipid concentrations and glucose concentrations in South African HIV patients treated with efavirenz-based ART is reported [28]. In our previous study, a higher prevalence of GMDs (27.6%) was reported among HIV patients on a long-term EFV-based regimen [11].

Altered efavirenz metabolism and disposition due to pharmacogenetic variation may influence susceptibility to treatment-induced glucose metabolic disorder. So far, no literature investigated the association of GMDs with functional variant alleles in genes relevant for EFV disposition among PLWH on long-term treatment. Therefore, in this case-control study, we investigated the association of genotype/haplotype variants with the incidence of GMDs among Ethiopian PLWH on EFV-based cARTs. The main finding of this study includes (1) *CYP3A* haplotype, particularly *CYP3A4*1B + 3A5*3 + 3A5*6* allele combinations, which were a significant predictor of IR, IFG, and overall GMD, and (2) *CYP2B6*6* is significantly associated with the risk of developing DM in EFV-based cART. To our knowledge, this is the first study to explore the importance of pharmacogenetic variation for susceptibility to antiretroviral treatment-induced GMD.

There was no statistically different mean in the age, duration of time since cART start, period on the current cART type, weight, waist circumference, and BMI between the case and the control groups, implying a matching between the case and the control groups in the study. We also showed that cases had significantly elevated HOMA-IR, FSG, FSI, and TG levels than controls, indicating the presence of GMDs. The overall observed genotype and allele frequency proportions were similar to the findings of previous studies from Ethiopia [29,30].

Interestingly, the distribution of *CYP3A5*6* allele and genotype frequencies between cases and controls were significantly different, being higher in cases than in controls (Table 2). *CYP3A* haplotype combination containing *3A5*6* significantly predicted the incidence of IR, IFG, and overall GMD than the wildtype or *CYP3A5*3* alone carriers (Table 3). case-control analysis showed the association of *CYP3A4*1B* and *CYP3A5* variants with the incidence of GMD, particularly when *3A4*1B* and *3A5*6* variants co-occur. Though patients on antiretroviral therapy were not stated to be included, a Japanese case-control study indicated that *CYP3A4* (*13989A>G*) polymorphism is associated with the prevalence of type 2 diabetes mellitus [31]. Additionally, another study reported the association of *CYP3A4*18B* with the incidence of tacrolimus-induced new-onset diabetes while reporting no association of *CYP3A5* variants with new-onset type 2 diabetes in renal transplant recipients [32], but these studies were on study participants with different disease conditions and types of treatment than our study participants that limited direct comparison.

The effect of the *CYP3A4*1B* genotype on enzyme activity is not fully understood, and data are inconclusive. As a proximal promoter variant, however, the *CYP3A4*1B* polymorphisms may influence protein expression more than the catalytic activity. While studies indicated reduced enzyme activity [33], there are also reports of higher expression and enzyme activities among carriers of *1B [34]. Nevertheless, *CYP3A4*1B* appears to have no or very limited functional impact on CYP3A4 enzyme activity, and variation in the overall CYP3A enzyme activity is mainly influenced by its linkage disequilibrium with *CYP3A5* variant alleles common in the black African population [35–37]. CYP3A enzyme activity and genetic variation display wide between-population variation even within black

Africans [35]. The major *CYP3A* haplotype in Tanzanians is *CYP3A4*1B* alone (34.2%), followed by its linkage with *CYP3A5*6* (17.6%) and *CYP3A5*3* (13.3%) [36]. *CYP3A5*7* is absent in Ethiopians. In our study, we found the major *CYP3A* haplotype among Ethiopians being *CYP3A4*3* alone (53.8%) and *CYP3A4*1B* alone (17.3%), followed by *CYP3A4*1B* linkage with *CYP3A5*6* (10.9%) or *CYP3A5*3* (6.3%). In the case of *CYP3A5*3*, as it has reduced activity, it may function better, unlike the null-functioning *CYP3A5*6* variant in metabolizing activity. Yet, the *CYP3A4*1B* + *CYP3A5*6* haplotype carriers have reduced active enzymes than carriers of *CYP3A5*3* allele or wildtype haplotype.

The higher association of GMD with the *3A4*1B* + *3A5*6* haplotype in our study may arise due to the impact of *CYP3A5*6* null-functional status. Supporting this assertion, the allele-based case-control analysis (Haploview) consistently showed the association of *CYP3A5*6* with GMDs. In general, the implication of the association of GMD with these variants could be due to a defective enzyme function which might result in higher plasma drug exposure linked with the risk of GMDs. Yet, the exact molecular mechanism that links *CYP3A* variants to GMD remains to be explored.

Notably, the *CYP2B6*6* genotype significantly predicted the incidence of DM. Similarly, the case-control analysis showed that the *CYP2B6*6* allele frequency was significantly higher in the GMD case group than in the control group implying the link between a defective enzyme function with the risk of DM in long-term EFV-based cART. To our knowledge, this is the first finding that showed a statistically significant association of the *CYP2B6* variant allele with the incidence of DM among long-term EFV-based regimens. The *CYP2B6*6* genotype determines interindividual variability of plasma efavirenz exposure. In our study, *CYP2B6*6* (*c.516G>T*) carriers had a four-fold higher risk of DM as compared to the wildtype carriers. Different from our findings, a previous study did not observe a significant association of *CYP2B6*6* with fasting glucose and 2-hour glucose concentration among patients on EFV-based regimens. The authors explained that the small sample size with the genotype data might have limited their study to detect statistically significant genetic associations [28].

In addition, *ABCB1 c.3435C>T* was associated with IR, where the T allele showed a more protective effect than the wildtype. A Turkish case-control study, despite methodological differences from our study, reported that the homozygous mutant of *ABCB1 c.3435C>T* is associated with higher BMI among the control group than in Type 2 DM cases [38], implying a protective role among the case group. Similarly, our study showed a trend that the T allele frequency is slightly higher among control groups than in the cases, although not statistically significant. However, the multivariate analysis revealed that the mutant variants protectively predicted IR. The explanation for this might be that the *ABCB1*, which encodes the efflux transporter P-glycoprotein is involved in the transport of substrates such as sugars, lipids, amino acids, and steroids, apart from their pharmacokinetic role. Functionally, studies suggest that variants with the T allele and the homozygous *ABCB1 c.3435C>T* mutant are linked with reduced expression and functioning of efflux transporters [39]. Thus, SNPs in these genes might alter substrate-related risk factors preventing the occurrence of metabolic conditions such as obesity, one of the predominant risk factors of IR and GMDs, resulting from altered hormonal or physiological biomolecules [40].

In our study, we employed a candidate gene association approach considering variant alleles that may affect the disposition of an EFV-based ART regimen. To our knowledge, for the first time, our study generated evidence of the association between pharmacogenetic variations in drug-metabolizing enzymes and drug transporters relevant for the disposition of antiretrovirals with metabolic disorders such as IR, IFG, and DM among PLWH. The inclusion of insulin and IR parameters as GMD would provide a cumulative prediction of such incidences among PLWH on long-term cART. One of the limitations of our study is that, although in at least 1–4 cases, the control ratio was maintained in most gene variants, the number of participants in the case group was relatively smaller than in the control groups. Our study also focused only on related pharmacokinetic genes and did not consider other SNPs of genes directly involved in carbohydrate metabolism and insulin release.

Therefore, we recommend confirmatory studies on a larger sample size with more extended and repeated follow-ups for future studies.

5. Conclusions

Our findings suggested that *CYP3A*, *CYP2B6*, and *ABCB1* genotypes predicted treatment-associated GMDs to a varying degree among HIV patients on long-term EFV-based combination antiretroviral therapies. Mainly, the defective functional *CYP3A5*6* allele and its haplotype combination (*3A4*1B/3A5*3/*3A5*6*) were independent predictors of the overall GMDs, IR, and IFG. Intriguingly, the *CYP2B6*6* genotype significantly predicted the occurrence of DM (increased risk) on long-term EFV-based cART. Further studies with a large sample size are needed to confirm our findings among PLWH on cART.

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PAPER III

High Plasma Efavirenz Mid-Dose Concentration Predicts Glucose Metabolism Disorders: a Case-Control Study

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Abstract

Objective: Efavirenz (EFV) use is linked with adverse effects, mainly because of its higher plasma level or long-term exposure. Higher plasma EFV concentration might be linked with glucose metabolism disorders (GMDs). Therefore, this study aimed to determine the association of plasma EFV concentration with the incidence of GMDs in patients on long-term therapy.

Methods: In this case-control study, 240 participants on EFV-based cART were recruited, and mid-dose plasma (CP12) efavirenz concentrations were determined using LC-MS/MS. GMD status of each participant was identified based on fasting glucose, fasting insulin, and HOMA-IR values. Genotyping for genes involved in EFV disposition was performed using Applied Biosystems 7500 Real-Time PCR system.

Results: a significant number of participants (31.6%) had supratherapeutic EFV level ($\geq$ 4000 ng/ml) and only 3.0% had a concentration <1000 ng/ml. Supratherapeutic EFV level among controls was 28.6% (46/161) while among cases 38.4% (28/73). Multivariate analysis revealed that gender, triglyceride level, and EFV Log CP12 $\geq$ 3.7 (5000) ng/ml were independent predictors of GMDs. Patients with EFV Log CP12 $\geq$3.7 (5000) ng/ml showed a 2.2-fold higher risk of GMD than EFV CP12 below 3.7 ng/ml (AOR=2.2, 95% CI 1.1-4.6, p=0.03). The *CYP2B6*6* allele independently predicted the log CP12 of EFV among GMD cases (β=0.23, 95%CI 0.10-0.35, p<0.001) and controls (β=0.20, 95%CI 0.12-0.23, p<0.001). GMD cases carrying the G allele of *ABCB1rs3842* also demonstrated a higher CP12 (p=0.02) than controls with the same allele.

Conclusion: The proportion of GMD cases was higher among participants with EFV log CP12 ≥ 3.7 (5000) ng/ml, and this CP12 level independently predicted GMDs. The classical supratherapeutic EFV concentration log CP12 ≥ 3.6 (or 4000) ng/ml failed to show a significant association with GMD. The *CYP2B6*6* and the *ABCB1B1 c.4036 (rs3842) G* alleles were independent predictors of CP12, and thus GMD among PLWH on long-term EFV.

Keywords: antiretroviral therapy; efavirenz; glucose metabolic disorder; HIV; plasma mid-dose concentration, pharmacogenetic variation; *CYP2B6*; ABCB1; genotype; Ethiopia

Introduction

Efavirenz (EFV) has been the preferred non-nucleoside reverse transcriptase inhibitor (NNRTI) component of the first-line combination antiretroviral therapy (cART) in resource-limited countries, until its very recent replacement by dolutegravir. Nevertheless, its use as a first-line agent would continue in resource-limited settings, as its full replacement might take some time. Moreover, it can be considered as a component of alternative first-line therapy in patients ineligible to dolutegravir. EFV has a longer half-life that guarantees its use as a once-daily dosing either in a fixed combination or alone. However, several studies linked EFV use with adverse effects, mainly because of its higher plasma level or its long-term exposure (1). One of the reasons for variation in plasma EFV concentration or exposure is polymorphism of genes involved in EFV disposition. Indeed, high plasma EFV concentrations and CYP2B6 genotype are considered as risk factors for central nervous system (CNS) disturbances during EFV therapy (2).

Evidence shows that the prevalence of glucose metabolism disorders (GMDs) is increasing among people living with HIV (PLWH), specifically those on EFV-based regimens (3-8). These adverse metabolic conditions are major public health concerns and may compromise treatment outcomes and patients' quality of life. The burden is alarming in the sub-Saharan African region, constituting 20-40% of glucose metabolism-related derangements (9).

Like CNS adverse effects, we speculate that glucose metabolism abnormalities may have a relationship with pharmacokinetic factors, which bring about higher plasma concentration or exposure. Several lines of evidence show that genetic variations affect EFV's plasma exposure and treatment outcomes (10). In this regard, the *CYP2B6* genotype

gained a huge attention due to its ubiquitous role in EFV metabolism and higher polymorphic nature (10–13). However, studies assessing the relationship of EFV concentration or genotypes involved in its disposition with the incidence of GMDs are quite limited. The available studies that attempted to address the relationship between EFV and GMD either determined prevalence or identified risk factors. Only one study considered glucose measurements, but not GMDs directly, while mainly focusing on other primary end-points such as lipodystrophy and hyperlipidemia in the analysis (14). The lack of consistent data about the link between EFV plasma concentration and the incidence of GMDs among PLWH is a notable research gap. Studies also barely assessed the effect of pharmacogenetic factors on the incidence of GMDs during long-term EFV-based cART.

Identifying important pharmacokinetic and pharmacogenetic risk factors in the incidence of glucose handling abnormalities among PLWH may have clinical benefits. The evidence generated from this study may help optimize and alert clinicians during regimen selection and follow-up, particularly in the high-risk group of PLWH for glucose metabolism abnormalities. The ultimate benefit would be for patients on long-term EFV-based cART as the findings could be used to improve quality of life and treatment outcomes. Therefore, this study was aimed to determine the association of plasma EFV concentration and related factors with the incidence of GMDs among Ethiopian patients on long-term efavirenz-based cART.

Methods

Study setting

The study was conducted at the HIV clinic of Tikur Anbessa Specialized Hospital (TASH), College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia. The HIV clinic at TASH provides HIV/AIDS prevention, patient care, and ART services. The ART pharmacy supply and dispense ARVs and related medications for patients through scheduled follow-up.

Study design and participants

The study design was a case-control study, and the study was done on sample population (240 participants on EFV) used for a previous cross-sectional study (8). The inclusion and exclusion criteria, treatment adherence, anthropometric measurements, HOMA-IR & body mass index (BMI) calculation, operational definition of GMDs, and determination cut-off points were clearly described in our previous study (8).

Cases were those individuals identified with GMDs and controls were individuals with normal fasting serum glucose, HOMA-IR, or serum insulin level, according to the stated cut-offs described previously (8). Out of the 240 participants, 75 formed the cases and 165 the controls.

The cases and controls were matched in terms of age, duration since cART start, duration on current cART, weight, waist circumference, and BMI. A structured questionnaire was

used to collect additional information, such as co-morbid conditions, concomitant medication use, time of last EFV dose, and BMI.

Sampling and quantification of efavirenz plasma concentration

Blood samples were collected at 12±2 h post-dose in EDTA tubes in duplicates and inverted 8-10 times for gentle mixing. Plasma was immediately isolated from one of the duplicates by centrifugation at 3,000 x *g* for 10 min. The other duplicate (whole blood sample) was stored for DNA extraction to do a pharmacogenetic study. Both plasma and whole blood samples were stored at -80 ºC and then transported in dry ice to the laboratory at Karolinska Institute, Sweden. The samples were then stored at -80 ºC until laboratory analyses.

Chemicals and reagents

EFV reference standard and deuterated-efavirenz (rac Efavirenz-d4) were purchased from Merck (Darmstadt, Germany) and Toronto Research Chemicals (Toronto, ON, Canada), respectively. LCMS analytical grade acetone, acetonitrile, ammonium acetate, isopropanol, methanol, and acetic acid were purchased from Merck (Darmstadt, Germany). Blank human plasma was obtained from the blood bank of the Karolinska University Hospital, Huddinge (Stockholm, Sweden).

LC-MS/MS method and validation

Plasma EFV concentration was determined by LC-MS/MS system consisting of an Acquity Ultra Performance LC-system coupled to a Xevo TQ-S Micro (Waters, Milford, MA, USA), as described by Chala *et al.* (16). The positive ion mode for MS/MS analysis was

selected, and data were processed by MassLynx 4.2 software (Waters). Fragment transitions of EFV and efavirenz-d4 were detected at m/z 316.15→168.15 and 320.05 →172.10, respectively. The chromatographic column consisted of Acquity UPLC BEH C18, 1.8 μ, 2.1 × 50 mm, reversed-phase column (Waters). The mobile phase A and B were 0.1% aqueous formic acid and MeOH, respectively. The initial composition of the mobile phase was 50% B, followed by a linear gradient to 99% in 1.5 min, with a flow rate of 0.4 mL/min. The chromatography run time was approximately 3 min for each sample. The lower limit of quantification (LLQ) was 15.8 ng/ml and the upper limit of quantification was 15.8 µg/ml.

Calibration samples were prepared by spiking blank plasma samples with EFV, included in each analytical run. The lower, middle, and higher concentration quality control samples were also prepared by spiking in blank plasma. Each plasma sample (50 µL) was precipitated in acetonitrile solution (200 µL), which contains the internal standard (500 ng/mL of efavirenz-d4 in methanol). Then, 5 µL of the supernatant was injected into the LC-MS system, after 30 s vortexing and centrifugation (2100x g for 5 min), to determine plasma EFV concentration.

The method was calibrated at each analysis. The calibration curve was calculated with linear regression based on the analyte/internal standard area ratios, weighted as 1/x. The method validation fulfilled the European Medicines Agency Guideline on bioanalytical criteria. The accuracy range was ±10% throughout the quantification range, and precision was <6% of the coefficient of variation (CV) except for LLQ (below 10 CV%). Log10 transformed EFV plasma concentrations values were used for statistical analysis.

DNA Extraction

Blood samples were transported in dry ice package to the Division of Clinical Pharmacology, Department of Laboratory Medicine, Karolinska Institute, Stockholm, Sweden. Genomic DNA was isolated from whole-blood samples using QIAamp DNA MidiKit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions. The purity and quantity of DNA were assessed using NanoDrop 2000 (Thermo Scientific, Saveen Warner, Sweden) based on the manufacturer's protocol. Allelic discrimination assay was performed using TaqMan drug metabolism genotyping assay (Applied Biosystems, CA, USA).

Selection of Genes

SNPs in genes encoding enzymes and drug transport proteins involved in the disposition of EFV (17–20) were selected based on functional significance. The SNPs were *CYP3A4*1B*, *CYP3A5*3*, *CYP3A*6*, *CYP2B6*6*, *UGT2B7*2*, *ABCB1,* and *SLCO1B1*. Genotyping was performed using TaqMan® allele-specific PCR (Applied Biosystems Genotyping Assays) with the following ID numbers for respective SNPs: C__7586657_20 for *ABCB1 3435C→T*, C:11711730_20 for *ABCB1c.4036*, C__7817765_60 for *CYP2B6 516G→T* (*CYP2B6*6*), C__30720663_20 for *UGT2B7 −327G→A* (*UGT2B7*2b, *2c, *2d, *2f*), C:9440184_20 for *UGT2B15*4*, C__26201809_30 for *CYP3A5 6986A→G* (*CYP3A5*3*), C__30203950_10 for *CYP3A5 14690G→A* (*CYP3A5*6*), C:1837671_50 for *CYP3A4* (*CYP3A4*1B*), C:_1901697_20 for *SLCO1B1*1B*, C:30633906_10 for *SLCO1B1*5*.

Genotype analysis

Genotyping was performed using the 7500 Real-Time PCR system (Applied Biosystems, United States). For each reaction, the final volume was 10 μL, consisting of 1 μL of genomic DNA and 9 μL of mix containing 5 μL TaqMan Universal PCR Master Mix® (Applied Biosystems, Waltham, MA, USA), 3.5 μL DNA/RNA free water, and 0.5 μL TaqMan 20× drug-metabolism genotyping assay mix (Applied Biosystems). The PCR conditions were as follows: an initial step at 60 °C for 30 s, hold-stage at 95 °C for 10 min and PCR stage for 40 cycles step 1 with 95 °C for 15 and step 2 with 60 °C for 1 min and after read-stage with 60 °C for 30 s (21).

Statistical analysis

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 26 (IBM® SPSS® Statistics). Baseline sociodemographic and laboratory parameters were described as means and standard deviation (SD) or medians, interquartile range (IQR) for continuous variables, and proportions for categorical variables.

The log-transformed EFV concentration was considered in statistical analysis. The effect of EFV concentration was analyzed among case and control groups using chi-square association by considering log EFV concentration ≥3.5 or 4000 ng/ml as toxic level and below that as non-toxic. Moreover, the same analysis was employed with a subsequent chi-square test by reducing the toxic concentration by log EFV concentration of 0.1 ng/ml to determine the minimum concentration significantly associated with GMDs. The mid-dose plasma (CP12) level of EFV was analyzed between cases and controls among alleles of the

different genotypes. To compare the mean CP12 of EFV between cases, controls, and the genetic variants, an independent t-test was used. To determine predictors of CP12 among cases and controls, a univariate followed by a multivariate linear regression analysis was performed. For this, clinical, organ function, and genetic markers were considered in the linear regression analysis against the log-transformed EFV plasma concentration. Variables with univariate analysis of $p<0.2$ were entered in the multivariate analysis. A stepwise conditional-backward analysis was employed to determine predictors of CP12 EFV concentration in cases and controls.

In all analyses, p-values were two-sided, and p-values of < 0.05 were considered statistically significant. The regression coefficient (β), Crude odds ratio (COR), and adjusted odds ratio (AOR) were recorded from univariate and multivariate logistic regression.

Ethical consideration

Ethical clearance was obtained from the Institutional Review Board of College of Health Sciences, Addis Ababa University (Protocol No. 019/19/SoP) and National Ethical Review Committee, Ministry of Science and Higher Education of Ethiopia (Ref. No. MoSHE/RD14.1/9324/20). Written informed consent was obtained from each study participant after fully explaining the purpose and nature of all procedures used. All participants were informed about their right to withdraw from the study at any stage. Study participants were also briefed that collected data and results would be kept confidential, coded, and no identifiers would be used in any form or publication. All data were kept safely in a locked cabinet and analyzed anonymously.

Results

Plasma samples were analyzed for CP12 EFV concentration for the 240 participants recruited. Of these, 6 samples, however, could not be included in the analysis because of plasma concentration falling below the LLQ or not detected by our method. Thus, a total of 234 PLWH on EFV-based regimen (163 female and 71 males) were included. Table 1 describes sociodemographic and clinical characteristics of the study participants. A large proportion of female participants (79.1%) were in the control group, while males (54.9%) were in the case group. The mean age of participants was 44.1±0.8 and 46.9±1.3 in controls and cases, respectively. None of the participants reported taking any other medication interacting with EFV and traditional medicine from the interview and, later confirmed from the patient medical chart review.

The mean baseline and recent CD4 counts did not significantly differ between cases and controls (p=0.43 and p=0.62, respectively) (Table1). The overall adherence for the treatment was 97.0%, with no apparent difference between the proportions in cases (100%) and controls (95.7%).

Table 1: Study population characteristics and comparison between GMD cases and controls, (n=234).

Demographic and treatment parameters	**Categories**	**Controls** n(%)	**GMD** n(%)	**p**
Age category	18≤45 years	97 (77.0)	29 (23.0)	<0.01
	≥46 years	64 (59.3)	44 (40.7)	
Gender	Female	129 (79.1)	34 (20.9)	<0.001
	Male	32 (45.1)	39 (54.9)	
Treatment adherence (3-day recall test)	Adhered	154 (95)	73 (100)	0.07
	Non-adhered	7 (4.3)	0 (0)	
Specific ARVs contained in cART	TDF containing	148 (70.8)	61 (29.2)	0.07
	AZT containing	14 (53.8)	12 (46.2)	
Age (years) (Mean ± SEM)		44.1±0.8	46.9±1.3	0.06
Time since HIV confirmed date (month)		131.3±3.7	140.5±6.1	0.17

Cumulative time on cART (month)		120.4±3.4	130.4±5.2	0.11
Cumulative time on EFV-based 1st-line (month)		99.1±3.4	106.6±4.9	0.21
Time on current cART regimen type (month)		92.3±3.1	97.8±5.0	0.34
Weight (Kg)		62.9±1.1	63.8±1.4	0.61
Waist circumference (cm)		34.4±0.4	34.7±0.7	0.60
Body Mass Index (Kg/m^2)		24.3±0.4	23.9±0.5	0.54
CD4+ (cells/ul) (baseline)		201.1±12.9	263.9±77.3	0.43
CD4+ (cells/ul) (recent)		534.7±29.3	517.1±19.6	0.62
Laboratory parameters		**$\bar{X}$±SEM**	**$\bar{X}$±SEM**	
Total cholesterol(mg/dL)		211.0±14.6	206.6±5.2	0.84
Triglyceride(mg/dL)		138.8±4.4	183.7±12.2	<0.001
HDL-C(mg/dL)		47.0±2.4	46.0±2.2	0.79
LDL-C(mg/dL)		134.2±10.3	141.0±18.9	0.74
Creatinine		0.98±0.3	2.0±1.3	0.30
BUN (Blood Urea/Nitrogen)		22.0±1.5	22.8±0.8	0.74
AST		22.9±0.9	21.3±1.1	0.29
ALT		18.4±3.7	28.7±9.7	0.22
ALP		116.3±3.9	118.2±5.8	0.79
Direct Bilirubin		0.11±0.009	0.15±0.018	0.04
Total Bilirubin		0.3±0.03	0.3±0.07	0.64
Fasting glucose (mg/dL)		94.3±0.6	118.4±5.0	<0.001
Fasting insulin (uU/ml)		7.2±0.3	16.7±2.4	<0.001
HOMA-IR (µU/ml)		1.7±0.1	5.2±1.0	<0.001
Genotypes	**Minor alleles**			
*CYP3A4*1B*	**1B*	0.57	0.64	0.30
*CYP3A5*3*	**3*	0.90	0.86	0.40
*CYP3A5*6*	**6*	0.24	0.40	0.01
*CYP2B6*6*	**6*	0.50	0.60	0.16
UGT2B7 g.-372 G>A	*A*	0.76	0.77	0.96
ABCB1B c.3435	*T*	0.45	0.41	0.60
ABCB1B c.4036 (rs3842)	*G*	0.36	0.31	0.50
*SLCO1B1*1B*	**1B (G)*	0.86	0.89	0.49
*SLCO1B1*5*	**5 (C)*	0.32	0.34	0.77
EFV concentration-related parameters				
Sampling time after the last dose of EFV (h:m)		12:51±1:02	12:49±1:05	0.79
log CP12 EFV concentration (ng/ml)		3.5±0.02	3.6±0.04	0.14
Mean CP12 EFV concentration (ng/ml)		3829.8±230.5	4934.8±536.9	0.03$^{\#}$
Participants with log CP12 EFV ≥3.6 (4000 ng/ml)), n (%)		46 (28.6)	28 (38.4)	0.14
Participants with log CP12 EFV ≥ 3.69 (5000 ng/mL), n (%)		26 (16.1)	21 (28.8)	0.026

n (%) are frequencies and percent proportions, $\bar{X}$±SEM=mean ± standard error of the mean, $^{\#}$ based on an independent t-test when equal variances assumed, HDL-C=High density lipoprotein Cholesterol, LDL-C= Low-density Lipoprotein Cholesterol, AST=Aspartate aminotransferase, ALT=alanine aminotransferase, ALP=alkaline phosphate, CP12= mid-dose EFV plasma concentration.

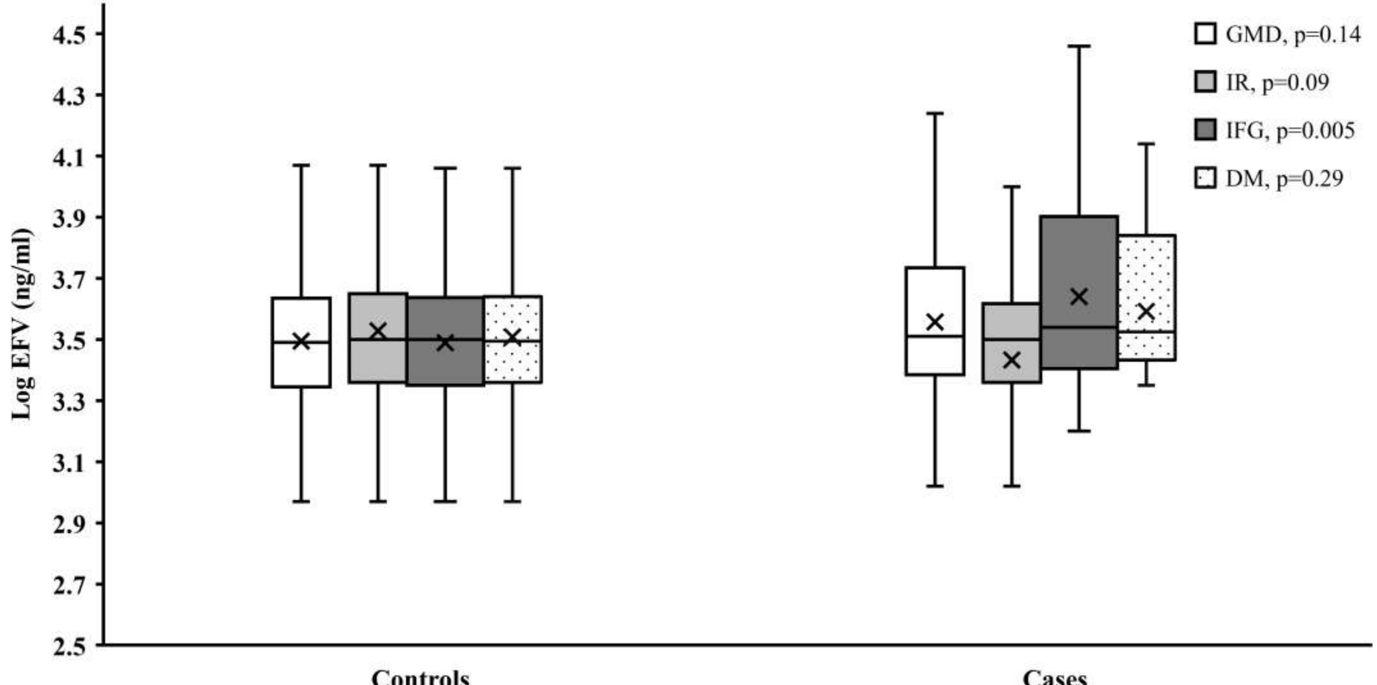
Mid-dose (CP12) EFV plasma concentration

Mean (±SEM) sample collection time for mid-dose interval EFV plasma concentration was 12:51±1:02 and 12:49±1:05 h for controls and cases, respectively (Table 1), and no detectable difference (p=0.79) in sampling time was observed between the two groups. The Log CP12 EFV concentrations for controls and cases were 3.5±0.02 and 3.6±0.04, respectively, and no statistically significant difference (p=0.14) was noted between the two groups (Table 1). But the geometric mean of the control (3829.8±230.5 ng/ml) and case (4934.8±536 ng/ml) group showed a significant difference (p=0.03) based on an independent t-test.

Of the 240 patients enrolled, about 3.0 (7/234), 65.4 (153/234), and 31.6% (74/234) had CP12 range of sub-therapeutic (below 1000 ng/ml), therapeutic (between 1000 and 4000 ng/ml), and supratherapeutic (4000 ng/ml and above) plasma EFV concentrations, respectively. The proportion of participants with CP12 above the recommended level (≥ 4000 ng/ml) was higher in cases (38.4%) than controls (28.6%) despite failing to demonstrate significance (p=0.14). On the other hand, the concentration of EFV ≥3.7 ng/ml, which corresponds to above 5000 ng/ml of geometric mean, was the minimum concentration to be significantly associated with the incidence of GMD. The proportion of participants with EFV log concentration of ≥3.7 ng/ml was significantly higher in cases (28.8%, p=0.026) than controls (16.1%).

Mean log CP12 EFV concentration was compared between controls and cases as well as cases with specific traits of GMDs (Diabetes mellitus, DM; Impaired fasting glycemia, IFG; and Insulin resistance, IR). As shown in Figure 1, no apparent difference was noted in mean CP12 between GMD cases and controls as well as controls and cases with IR and DM. By contrast, a significantly greater (p=0.005) mean log CP12 (3.62±0.05) (4168.9 ng/ml) was found among IFG cases when compared to the controls (3.50±0.03), which

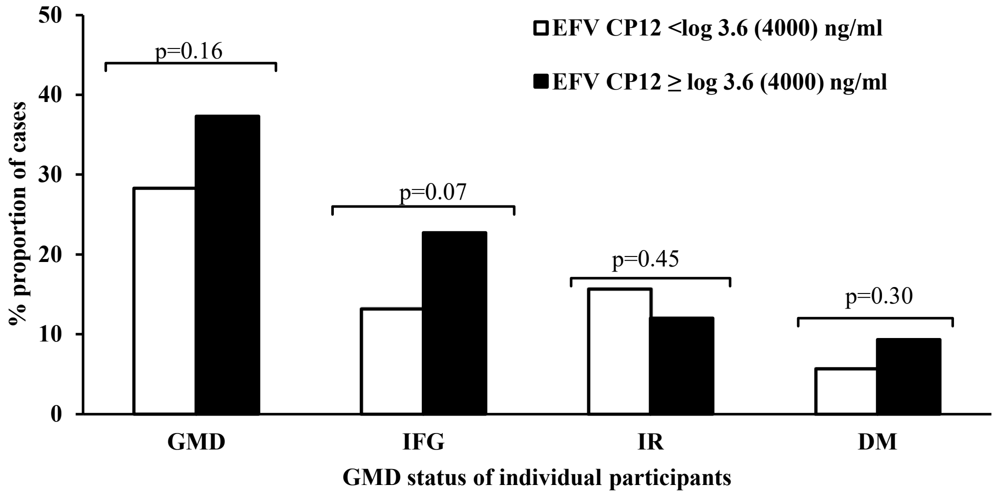
corresponds to 3162.3 ng/ml. Compared with controls, the GMD cases failed to show a significant mean difference in terms of log CP12 (p=0.14). However, the geometric mean showed a significantly elevation of CP among GMD cases (4,934.8±536.9 ng/ml) than the controls (3829.8±230.5) (p=0.03 when equal variance is assumed).



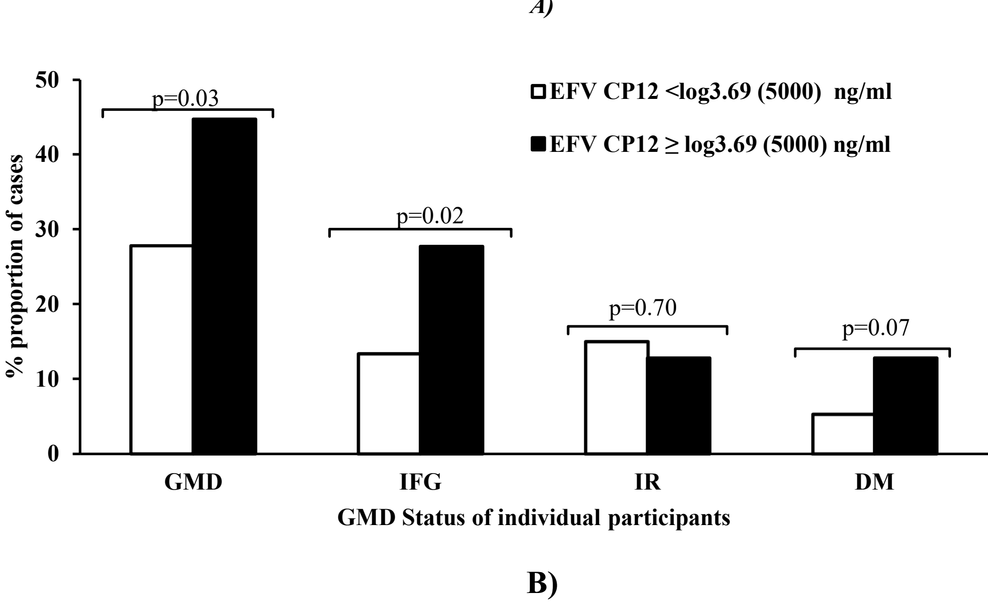
fasting glycemia, insulin resistance, and diabetes: the 'x' in the box represents the mean, boxes represent the log EFV concentration range with the horizontal line within the box represent the median CP12, and the upper and lower horizontal edges of the box represent the interquartile ranges (Q1-Q3), and the whiskers are 95% confidence interval. GMD=glucose metabolism disorder, IR=insulin resistance, IFG=impaired fasting glucose, DM=diabetes Mellitus, and p=level of statistical significance. p= the level of statistical significance mean difference in log EFV concentration between individual cases and controls.

An attempt was also made to see the distribution of EFV concentration among cases and cases with specific traits of GMDs using the cut-off CP12 concentrations (Figure 2). Apparently, no significant difference was observed in the proportion of all forms of GMD when log 3.6 (or 4000) ng/ml concentrations were used to compare respective controls (Figure 2A). Interestingly, the proportion of participants with CP12 ≥ log 3.69 (5000)

ng/ml was significantly higher in cases with GMD (p=0.03) and IFG (p=0.02) than the control (Figure 2B).



A)



B)

Figure 2: Proportion of GMDs among study participants with EFV concentrations cut-off values of: (A) below log3.6 vs log 3.6 and above (4000) ng/ml. (B) below log 3.69 vs log 3.69 and above (5000) ng/ml: p=level of statistical significance for comparison between case and controls, GMD=Glucose metabolism disorders, IFG=impaired fasting glycemia, IR=insulin resistance, and DM=Diabetes Mellitus.

Pharmacokinetics related predictors of GMDs

Multivariate analysis of pharmacokinetic factors of EFV revealed that gender, triglyceride level, and EFV log CP12≥3.69 ng/ml were independent predictors of GMDs among patients on EFV-based cART (Table 2). Males had about 3.8 times higher risk of GMDs than female patients on EFV (AOR=3.8, 95%CI 2.0-7.1, p<0.001). A unit increase in triglyceride level showed a 0.06% increase in GMD cases (AOR=1.006, 95%CI 1.002-1.010, p<0.00). Patients with EFV Log CP12≥3.69 ng/ml showed a 2.2 times higher risk of GMDs than EFV CP12 below 3.69 ng/ml (AOR=2.2, 95% CI 1.1-4.6, p=0.03). In the multivariate analysis, replacing CP12≥3.69 ng/ml with CP12≥4.0 (geometric mean ≥10,000) ng/ml doubled the risk of GMDs (β=1.4, AOR=4.2, 95%CI 1.4-13.0, p=0.01).

Table 2 Pharmacokinetics related predictors of GMDs among study participants on long-term EFV-based cART (N=234).

Variables		**Univariate analysis**			**Multivariate analysis**		
		β	**COR (95%CI)**	**p**	**β**	**AOR (95%CI)**	**p**
Age		0.03	1.03(1.0, 1.1)	0.06			
Sex F=reference		1.5	4.6(2.5, 8.4)	<0.00	1.3	3.8 (2.0, 7.1)	<0.00
Weight		0.01	1.0(0.98, 1.03)	0.65			
Total cholesterol		0.00	1.0(0.99, 1.00)	0.84			
Triglyceride		0.01	1.008(1.004,1.012)	<0.00	0.006	1.006(1.002, 1.01)	<0.00
LDL		0.00	1.0(0.99, 1.002)	0.74			
HDL		-0.002	0.99(0.99, 1.01)	0.79			
Creatinine		0.02	1.02(0.98, 1.06)	0.35			
Urea		0.003	1.003(0.99, 1.02)	0.74			
AST		-0.02	0.98 (0.95, 1.02)	0.30			
ALT		0.003	1.003(0.998, 1.007)	0.26			
ALP		0.001	1.001(0.995, 1.006)	0.79			
Direct Bilirubin		2.2	9.4(0.9, 100.6)	0.06			
Total Bilirubin		0.14	1.2(0.6, 2.1)	0.64			
Genotype and CP12 variables							
*CYP3A4*1B*	**1B*	0.30	1.4(0.8, 2.4)	0.30			
*CYP3A5*3*	**3*	-0.4	0.7(0.3, 1.6)	0.40			
*CYP3A5*6*	**6*	0.8	2.1(1.2, 3.9)	0.01			

*CYP2B6*6*	**6*	0.4	1.5(0.9, 2.6)	0.16			
UGT2B7 g.-372 G>A	*A*	0.02	1.02(0.53, 1.97)	0.99			
ABCB1B 3435	*T*	-0.15	0.9(0.5, 1.5)	0.61			
ABCB1B c.4036 (rs3842)	*G*	-0.2	0.8(0.4, 1.5)	0.50			
*SLCO1B1*1B*	**1B G*	0.3	1.4(0.6, 3.2)	0.50			
*SLCO1B1*5*	**5 C*	0.1	1.1(0.6, 2.0)	0.77			
Log CP12≥3.6 (4000) ng/ml		0.41	1.5 (0.8, 2.7)	0.17			
Log CP12≥3.7 (5000) ng/ml[§]		0.74	2.1(1.1, 4.0)	0.03	0.8	2.2(1.1, 4.6)	0.03

[§]when log CP12≥3.6 (4000) ng/ml was replaced by Log CP12≥3.7 (5000) ng/ml

Association of EFV Log CP12 and Genotype variants

In Figure 4, the mean CP12 of EFV is plotted against genotype variants in both cases and controls. The log CP12 was found to be significantly higher in both cases (p=0.002) and controls (p=0.000) carrying *CYP2B6*6* compared to their corresponding wildtype allele (Figure 4A). However, no apparent difference in concentration was observed between the two groups carrying the same allele.

As depicted in Figure 4B, no apparent difference in CP12 levels was noted between cases and controls carrying the G allele of *ABCB1rs3842* when compared to their corresponding wild type allele, although cases with G allele tended to increase CP12 levels, which failed to reach statistical significance. Within the same allele, no difference was noted between the two groups in those carrying the wild type allele. However, in the G allele, cases produced a significantly higher (p=0.02) CP12 concentration compared to controls.

Cases with *SLCO1B1*1B* A allele showed a significantly higher EFV CP12 (p<0.01) compared to controls with the same allele as well as cases with the G allele (p<0.01). In contrast, cases and controls with the G allele did not significantly differ in their CP12 levels. Likewise, no apparent difference was noted between controls carrying A and G alleles (Figure 3C).

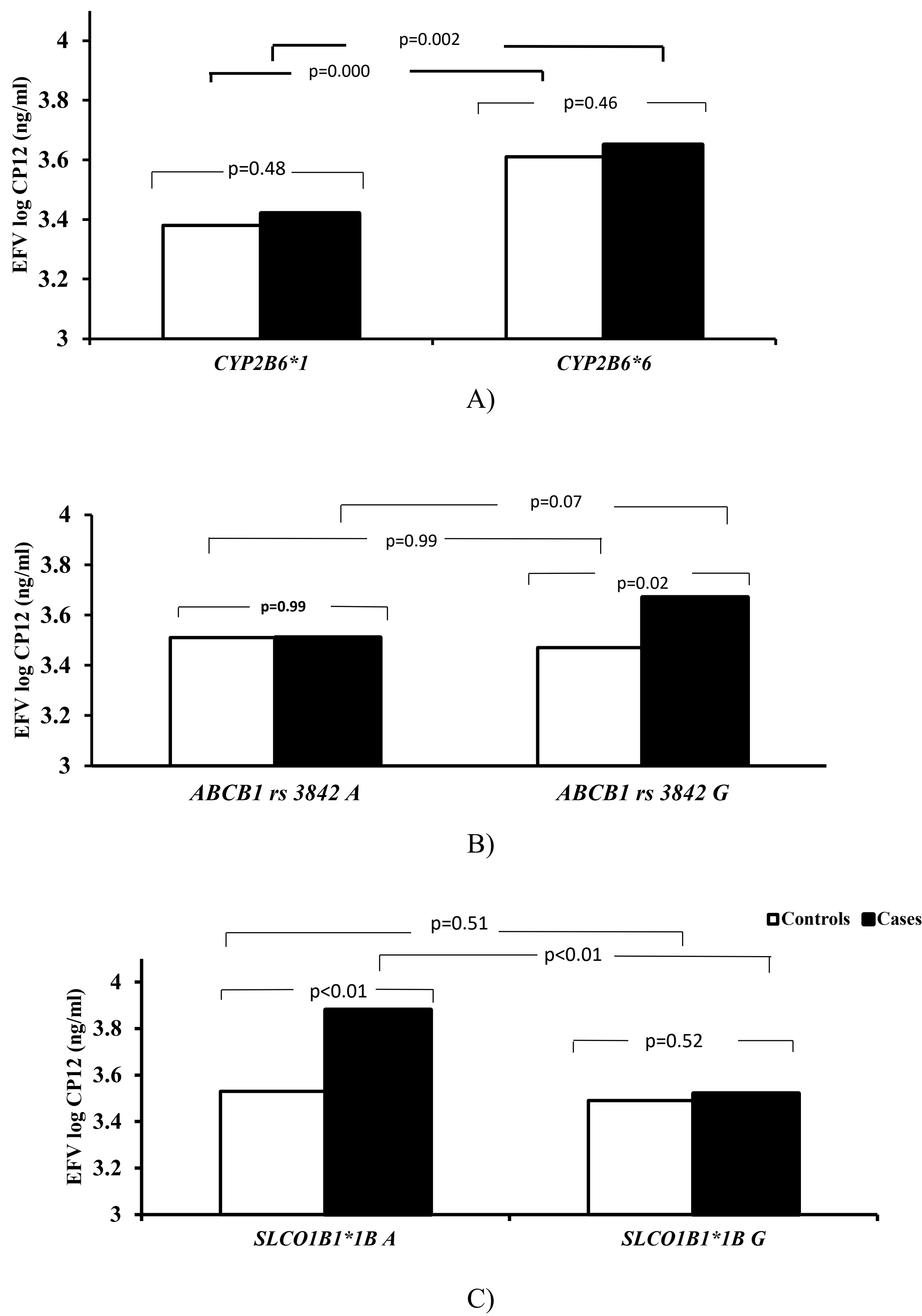


Figure 3: Log CP_{12} EFV concentration (Log ng/ml) vs *CYP2B6* (A), *ABCB1c.4036 (rs3842)* (B), and *SLCO1B1*1B* (C) alleles in cases and controls.

Predictors of Log CP12 of EFV

Overall study participants

Table 3 presents a univariate and multivariate linear regression analysis of EFV log CP12 among all cases and controls. The multivariate analysis revealed that whilst age (β=0.005, 95%CI 0.001-0.008, p<0.01) and *CYP2B6*6* (β=0.215, 95%CI 0.143-0.288, p<0.001) were positively associated, weight (β=-0.003, 95%CI -0.006-0.000, p=0.03) was negatively associated with CP12 of EFV.

Among cases and controls

Table 4 depicts the linear regression analysis of EFV CP12 for cases and controls. The *CYP2B6*6* allele positively predicted log CP12 of EFV in both cases (β=0.23, 95%CI 0.10-0.35, p<0.001) and controls (β=0.20, 95%CI 0.12-0.23, p<0.001). On the other hand, *CYP3A5*6* showed a negative association (β=-0.1, 95%CI 0.2-0.01, p=0.03) with log CP12 of EFV only in controls.

Variables such as age, HDL, ALP, *CYP2B6*6,* and G allele of *ABCB1B rs 3842* were identified to be independent predictors of log CP12 of EFV among cases in a positive manner. On the other hand, direct bilirubin levels were negatively associated with CP12 among cases (Table 4).

Table 3: Predictors of mid-dose EFV log-concentration among the overall study participants, with and without GMD, N=234.

Variables		r^2	**Univariate analysis**		**Multivariate analysis**	
			Beta coefficient (95%CI)	**p**	**Beta coefficient (95%CI)**	**p**
Age		0.03	0.005(0.001, 0.008)	0.01	0.005 (0.001, 0.008)	<0.01
Sex F=reference		0.00	-0.002(-0.088, 0.083)	0.95		
Weight		0.02	-0.003(-0.006, 0.000)	0.05	-0.003 (-0.006, 0.000)	0.03
Fasting serum glucose		0.01	0.001 (0.000-0.002)	0.18	0.001 (-0.001, 0.003)	0.18
Fasting serum insulin		0.02	-0.004 (-0.007, 0.000)	0.03		
HOMA-IR		0.01	-0.005(-0.013-0.002)	0.19	-0.009 (-0.019, 0.000)	0.06
Total cholesterol		0.01	0.000 (0.000, 0.000)	0.29		
Triglyceride		0.00	0.00002 (0.000, 0.001)	0.94		
LDL		0.01	0.000 (0.000, 0.000)	0.17		
HDL		0.01	0.001 (0.000, 0.003)	0.12		
Creatinine		0.01	0.005 (0.000, 0.01)	0.07	0.004 (-0.001, 0.009)	0.11
Urea		0.00	0.000 (-0.002, 0.002)	0.74		
AST		0.01	0.003(-0.001, 0.006)	0.29		
ALT		0.00	0000(-0.001, 0.001)	0.22		
ALP		0.03	0.001(0.000, 0.002)	<0.01		
Direct Bilirubin		0.00	-0.115(-0.423, 0.193)	0.46		
Total Bilirubin		0.00	-0.008 (-0.098, 0.082)	0.86		
Genotype variables						
*CYP3A4*1B*	**1B*	0.00	-0.01(-0.09, 0.07)	0.75		
*CYP3A5*3*	**3*	0.00	-0.02(-0.15, 0.10)	0.75		
*CYP3A5*6*	**6*	0.001	-0.03(-0.11, 0.06)	0.57		
*CYP2B6*6*	**6*	0.15	0.23(0.16, 0.30)	<0.001	0.215(0.143, 0.288)	<0.001
UGT2B7 g.-372 G>A	*A*	0.002	-0.03(-0.12, 0.06)	0.52		
ABCB1B c.3435	*T*	0.001	-0.02(-0.10, 0.06)	0.65		
ABCB1B c.4036 (rs3842)	*G*	0.001	0.02(-0.06, 0.10)	0.66		
*SLCO1B1*1B*	**1B G*	0.02	-0.12(-0.24, -0.01)	0.04		
*SLCO1B1*5*	**5 C*	0.00	0.004(-0.08, 0.09)	0.92		

Table 4: Predictors of EFV mid-dose log-concentration between the cases (n=73) and the controls (161) of study participants on long-term EFV-based cART (N=234).

Variables		**Controls**					**Cases**				
		r^2	**Univariate analysis**		**Multivariate analysis**		r^2	**Univariate analysis**		**Multivariate analysis**	
			Beta coefficient (95%CI)	**p**	**Beta coefficient (95%CI)**	**p**		**Beta coefficient (95%CI)**	**p**	**Beta coefficient (95%CI)**	**p**
Age		0.00	0.00(-0.003, 0.01)	0.71			0.12	0.011(0.004, 0.017)	<0.01	0.009(0.004, 0.015)	<0.01
Sex F=reference		0.00	-0.005(-0.094, 0.083)	0.91			0.26	-0.181(-0.344, -0.018)	0.03		
Weight		0.01	-0.002(-0.005, 0.001)	0.13	-0.002(-0.01, 0.0)	0.1	0.02	-0.004(-0.011, 0.003)	0.22		
Fasting serum glucose		0.02	0.005(-0.001, 0.01)	0.12			0.00	0.000(-0.002, 0.002)	0.70		
Fasting serum insulin		0.00	-0.003(-0.015, 0.01)	0.67			0.09	-0.005(-0.009, -0.001)	0.01		
HOMA-IR		0.00	-0.008(-0.06, 0.04)	0.75			0.04	-0.008(-0.02, 0.002)	0.11	-0.01(-0.01, 0.00)	0.15
Total cholesterol		0.06	0.000(0.00, 0.00)	0.47			0.27	0.002(0.00, 0.004)	0.02	0.001(0.00, 0.003)	0.09
Triglyceride		0.01	0.001(0.000, 0.001)	0.21			0.02	0.000(-0.001, 0.000)	0.25		
LDL		0.02	0.000(-0.001, 0.000)	0.09			0.00	0.000(-0.001, 0.000)	0.76		
HDL		0.00	0.000(-0.001, 0.002)	0.52			0.07	0.005(0.001, 0.009)	0.02	0.001(0.003, 0.01)	<0.001
Creatinine		0.02	0.009 (-0.001, 0.018)	0.08	0.01(-0.003, 0.015)	0.17	0.01	0.003(-0.004, 0.01)	0.37		
Urea		0.00	0.000(-0.002, 0.002)	0.82			0.01	0.006(-0.006, 0.017)	0.32		
AST		0.02	0.003(-0.001, 0.007)	0.12			0.01	0.003(-0.006, 0.011)	0.57		
ALT		0.00	0.000(-0.001, 0.001)	0.82			0.01	0.000(-0.001, 0.001)	0.44		
ALP		0.03	0.001(0.000, 0.002)	0.05			0.05	0.002(0.000, 0.003)	0.05	0.001(0.000, 0.003)	0.02
Direct Bilirubin		0.02	0.33 (-0.04, 0.71)	0.08			0.10	-0.75(-1.3, -0.2	<0.01	-0.73 (-1.1, -0.34)	<0.001
Total Bilirubin		0.01	0.09(-0.03, 0.22)	0.14	0.1(-0.01, 0.21)	0.09	0.02	-0.09(-0.23, 0.05)	0.21		
*CYP3A4*1B*	**1B*	0.00	0.001(-0.09, 0.09)	0.98			0.01	-0.06(-0.23, 0.11)	0.49		
*CYP3A5*3*	**3*	0.00	0.02 (-0.12, 0.17)	0.77			0.01	-0.08 (-0.32, 0.16)	0.53		
*CYP3A5*6*	**6*	0.01	-0.07 (-0.17, 0.03)	0.16	-0.1(-0.2, -0.01)	0.03	0.00	0.02(-0.15, 0.19)	0.79		
*CYP2B6*6*	**6*	0.16	0.23(0.15, 0.30)	<0.001	0.2(0.12, 0.23)	<0.001	0.11	0.24(0.08, 0.39)	<0.01	0.23 (0.10, 0.35)	<0.001
UGT2B7 g.-372 G>A	*A*	0.01	-0.06(-0.16, 0.05)	0.28			0.00	0.03(-0.17, 0.22)	0.79		
ABCB1B c.3435	*T*	0.00	-0.01(-0.1, 0.07)	0.75			0.00	-0.02(-0.19, 0.15)	0.81		
ABCB1B c.4036 (rs3842)	*G*	0.00	-0.04(-0.13, 0.05)	0.41			0.05	0.16(-0.01, 0.33)	0.07	0.14 (0.01, 0.27)	0.04
*SLCO1B1*1B*	**1B*	0.00	-0.01(-0.08, 0.06)	0.74			0.06	-0.1(-0.26, -0.004)	0.04		
*SLCO1B1*5*	**5*	0.00	-0.01(-0.11, 0.08)	0.76			0.00	0.04(-0.13, 0.21)	0.64		

Discussion

The present study aimed at determining the plasma CP12 and other factors associated with the incidence of GMD among PLWH on long-term EFV-based cART. This is the first study generating evidence linking the plasma EFV concentration and genotype of metabolizing enzymes as well as drug-transporters with GMDs such as IR, IFG, and DM among PLWH.

Among overall study participants, 65.4% had CP12 of EFV within therapeutic range and only 3% showed sub-therapeutic plasma concentration. While 31.6% of the study participants had supratherapeutic plasma EFV concentrations. Compared to a study from Tanzania, the proportion of patients with sub-therapeutic EFV concentration is lower in our study (3.0 vs 20.5%) (23). While the proportion of patients with therapeutic (65.4 vs 56.2) and supra-therapeutic (31.6 vs 23.3%) concentrations is slightly higher in our study than the Tanzanian study (23).

The overall supratherapeutic and subtherapeutic proportions reported in our study were different compared to other earlier studies (22–24). The reason for this could be due to differences in study participant composition, sampling time, adherence, or genotype frequency affecting EFV CP12. For instance, one of the studies (22), included PLWH on EFV and anti-TB drugs (vs. only EFV-based cART in our study) and the sampling time was 14±2 h post-dose (vs. 12±2 h). Other probable reasons could be the difference in participants' duration of time on ART or treatment adherence issues. In one of the studies (23), the median duration was 40 months (vs. 111.5 months) and the level of non-adherence was 15.8% (vs. 3%). A difference in *CYP2B6*6* allele frequency could also be a main

reason for such disparity. One study (24), for example, used a higher frequency of *6 (T) allele for comparison than our study.

Notably, the proportion of supratherapeutic plasma concentration was higher among cases (38.4%) than controls (28.6%), when within group proportion is calculated. A similar trend of GMD proportions was observed among those with EFV log CP12 ≥ 3.7 (or 5000 and above) ng/ml (44.7%) than those with subtoxic levels (27.8%) with significant association (p=0.026). GMDs were observed in a higher proportion among individuals with supratherapeutic plasma levels. This finding is consistent with the findings that state the incidence of adverse effects, especially the CNS side effects, are associated with supratherapeutic levels (22,24,25).

Importantly, we demonstrated that PLWH with EFV log CP12 above 3.7 ng/ml (or above 5000 ng/ml) had a significant risk for GMDs. Multivariate analysis revealed that EFV log CP12 >3.7 ng/ml was an independent predictor of GMDs among patients on long-term EFV. This suggests that EFV concentration higher than the classical supratherapeutic concentration (≥4000 ng/ml) might highly contribute to the incidence of GMDs. The risk of GMDs increased when the EFV log CP12 increased. The multivariate analysis supported this suggestion in that the risk of GMDs among those with EFV log CP12≥3.7 (5000) ng/ml (AOR=2.2, 95%CI 1.1-4.6, p=0.03) was significantly higher than the risk in the log CP of 3.6 (or 4000) ng/ml concentrations. This might indicate that increased exposure and high plasma concentration of EFV could have a role in GMDs incidence among PLWH on long-term EFV.

Our study identified *CYP2B6*6* as a marker of higher EFV CP12 in PLWH in both cases and controls. Similarly, GMD cases with *ABCB1c.4036 (rs3842)* G allele exhibited a significant elevation of EFV log CP12 compared to cases with the same allele. Moreover, a significantly higher EFV log CP12 was associated with *SLCO1B1*1B A* allele in GMD cases than controls carrying the same allele and the G allele carriers in both case and control groups. Unlike the *CYP2B6*6* and the *ABCB1c.4036* (rs3842) G alleles, the *SLCO1B1*1B A* allele did not show a significant predictive association with CP12 with GMD in the multivariate linear regression. Different studies generated evidence that *CYP2B6*6* and *ABCB1 c.4036* (rs3842) genotypes are major factors associated with elevated log EFV CP12 and incidence of adverse effects, particularly the CNS related effects (10,12,13,16,26). The fact that genotypes associated with higher EFV CP12 were also associated with higher EFV CP12 among GMD cases than controls may imply that GMD could result from elevated plasma concentrations of EFV. Like the CNS adverse effects, we speculate that higher EFV concentration might be the culprit for the incidence of GMDs among PLWH on EFV-based regimens.

Our study also revealed that age, *CYP2B6*6* allele, and body weight were significant predictors of CP12 of EFV among the overall study participants. Whereas specific to GMD cases, age, HDL, ALP, direct bilirubin, *CYP2B6*6,* and *ABCB1B rs3842* (G allele) genotypes were predictors of the log EFV CP12. A similar finding was reported by Huang et al. (27). Though GMD status was not considered, CYP2B6*6 and weight were demonstrated to be a positive and negative predictor of EFV CP12, respectively. Other studies also showed that body weight has a negative trend of association with EFV CP12 (27,28) and this is consistent with our finding in the overall study participants, although

the association was lost when disaggregated into cases and controls. Taken together, these findings collectively reinforce the notion that *CYP2B6*6* can be used as a possible marker for predicting CP12 concentration in HIV patients on long-term EFV therapy.

In participants with GMDs, factors such as age and biochemical markers (HDL, ALP, and direct bilirubin) predicted EFV log CP12. A direct correlation between HDL-C and EFV plasma concentration was reported. A concentration-dependent change in lipid profile, specifically an increase in HDL-C, was suggested as a beneficial effect of long-term EFV therapy (28,29). A recent multi-centered prospective study conducted on HIV-infected children reported that low pre-treatment LDL-C level, which has an inverse relation with HDL-C, predicted an increase in plasma EFV exposure (16). Consistent with our data, *i.e.,* a negative predictor effect of direct bilirubin with EFV CP12, a study conducted on a small number of healthy volunteers, though GMD was not in focus, indicated that EFV reduced the level of conjugated (direct) bilirubin (30). In fact, in this study, it was stated that the total bilirubin, unlike our data, was also negatively affected by EFV (30). Similar studies also indicated that monotherapy of EFV resulted in a significantly lower plasma total bilirubin through hemoglobin independent effect (31). The negative trend of association between EFV concentration and bilirubin was also consistent with other studies (30,31). ALP also predicted the Log EFV mid-dose concentration positively, specifically among participants with GMDs. No other study reported a significant association of ALP with EFV CP12 making comparison difficult.

The study has some limitations. Timing of EFV dose was based on participants' reports of the last dose. However, most participants reported that they took their medications in the evening (20:00-22:00), which allowed a suitable sampling time the next morning. We have

also used a one-time sampling and follow-up for diagnosis and measurement of lab parameters. Yet, the average value of two to three repeated test results was considered for analysis, which might offset the stated limitation. Our study also focused only on genes important in the pharmacokinetics of EFV and did not consider phenotypic parameters such as detecting the level of EFV metabolites. Therefore, we recommend confirmatory studies on a larger sample size with more extended and repeated follow-up for future studies.

Conclusion

This study revealed that mid-dose plasma EFV concentration (CP12) predicts GMDs among PLWH on long-term EFV-based cART. GMD cases were significantly higher among participants with higher EFV log CP12≥3.7 (5000) ng/ml. EFV log CP12≥3.7 ng/ml independently predicted GMDs, and increasing doses beyond this level resulted in an increased risk of GMDs while the classical supra-therapeutic EFV concentration, *i.e.* log CP12≥3.6 or 4000 ng/ml, failed to show significant association with GMD. *CYP2B6*6* and the *ABCB1B1 c.4036 (rs3842) G* alleles were associated with high EFV log CP12 and identified as an independent predictor of GMD occurrence among PLWH on long-term EFV-based cART. If introduced into clinical practice, plasma EFV concentration measurements may help determine GMDs risk and early detection and intervention.

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Author's contribution

WT, EA, EE, and WA conceived and designed the study. WT and WA were responsible for patient enrollment and supervision at the study site. WT, EM, and NF were responsible for sample preparation, sample analysis, and data recording during genotyping and LCMS/MS analysis. EA supervised the genotyping, LCMS/MS analysis, and data analysis. EA and WS facilitated the acquisition of grants. WT was responsible for data analysis and drafting the manuscript. EE, WS, WA, and EA supervised the overall study. All co-authors revised the manuscript and read and approved the final version.

Declaration of interest

The authors declare that there is no competing interest.

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PAPER IV

Pharmacogenetic marker for glucose metabolism disorders among patients on atazanavir/ritonavir-based antiretroviral therapy: the implication of *CYP3A* and *SLCO1B1*5*

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Abstract

Objective: HIV-infected patients on long-term combination antiretroviral treatment (cART) are at a higher risk of developing glucose metabolism disorders (GMDs). Genetic variation in drug-metabolizing CYP enzymes and transporter proteins may influence antiretrovirals' disposition and adverse metabolic effects. We investigated the association between polymorphisms in relevant drug-metabolizing and transporter genes with GMDs among HIV patients on cART.

Methods: This was a case-control study that included people living with HIV (PLWH) on ATV/r-based cART (n=111), including patients with GMD (cases, n=22) and those without GMD (controls n=89). GMDs were defined as the presence of impaired fasting glucose, insulin resistance (IR), or diabetes mellitus (DM). Participants were genotyped for *CYP3A (4*1B*, *5*3*, *5*6)*, *ABCB1* rs3435, *ABCB1* rs3842, and *SLCO1B1(*1b*, **5)*. Then, case-control genotype association and logistic regression analysis were adjusted for age, sex, and BMI.

Results: The overall study participants' mean age (SEM) was 39.9±1.0 years, with the majority being female (73.9%, 82/111). The proportion of males (25.8 vs. 27.3%) and females (74.2 vs. 72.7%) was relatively similar between the cases and the control groups (p=0.89). All genotype frequencies followed the Hardy-Weinberg Equilibrium (HWE) (p>0.05) between the case-control groups. The haplotype analysis of *CYP3A4*1B*, *CYP3A5*6*, and *5*3* showed that *CYP3A5*3* alone is the major haplotype (49.1%), followed by *CYP3A4*1B* alone (19.2%), and then **1B* linkage with **3* (9.8%).

In the multivariate analysis, the SL_CO1B1*5 c.521 T >C_ genotype significantly predicted GMD that the C allele carriers demonstrated a 2.9 times higher risk of GMD [AOR=2.9; 95% CI 1.03-8.1, p=0.04] than the wildtype allele carriers. A respective 80.0% (p=0.03) and 90.0% (p=0.01) protection from GMD was recorded for haplotypes containing any _*6_ and only _*3_ of _CYP3A_ than the wildtype combination haplotypes after multivariate analysis. In contrast, a 90% protection from IR was recorded for both haplotype combinations containing any _*6_ (p=0.03) and only _*3_ (p=0.01) types than the wildtype haplotype combinations. Also, the G allele carriers of _ABCB1 c.4036_ demonstrated an 80.0% protection of IR (p=0.05) than the wildtype allele.

Conclusions: The _CYP3A_ haplotype combination consisting of *6 and *3-only of CYP3A 4*1B/5*3/5*6 was a protective predictor of GMD and IR among patients with ATV/r-based cART. Also, carriers of the G allele of _ABCB1 c.4036_ were more protected from IR than the wildtype carriers. Whereas carriers of the C allele of _SLCO1B*5_ were at high risk of developing glucose metabolism disorders than wildtype carriers on ATV/r-based cART.
Keywords: antiretroviral therapy; atazanavir; ritonavir; glucose metabolic disorder; HIV; pharmacogenetic variation; _CYP3A_; _CYP3A5*3_; _CYP3A5*6_; _SLCO1B*5_; genotype; Ethiopia

Introduction

Antiretroviral therapy (ART) dramatically arrested the devastating sequelae of HIV infection, improving the longevity and the quality of life of People Living with HIV (PLWH) (1). The most effective and efficient treatment strategy relies on combination ARTs, consisting of at least three antiretrovirals (ARVs) targeting the different viral replication processes. As a first-line regimen, two nucleoside reverse transcriptase inhibitors (NRTIS) and one non-nucleoside reverse transcriptase inhibitors (NNRTI) were the commonest combination antiretroviral therapy (cART). However, recently integrase strand transfer inhibitors, like dolutegravir, have replaced NNRTIs, specifically efavirenz, from the first-line regimen in all guidelines. In situations of treatment failure and severe adverse effects, a second-line alternative can replace first-line regimens consisting of protease inhibitors (PIs)─the cornerstone of a second-line cART. Currently, ritonavir-boosted atazanavir (ATV/r)-based combination antiretroviral therapies (cART) are widely employed as second-line regimens (2).

Studies have shown that PIs may cause metabolic disorders, including abnormal glucose metabolism ranging from insulin resistance, impaired glucose tolerance, and impaired fasting glucose to type 2 diabetes (3,4). Older PIs such as lopinavir and ritonavir are highly associated with these effects (5). A cross-sectional study reported a five to nine-fold elevated prevalence of type 2 DM among HIV-infected patients with protease inhibitors (6). Similarly, another study that evaluated glucose handling factors in 80 Thai adolescents on PI-based ART showed about 22.1% and 3.8% prediabetes and overt type 2 diabetes mellitus states. Although the older PIs were studied well, newer PIs [such as atazanavir

(ATV)] are less studied. ATV is a first-line PI with relatively less side effects and a better tolerability profile (7). However, its use commonly requires a booster ritonavir combination associated with some adverse effects such as hyperlipidemia and hyperglycemia (8,4). The association with metabolic and DM-associated adverse effects needs to be studied to determine the various risk and factors during ATV-based cART, including pharmacokinetic variability and genetic polymorphisms significant to their disposition.

The metabolism of atazanavir (ATV) is mediated through *CYP3A,* which includes *3A4* and *3A5*, and *UGT1A1* enzymes, while *ABCB1* and *SLCO1B* transporters are involved in its disposition (4,9–11). Polymorphisms in genes responsible for drug metabolism and transport can alter plasma concentration and treatment exposure, affecting treatment outcomes and PI-related metabolic abnormalities. SNPs of *CYP*, *UGT*, and *ABCB1* genes essential in ATV (12) disposition are associated with variable plasma concentrations, thereby altering the incidence of ADRs of ATV-based regimens. The best example indeed was the association of hyperbilirubinemia had been attributed to polymorphism of (*ABCB1*, *3435 C> T*) among patients on ATV-based therapies (13).

Accumulating evidence in the literature indicates that long-term cARTs are associated with GMDs in PLWH (14–18). Like the case of hyperbilirubinemia, ATV-based regimens might predispose GMDs and lipid abnormalities due to single nucleotide polymorphisms primarily related to ATV disposition. Not only among PLWH, but the literature is also scanty concerning the association of drug-metabolizing enzyme and transporter genes with glucose metabolism disorders (GMDs) among HIV-negative individuals. Yet, a few studies showed that transporter genes, which are involved in the disposition of ATV/r, may link

with GMDs. A Chinese study reported susceptibility to type 2 diabetes mellitus (T2DM) in individuals carrying the ABC variants rs1800977 and rs4149313 (19). However, no data exist *SLCO1B1* SNPs to glucose metabolism abnormalities either in T2DM or PLWH with T2DM.

To our best knowledge, the plasma concentration and pharmacogenetics of ATV-based regimens have not been previously linked with the incidence of GMDs among PLWH. The genes of drug-metabolizing enzymes and transporters have not been studied as a factor for predisposition or resistance to GMDs (Insulin resistance, IR, or impaired fasting glycemia, IFG) in antiretroviral therapy (ART), so far. No studies have been conducted to assess the association of GMDs with the polymorphism of genes involved in the disposition of ATV. However, such studies could provide a means to predict specific ADRs associated with ATV/r-based regimens. Thus, this study aims to determine the association of common SNPs of metabolic enzymes (*CYP3A4* and *CYP3A5*) and transporters (*ABCB1B1* and *SLCO1B)* with the incidence of GMDs among patients on ATV-based cART.

Methods

Study setting

The study was conducted at the HIV clinic of Tikur Anbessa Specialized Hospital (TASH), College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia. TASH is the largest tertiary, referral, and teaching hospital in Ethiopia. With more than 800 beds, it provides various specialized clinical services for about 500,000 patients. This setting regularly receives referred patients from different corners of the country, representing

diverse ethnic populations and socioeconomic statuses. The HIV clinic at TASH provides HIV/AIDS prevention, patient care, and ART services.

Study design and participants

The study design was a matched case-control study and the population was selected from a previous cross-sectional study (20). A total of 111 participants were recruited based on the stated screening study and GMD data were considered for analysis. The inclusion and exclusion criteria and the HOMA-IR calculation and GMDs operational definition were described in our earlier study (20).

Individuals with GMDs were compared with Controls. Controls were those individuals with normal fasting serum glucose, HOMA-IR, or serum insulin level based on cut-off values stated in the operational definition. The case and control groups were matched in terms of age, gender, duration since cART start, duration on current cART, weight, waist circumference, and BMI.

Sample collection

Blood samples were collected for genotype analysis in EDTA coated vacutainer tube and inverted 8 to 10 times for thorough mixing of EDTA. The samples were then transferred to cryotubes and stored at -80°C until genotyping analysis.

DNA extraction

Blood samples were transported in dry ice packages to the Division of Clinical Pharmacology, Department of Laboratory Medicine, Karolinska Institute, Stockholm, Sweden, for genotyping. According to the manufacturer's instructions, genomic DNA was

isolated from whole-blood samples using QIAamp DNA MidiKit (QIAGEN GmbH, Hilden, Germany). The purity and quantity of DNA were assessed using NanoDrop 2000 (Thermo Scientific, Saveen Warner, Sweden) based on the manufacturer's protocol. Allelic discrimination assay was performed using TaqMan drug metabolism genotyping assay (Applied Biosystems, CA, USA).

Selection of genes

SNPs in genes encoding enzymes and drug transport proteins involved in the disposition of EFV (21–24) and ATV/r (9,13,25,26) were selected based on functional significance. The SNPs were *CYP3A4*1B*, *CYP3A5*3*, *CYP3A*6*, *ABCB1,* and *SLCO1B1*. Genotyping was performed using TaqMan® allele-specific PCR (Applied Biosystems Genotyping Assays) with the following ID numbers for respective SNPs: C__7586657_20 for *ABCB1 3435C→T*, C_11711730_20 for *ABCB1c.4036*, C__26201809_30 for *CYP3A5 6986A→G* (*CYP3A5*3*), C__30203950_10 for *CYP3A5 14690G→A* (*CYP3A5*6*), C_1837671_50 for *CYP3A4* (*CYP3A4*1B*), C_1901697_20 for *SLCO1B1*1B*, C_30633906_10 for *SLCO1B1*5*.

Genotype analysis

The 7500 Real-Time PCR system (Applied Biosystems, United States) was used for genotyping. The final volume was 10 µL for each reaction, consisting of 9 µL of TaqMan Universal PCR Master Mix® (Applied Biosystems, Waltham, MA, USA), DNA/RNA free water, TaqMan 20× drug-metabolism genotyping assay mix (Applied Biosystems), and 1 µL of genomic DNA. The PCR conditions were as follows: an initial step at 60 °C for 30

s, hold-stage at 95 °C for 10 min and PCR stage for 40 cycles step 1 with 95 °C for 15 and step 2 with 60 °C for 1 min and after read-stage with 60 °C for 30 s (27).

Statistical analysis

Hardy-Weinberg equilibrium was assessed by the chi-square test for each tag SNP to determine normal genotype distributions among control and case groups (P-value <0.05 was considered statistically significant). Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 26 (IBM® SPSS® Statistics) and Haploview version 4.2 (Broad Institute, Cambridge, USA).

Determination of GMDs status was discussed in our previous publication (20). Baseline sociodemographic and laboratory parameters were described as means with standard deviation (SD) or medians with interquartile range (IQR) for continuous variables and proportions for categorical variables. An independent t-test statistics was used to compare contineuous variables between the case and the control groups.

Haploview version 4.2 was employed to determine the association and case-control analysis of genotypes and haplotypes. Also, using SPSS version 26, the association of GMDs with genetic variants was determined through logistic regression analysis. Univariate followed by a multivariate logistic regression model was used to assess the association of GMD with genotype variants after adjusting for factors such as age, sex, and BMI among the groups. Genotypes with univariate analysis of p<0.2 were entered in the multivariate analysis. A stepwise conditional-backward analysis was employed when three or more genotypes meet the stated entry criteria for multivariate analysis. When only one or two genotypes meet the entry criteria, a direct enter method (without a stepwise) was

used in the multivariate analysis. In all analyses, p-values were two-sided, and p-values of < 0.05 were considered statistically significant. The regression coefficient (β), crude odds ratio (COR), and adjusted odds ratio (AOR) were recorded from univariate and multivariate logistic regression.

Ethical consideration

Ethical clearance was obtained from the Institutional Review Board of College of Health Sciences, Addis Ababa University (Protocol No. 019/19/SoP) and National Ethical Review Committee, Ministry of Science and Higher Education of Ethiopia (Ref. No. MoSHE/RD14.1/9324/20). Written informed consent was obtained from each study participant after fully explaining the purpose and nature of all procedures used. All participants were informed about their right to withdraw from the study. Study participants were also briefed that collected data and results would be kept confidential, coded, and no name would be used in any form or publication. All data were kept safely in a locked cabinet and analyzed anonymously.

Results

Characteristics of study participants

A total of 111 participants were recruited, whereby 73.9% (82/111) were females and 26.1% (29/111) were males. As depicted in Table 1, the overall mean age of the study participants was 39.9±1.0 years with no difference between cases and controls. Proportional distribution of males (25.8 vs 27.3%) and females (74.2 vs 72.7%) was observed between the control and the case groups (p=0.89). The cumulative time on cART since initiation did not significantly differ between the groups (112.0±4.8 vs 116.5±10.5 months, p=0.69). Similarly, the duration of time on ATV/r-based cART between the groups did not show a significant difference (p=0.22) between the control (37.5±3.6) and the case (28.2±4.5 months) groups. Comparative clinical laboratory data for lipid measurements, fasting serum glucose, fasting serum insulin, and HOMA-IR values are shown in Table 1. A significant mean difference (p<0.001) was observed in the biochemical markers of GMD between the control and the case groups (Table 1).

Table 1 Comparison of sociodemographic and biochemical parameters among HIV patients on atazanavir/ritonavir (**ATV/r**) based ART with glucose metabolic disorders (cases) versus without (controls).

Variables	**ATV/r**		
	Control	**Case**	***p***
Sample (n) (case:control ratio)	89	22	(1:4)
Gender n(%)			
Male	23 (25.8)	6 (27.3)	0.89
Female	66 (74.2)	16 (72.7)	
Age (years)	39.9±1.0	43.5±2.9	0.16
Duration since HIV confirmed date (Month)	125.8±5.4	121.7±11.0	0.74
Cumulative time on cART (month) [§]	112.0±4.8	116.5±10.5	0.69

Time on current cART regimen type (month) [§]	34.1±3.1	35.5±5.9	0.85
Cumulative time on EFV-based 1st-line (month) [§]	39.5±4.6	45.9±10.0	0.55
Time on prior cART regimen types (month) [§]	92.6±14.6	80.4±10.8	0.68
Time on NVP-based 1st-line (Prior to EFV) (month) [§]	33.0±4.9	31.6±8.7	0.9
Cumulative time on ATV/r-based 2nd-line (month) [§]	37.5±3.6	28.2±4.5	0.22
Time on LPV/r-based 2nd-line (prior to ATV/r) (month) [§]	2.9±1.5	10.7±5.6	0.19
Weight	60.8±1.5	60.2±2.9	0.86
Waist circumference (cm)	33.0±0.5	35.1±1.0	0.05
BMI (Kg/m2)	23.5±0.6	24.3±0.9	0.54
Total cholestrol (mg/dL)	184.1±4.9	188.1±6.6	0.62
Triglyceride (mg/dL)	156.8±6.6	195.3±18.5	0.02
HDL-C (mg/dL)	44.3±4.4	39.3±1.7	0.58
LDL-C (mg/dL)	106.0±4.0	106.2±6.3	0.99
Fasting serum glucose (mg/dL)	88.1±1.1	119.8±11.6	<0.001
Fsting serum Insulin (U/	6.4±0.3	15.5±1.6	<0.001
HOMA-IR	1.4±0.1	4.5±0.8	<0.001

Genotype, allele, and haplotype frequencies

The CYP *3A4*1B, CYP3A5*3, CYP3A5*6, ABCB1 c.3435 C>T, ABCB1 c.4036 A>G, SLCO1B1*1B*, and *SLCO1B1*5* genotype and allele frequencies are depicted in Table 2 comparing the control and the case groups. All genotype frequencies were as per Hardy-Weinberg Equilibrium (HWE) (p>0.05) in the case and control groups.

The **3* alleles of *CYP3A5*3* c.6986A>G showed a significantly higher frequency among the control (0.91) than in the case group (0.73) (p=0.02) (Table 2). The C allele of *SLCO1B1*5 c.521 T>C* demonstrated a marginally significant (p=0.07) high frequency (0.46) in the case than in the control group (0.26).

Table 2 Genotype and variant allele frequency distribution and association with glucose metabolism disorders (GMD) among PLWH (N=111).

Genotypes	Variants	ATV/r Based		
		Control, n (%)	**Case, n (%)**	**p**
CYP 3A4*1B (−392A>G)	*1/*1	35 (39.3)	11 (50.0)	0.01
	*1/*1B	46 (51.7)	5 (22.7)	
	*1B/*1B	8 (9.0)	6 (27.3)	
CYP3A5*3 c.6986A>G	*1/*1	8 (9.0)	6 (27.3)	0.07
	*1/*3	47 (52.8)	9 (40.9)	
	*3/*3	34 (38.2)	7 (31.8)	
CYP3A5*6 c.14690G>A	*1/*1	66 (74.2)	17 (77.3)	0.77
	*1/*6	21 (23.6)	5 (22.7)	
	*6/*6	2 (2.2)		
ABCB1 c.3435 C>T	CC	45 (50.6)	13 (59.1)	0.55
	CT	39 (13.8)	7 (31.8)	
	TT	5 (5.6)	2 (9.1)	
ABCB1 c.4036 A>G	AA	56 (62.9)	16 (72.7)	0.54
	AG	30 (33.7)	6 (27.3)	
	GG	3 (3.4)		
SLCO1B1*1B c.388A > G	AA	16 (18.0)	2 (9.1)	0.60
	AG	43 (48.3)	12 (54.5)	
	GG	30 (33.7)	8 (36.4)	
SLCO1B1*5 c.521T > C	TT	66 (74.2)	12 (54.5)	0.19
	TC	20 (22.5)	9 (40.9)	
	CC	3 (3.4)	1 (4.5)	
	Minor Allele	**Frequency**		**p**
CYP 3A4*1B (−392A>G)	*1B	0.61	0.5	0.36
CYP3A5*3 c.6986A>G	*3	0.91	0.73	0.02
CYP3A5*6 c.14690G>A	*6	0.26	0.23	0.76
ABCB1 c.3435 C>T	T	0.49	0.41	0.47
ABCB1 c.4036 A>G	G	0.37	0.27	0.39
SLCO1B1*1B c.388 A>G	G	0.82	0.91	0.31
SLCO1B1*5 c.521 T>C	C	0.26	0.46	0.07

The *CYP3A* (CYP*3A4*1B, CYP3A5*3,* and *CYP3A5*6*) haplotype linkage disequilibrium (LD) analysis is shown in Figure 1. The most robust linkage among the three CYP3A SNPs is between *CYP3A5*6* and *5*3*. Besides, Table 3 shows the frequency distribution of *CYP3A* haplotype and its association with GMD. The major *CYP3A* haplotype was *CYP3A5*3* alone (49.1%), followed by *CYP3A4*1B* alone (19.2%), and then by **1B* linkage with **3* (9.8%). Based on haploview analysis, the **1B*-alone haplotype was significantly associated with IR (p=0.03) (Table 3), while other studied alleles failed to show a significant association with overall and specific forms GMDs.

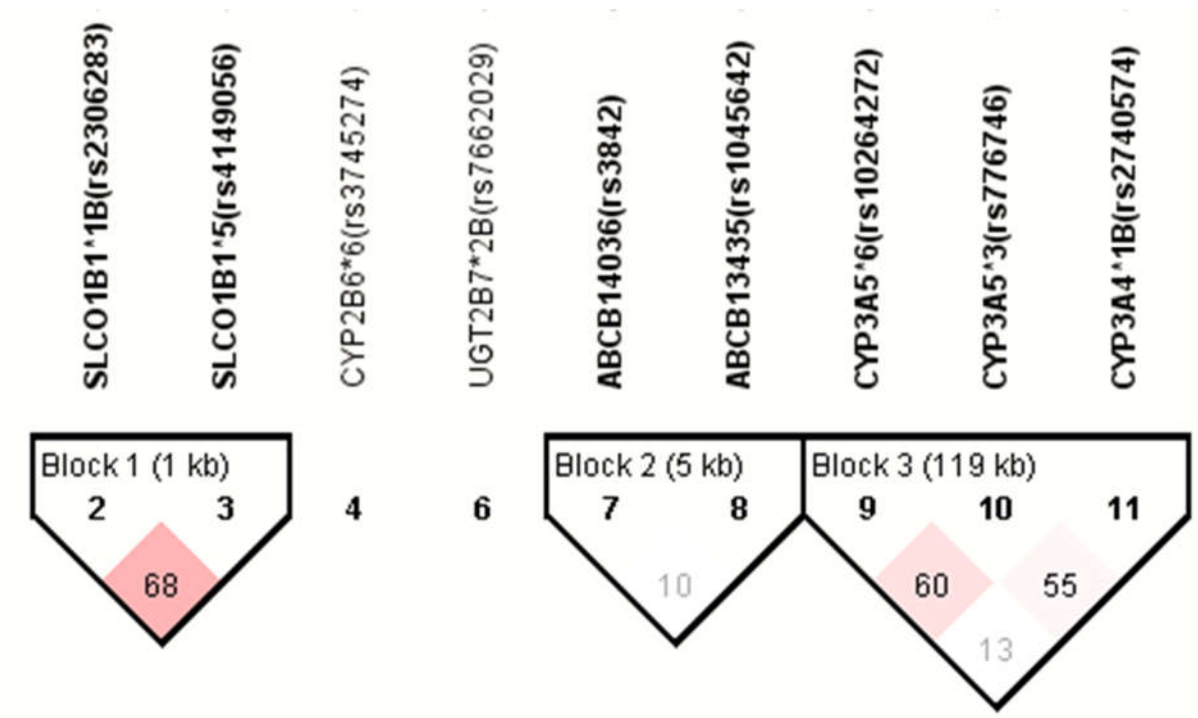

Figure 1 Linkage disequilibrium (LD) plot of the studied genotype variants among Ethiopian PLWH on ATV/r-based cART. The triangular blocks show the LD values for closely related gens (Block 1 for SLCO1B1, Block 2 for ABCB, and Block 3 for CYP3A genotypes). The values in the diagonal square boxes represent the respective observed D′ values of the pair-wise LD relationship between two SNPs. The red color gradient indicates higher LD values while the white represents lower LD values (D′ 1–0).

Table 3 *CYP3A* haplotype distribution and the association of respective haplotypes with overall and specific type of GMDs based on case-control analysis by Haploview (v 4.2) among PLWH on ATV/r-based cART.

***CYP3A* haplotypes**	***CYP3A* SNP combinations**			**F (%)**	**p-values**			
	CYP3A4*1B -392A>G	***CYP3A5*3 c.6986 A>G***	***CYP3A5*6 c.14690 G>A***		**GMD**	**IFG**	**IR**	**DM**
AGG	wt	*3	Wt	49.1	0.43	0.88	0.58	0.51
GAG	*1B	wt	Wt	19.2	0.12	0.99	0.03	0.62
GGG	*1B	*3	Wt	9.8	0.26	0.22	0.26	0.79
AAG	wt	wt	Wt	8.4	0.33	0.08	0.36	0.08
GAA	*1B	wt	*6	6.5	0.81	0.77	0.65	0.45
AAA	wt	wt	*6	3.7	0.60	0.62	0.94	0.57
AGA	wt	*3	*6	3.3	0.99	0.72	0.54	0.14

F (%) represents haplotype frequency distribution; level of significance (p-values) are based on haploview case-control analysis for *CYP3A* haplotypes; Wt=wildtype, GMD=glucose metabolism disorder, IFG=impaired fasting glycemia, IR=insulin resistance, DM=Dibetus Mellitus.

Association with GMD

As shown in Table 4, the SL*CO1B1*5 c.521 T >C* genotype significantly predicted GMD that the C allele carriers demonstrated a 2.9 times higher risk of GMD [AOR=2.9; 95% CI 1.03-8.1, p=0.04] than the wildtype allele carriers. While any **6* and only **3* containing haplotypes of *CYP3A* demonstrated a respective significant 80.0% (p=0.03) and 90.0% (p=0.01) protection from GMD than the wildtype combination haplotypes (Table 4).

Regarding IR, the multivariate analysis showed that any **6* and only **3* haplotype combinations significantly predicted the incidence of IR in a protective manner (Table 4). The IR risk was reduced by 90% in both any-**6* (p=0.03) and only-**3* (p=0.01) than the wildtype haplotype combinations. Also, the G allele carriers of *ABCB1 c.4036* demonstrated an 80.0% protection of IR (p=0.05) than the wildtype allele (Table 4).

Table 4 Regression analysis of drug-metabolizing and transporter genotypes with glucose metabolism disorders (GMD) and insulin resistance (IR) among study participants on ATV/r-based cART (n=111).

Genotype		**GMD among ATV/R-based cART**				**IR among ATV/R-based cART**			
		COR (95%CI)	***p***	**AOR (95%CI)**	***p***	**COR (95%CI)**	***p***	**AOR (95%CI)**	***p***
*CYP 3A4*1B/5*3/ *6 Haplotypes*	**1/*1/*1 & *1B/*1/*1*	1		1		1		1	
	Any **6* carriers	0.2(0.03, 0.9)	0.04	0.2(0.03, 0.9)	0.03	0.2(0.04, 1.1)	0.07	0.1(0.02, 0.9)	0.03
	**3* only	0.2(0.04,0.7)	0.01	0.1(0.03, 0.6)	0.01	0.1(0.03, 0.6)	0.01	0.1(0.02, 0.5)	0.01
ABCB1 c.3435 C>T	*CC*	1				1			
	CT or TT	0.7(0.3, 1.8)	0.47			0.5(0.2, 1.6)	0.24		
ABCB1 c.4036 A>G	*AA*	1				1		1	
	AG or GG	0.6(0.2, 1.8)	0.39			0.2(0.1, 1.1)	0.07	0.2(0.04, 1.0)	0.05
*SLCO1B1*1B c.388 A>G*	*AA*	1				1			
	AG or GG	2.2(0.47, 10.3)	0.32			3.0(0.4, 24.5)	0.30		
*SLCO1B1*5 c.521 T >C*	*TT*	1		1		1			
	TC or CC	2.4(0.9, 6.3)	0.08	2.9(1.03, 8.1)	0.04	1.7(0.6, 5.2)	0.35		

Discussion:

The aim of our study was to investigated the association of genotype variants important in the disposition of ATV/r with the incidence of GMDs among Ethiopian PLWH on ATV/r-based cARTs. Long-term ART has been associated with several metabolic adverse effects including GMDs (*i.e.,* IR, IFG, or T2DM). Our previous study reported a higher prevalence of GMDs (19.8%) among PLWH on ATV/r-based cART (20). As treatment outcomes, adverse effects, and toxicities are dependent on pharmacokinetics, variation in genes encoding metabolizing enzymes and transporter proteins that are important in the pharmacokinetics of antiretrovirals may influence. It could be a risk factor for such outcomes (28,29). So far, no literature directly assessed the association of GMDs with functionally prominent SNPs in these genes that encode proteins involved in the disposition of ATV/r among PLWH on long-term treatment.

For this purpose, functional variants in *CYP450* (*CYP3A4, CYP3A5*), *ABCB1*, and *SLCO1B1* genes are involved in the disposition of ATV/r (9,13,25,26) were assessed. There was no statistically significant difference in the mean age, duration of time since cART start, period on the current cART type, weight, and BMI between the case and the control groups, implying a matching between the case and the control groups in the study. We also showed that cases had elevated HOMA-IR, FSG, FSI, and TG levels than controls, indicating the presence of GMDs. The observed genotype and allele frequency proportions were similar to previous studies from Ethiopia (27,30). The main finding of this study was that, first, the C allele of *SLCO1B1*5 c.521 T >C* is a significant predictor of the overall GMD in ATV/r-based cART. Secondly, the case-control and multivariate analyses showed

that carriers of the wild type were at higher risk of IR than any *6 or *3-only haplotype combinations of CYP3A, which in other way is stated as those carriers of the mutant haplotype combinations were protective from GMD than wildtype carriers.

The *CYP3A4*1B*-alone mutant haplotype is associated with the incidence of IR based on the haploview analysis. While haplotype combinations of *CYP3A* with any **6* and **3* were an independent predictor of both IR and GMD protectivity compared to the wildtype haplotype combinations. A study showed that *CYP3A5* expressors had a faster atazanavir clearance and lower minimum trough concentration than non-expressors, while ritonavir reduced the *CYP3A5* expressor effect (31). Another study stated that ATV alone reportedly has no significant effect on blood glucose or insulin levels (32). However, evidence indicates that dyslipidemia and insulin resistance were associated with ritonavir-boosted ATV therapy (33). An *in vitro* investigation also showed that ATV inhibited glucose uptake significantly but less than other PIs such as lopinavir and ritonavir (34). When RTV was combined with ATV, the same study showed that the combination did not inhibit glucose uptake, and the area under the curve of glucose did not increase with ATV/r, unlike lopinavir/ritonavir (34). Also, the same study reported that the decrease in insulin sensitivity with ATV/r was significantly lesser than lopinavir/ritonavir combination (34). Therefore, based on the stated evidences from these studies and our finding, ATV combinations might play a protective role from IR or GMD.

Also, our study showed that SNPs of *SLCO1B1,* a gene encoding the uptake (influx) transporter OATP1B1, predicted the incidence of GMD in patients on ATV/r-based cART. Explicitly, *SLCO1B1*5 c.521 T >C* (the C allele than the wildtype) predicted a higher incidence of GMD among cases. Evidence from the literature indicates that SNPs of both

c.521 and c.388 have a major effect on OATP1B1 activity by reducing the uptake transport of substrates to the liver, especially the c.521 (36). Indirectly, SNPs in uptake transporters like OATP1B1 may impact plasma exposure of substrate drugs leading to reduced metabolism by the liver. A study showed that the C allele carriers of *SLCO1B1*5 c.521* had a significantly higher ritonavir intracellular concentration than T allele carriers (37). This could also be associated with higher plasma exposure of PIs, as reports indicated that these SNPs are associated with increased plasma PIs exposure (13,38), and IR is associated with PI use (39). The effect by the variant c. 521 appears to dominate over that of the c.388 A>G SNP in resulting in a variant protiens with decreased activity (36). Therefore, it is probably due to the *SLCO1B1*5 c.521* vairiant's reduced activitiy that the increased risk of GMD is observed among the C allele carriers than the wildtype in our study.

One of the strengths of our study is that we considered SNPs of candidate genes with already determined association with pharmacogenomics that affects the disposition of EFV and ATV/r. For the first time, our study generated evidence about the association of SNPs in the genes of metabolizing enzymes and drug-transporters, which are involved in the disposition of antiretrovirals, with metabolic disorders such as IR IFG, and DM among PLWH. The inclusion of insulin and IR parameters as GMD would provide a cumulative prediction of such incidences among PLWH on long-term cART. One of the limitations of our study is that, although in at least 1 to 4 cases: the control ratio was maintained in most gene variants, the number of participants in the case group was relatively small for a few genetic variants relative to the control groups. Moreover, we have used one-time sampling and follow-up to diagnose and measure laboratory parameters. Our study also focused on related pharmacokinetic genes and did not consider other SNPs of genes directly involved

in carbohydrate metabolism and insulin release. Therefore, we recommend confirmatory studies on a larger sample size with more extended and repeated follow-ups for future studies.

Conclusion

Our findings suggested that the haplotype consisting any-*6 and *3-only of *CYP3A 4*1B/5*3/5*6* were an independent predictor of GMD and IR protectively in patients on ATV/r-based cART. Also, carriers of G allele of *ABCB1 c.4036* demonstrated protection from IR than the wildtype carriers. Whereas, carriers of the C allele of *SLCO1B*5* were at high risk of developing glucose metabolism disorders than wildtype carriers on ATV/r-based cART. Further studies with a large sample size are needed to confirm the observation as risk factors for T2DM among PLWH on cART.

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PAPER V

The Influence of Pharmacokinetic and Pharmacogenetic Factors on the Incidence of Glucose Metabolism Disorder in Patients Receiving Long-term Atazanavir-based Antiretroviral Therapy

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Abstract

Objective: The purpose of this study was to determine the relationship between plasma atazanavir/ritonavir (ATV/r) concentrations and pharmacogenetic factors in the incidence of glucose metabolism disorders (GMDs) among people living with HIV on ATV-based cART.

Method: A total of 111 study subjects, 89 controls and 22 cases, on ATV/r were recruited and mid-dose plasma (CP12) concentrations were analyzed using LC-MS/MS. GMD status was determined based on fasting glucose, fasting insulin, and HOMA-IR values. The cases were individuals with GMD while the controls were without GMD. Genetic variants important in ATV/r disposition were analyzed using real-time PCR. Logistic and linear regression analyses were used to determine the association of ATV/r plasma concentrations and pharmacogenetic variants with GMDs.

Results: a total 90 samples (cases, n=16 and controls, n= 74) were considered for statistical analysis. The mean logCP12 of ATV was 3.24 ng/ml (SEM = =0.04) for controls and 3.52 ng/ml (SEM=0.06) for cases. Majority of the subjects (88.9%) had ATV concentration >850 ng/ml. But, the logCP12 of both ATV and Ritonavir (RTV) failed to show significant association with the GMD types. By contrast, *CYP3A5*3* allele lowered the incidence of GMD by 20% (AOR=0.2, 95% CI 0.03-0.9, p=0.04) and IR by 10% (AOR=0.1, 95%CI 0.02-0.9, p=0.04, while the C allele of *SLCO1B1*5* increased the incidence of overall GMD by about nine-fold (AOR= 8.8, 95%CI 1.8-41.7, p=0.006) than the respective wildtype alleles. From the liver function panels, ALP (AOR=1.03, 95%CI 1.01-1.05), p<0.05), ALT (AOR=1.1,95%CI 1.0-1.3, p=0.03), and direct bilirubin levels (AOR=31.3, 95%CI 2.9-341.9, p=0.03) predicted the occurrence of GMD.

In a similar analysis, BMI (p=0.036), creatinine (p=0.014), direct bilirubin (p=0.022), HOMA-IR (p=0.026), *CYP3A5*3* (p=0.047), and *ABCB1 c.4036A>G* (p=0.019) alleles were positively corelated with the logCP12 of ATV. Whereas; duration on prior 1st line cART (p=0.005), ALT (p=0.007), and fasting serum insulin (0.029) were negatively correlated with the logCP12 of ATV.

Conclusion: The logATVCP12 level did not predict the occurrence of GMDs. Whereas, the *CYP3A5*3* allele demonstrated a protective association while the SLCO1B1*5 increased the risk of GMD than their respective wildtype alleles. Moreover, the *CYP3A5*3* and *ABCB1B c. 4036* alleles were correlated with the logCP12 of ATV. Our findings warrant further research with a larger sample size.

Keywords: antiretroviral therapy; atazanavir; ritonavir; glucose metabolic disorder; HIV; mid-dose plasma concentration; pharmacogenetic variation; *CYP3A*; *CYP3A5*3*; *SLCO1B*5*; *ABCB1B;* genotype; Ethiopia

Introduction

Protease inhibitor (PI)-based antiretroviral therapy (ART) is a second-line option in the treatment of HIV infection in sub-Saharan Africa. Combination antiretroviral therapies (cART) based on ritonavir-boosted atazanavir (ATV/r) are widely used as second-line treatment options in cases of treatment failure and severe adverse drug reactions in most resource-limited settings [1,2]. ATV is the preferable alternative among PIs, especially when boosted, due to its once-daily dose, lower metabolic adverse effects, and higher barrier to drug resistance [3,4].

PIs have been linked to metabolic disorders such as abnormal glucose metabolism, which can range from insulin resistance through impaired glucose tolerance and fasting glucose to type 2 diabetes [5,6]. Older PIs, such as lopinavir and ritonavir (RTV), are strongly linked to these side effects [7,8]. A case- control study reported at least a 21-fold increase in diabetes among HIV-infected patients taking PIs than other fixed dose combination therapies [9]. Another study that assessed glucose handling factors in 80 adolescent participants on PI-based ART reported a 22.1% prediabetes and 3.8% overt type 2 diabetes mellitus (DM) [10]. Although older PIs have received extensive research attention, recently introduced PIs (such as ATV) did not, particularly in resource-limited health settings. Thus, explorative studies are required to understand the association of their long-term use with incidence of metabolic adverse effects.

The existing body of evidence consistently indicates that ATV/r has less metabolic adverse effects than older PIs [11,12]. However, clinically, the effect of the booster dose of RTV on lipid and glucose metabolism on long-term use has not been well studied. In this regard, a study reported that there was an increase in glucose area under the curve (AUC) from the baseline only when RTV is combined with lopinavir. The same study showed also that the HOMA-IR index and insulin AUC were elevated for both ATV/r and LPV/r [13]. Another study also suggested that higher plasma levels of ATV could be a risk for metabolic conditions like dyslipidemia [14]. Investigating the relationship of pharmacokinetic variability and genetic polymorphisms important to ATV/r disposition with glucose metabolism may thus be required.

ATV metabolism is mediated by CYP3A, which includes 3A4 and 3A5, and UGT1A enzymes, while ABCB1 and SLCO1B transporters are involved in its disposition [3,4,15].

Polymorphisms in genes involved in drug metabolism and transport can lead to varied plasma concentrations and treatment exposure, influencing treatment outcomes and PI-related metabolic abnormalities [3] To this effect, the association of hyperbilirubinemia with polymorphism of *ABCB1 3435C>T*[16] and *UGT1A1*28* [17] variants among patients receiving ATV-based therapies can be cited as an example.

ATV-based regimens may predispose people living with HIV/AIDS (PLWH) to glucose metabolism disorders (GMDs) due to higher plasma concentration and single nucleotide polymorphisms (SNPs) primarily related to ATV disposition. To the best of our knowledge, the plasma concentration and related factors of ATV-based regimens have not been well studied in association with the occurrence of GMDs in PLWH, at least in Ethiopia. There is a paucity of local data on ATV pharmacokinetics and pharmacogenetics, which could have helped in predicting GMD-related ADRs associated with ATV/r-based regimens. Thus, the purpose of this study was to determine the relationship between pharmacokinetic/pharmacogenetic factors and the occurrence of GMDs in patients on ATV-based cART.

Methods

Study setting

The investigation was carried out at the HIV clinic of Tikur Anbessa Specialized Hospital (TASH), College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia. TASH's HIV clinic provides treatment, care, and support services for PLWH. The ART pharmacy provides ARVs and related medications to patients through scheduled follow-up.

Study design and participants

The study used a case-control design with a sample population (111 participants on ATV/r) from previous cross-sectional and pharmacogenomic studies [1]. Additional information was gathered using a structured questionnaire, consisting of co-morbid conditions, concurrent medication use, time of last ATV/r dose, and substance use information (cigarette and alcohol use). Other information, including treatment adherence, anthropometric measurements, HOMA-IR & BMI calculation are described in our previous studies [1,18].

The inclusion and exclusion criteria were also stated in detail in our previous publications [1,18]. In brief, PLWH on ATV/r-based cART at least for one year with age ≥ 18 years were included. While, PLWH diagnosed with conditions, such as recorded DM prior to ART initiation, pregnancy, cancer, renal disease, liver disease, un-controlled hypertension, and heart failure were excluded. Besides, patients on concomitant treatment with antipsychotics, anti-cancer agents, anti-TB, corticosteroids, hormonal agents, and antidiabetic agents were excluded. Moreover, participants with plasma concentrations

bellow the lower limit of quantification (LLQ) were further excluded after the pharmacokinetic analysis. The participants who met the inclusion criteria were then divided into case and control groups.

As described in our previous study [1,18], the operational definitions used to define cases were:

- DM was defined as a fasting glucose level of 126 mg/dL or higher
- IFG was defined as a fasting glucose level between 110 and 125 mg/dL
- IR was diagnosed by either Homeostasis model assessment insulin resistance (HOMA-IR) value of ≥3.8, Fasting plasma insulin of ≥ 20 μU/ml, or fasting glucose/insulin ratio of ≥4.5'
- GMDs were defined as the presence of IFG, IR, or DM

Controls were those individuals with normal fasting serum glucose (70-109 mg/dL), HOMA-IR, or serum insulin level based on the above stated cut-off values. The cases were those individuals with at least one diagnosis of insulin resistance, impaired fasting glycemia or DM. Therefore, plasma samples of 22 cases and 89 controls were recruited from the previous cohort [1] for plasma concentration analysis.

Quantification of ATV/r plasma concentration

Blood samples were collected in 2 EDTA and 1 SST tubes, 2 ml each, 12 h after the last dose and the EDTA tubes were inverted 8-10 times for gentle mixing. Plasma was extracted from one of the EDTA tubes immediately separated by centrifugation at 3,000 x g for 10 min. Similarly, serum was separated from the SST tube for biochemical analyses. The other EDTA (whole blood sample) was saved for DNA extraction for a pharmacogenetic study.

Both plasma and whole blood samples were stored at -80 ºC before being transported in dry ice to the laboratory at Karolinska Institute, Stockholm, Sweden. The samples were then stored at -80 ºC until analysis.

Chemicals and reagents

ATV reference standard, deuterated-atazanavir (atazanavir-d_5), was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Also, RTV reference standard, deuterated-ritonavir (ritonavir-d_6), was obtained from trc-Canade (North York, ON, Canada). LCMS analytical grade acetone, acetonitrile, ammonium acetate, isopropanol, methanol, and acetic acid were purchased from Merck (Darmstadt, Germany). Blank human plasma was obtained from the blood bank of the Karolinska University Hospital, Huddinge (Stockholm, Sweden).

LC-MS/MS method and validation

Plasma ATV concentration was determined using an LC-MS/MS system comprised of an ACQUITY Ultra Performance LC-system coupled to a Xevo TQ-S Micro (Waters, Milford, MA, USA). The positive ion mode was selected for MS/MS analysis, and data were processed using the MassLynx 4.2 software (Waters). ATV, atazanavir-d_5, RTV, and ritonavir-d_6 fragment transitions were detected at m/z 705.32, 335.27, 721.28, and 296.17, respectively. ACQUITY UPLC BEH C18, 1.8, 2.1 50 mm reversed-phase column was used as the chromatographic column (Waters). Aqueous formic acid and MeOH were used as mobile phases A and B, respectively. The mobile phase's initial composition was 50% B, followed by a linear gradient to 99% in 1.5 min at a flow rate of 0.4 mL/min. For each sample, the chromatography run time was approximately 3 min. The LLQ was 15.8 ng/ml, while the upper limit of quantification (ULQ) was 15.8 g/ml for both ATV and RTV.

Calibration samples were made by spiking blank plasma samples with ATV or RTV and incorporating them into each analytical run. Spiking in blank plasma was also used to prepare the lower, middle, and higher concentration quality control samples. Each plasma sample (50 μL) was precipitated in a 200 μL acetonitrile solution containing the internal standard (500 ng/mL atazanavir-d_5 and ritonavir-d_6 in methanol). After 30 sec of vortexing and centrifugation (2100x g for 5 min), 5 μL of the supernatant was injected into the LC-MS system to determine plasma ATV and RTV concentrations.

At each analysis, the method was calibrated. The calibration curve was calculated using linear regression and weighted as 1/x analyte/internal standard area ratios. The method validation met the European Medicines Agency's bioanalytical criteria. Except for LLQ (below 10 CV percent), the accuracy range was ± 10% throughout the quantification range, and precision was <6% of the coefficient of variation (CV). For statistical purposes, log10 transformed atazanavir and ritonavir plasma concentrations were used.

DNA extraction

QIAamp DNA MidiKit (QIAGEN GmbH, Hilden, Germany) was used to isolate genomic DNA from whole-blood samples, according to the manufacturer's instructions. The purity and quantity of DNA were determined using the NanoDrop 2000 (Thermo Scientific, Saveen Warner, Sweden) protocol. TaqMan drug metabolism genotyping assay was used for allelic discrimination (Applied Biosystems, CA, USA).

Selection of genes

SNPs in genes encoding enzymes and drug transport proteins involved in the disposition of ATV [19–22] were selected based on functional significance. The SNPs were

*CYP3A4*1B*, *CYP3A5*3*, *CYP3A*6*, *UGT2B7*2*, *ABCB1,* and *SLCO1B1*. Genotyping was performed using TaqMan® allele-specific PCR (Applied Biosystems Genotyping Assays) with the following ID numbers for respective SNPs: C__7586657_20 for *ABCB1 3435C→T*, C:11711730_20 for *ABCB1c.4036*, C__30720663_20 for *UGT2B7 −327G→A* (*UGT2B7*2b, *2c, *2d, *2f*), C:9440184_20 for *UGT2B15*4*, C__26201809_30 for *CYP3A5 6986A→G* (*CYP3A5*3*), C__30203950_10 for *CYP3A5 14690G→A* (*CYP3A5*6*), C:1837671_50 for *CYP3A4* (*CYP3A4*1B*), C:_1901697_20 for *SLCO1B1*1B*, C:30633906_10 for *SLCO1B1*5*.

Genotype analysis

Genotyping was performed using the 7500 Real-Time PCR system (Applied Biosystems, United States). For each reaction, the final volume was 10 µL, consisting of 1 µL of genomic DNA and 9 µL of mix containing 5 µL TaqMan Universal PCR Master Mix® (Applied Biosystems, Waltham, MA, USA), 3.5 µL DNA/RNA free water, and 0.5 µL TaqMan 20× drug-metabolism genotyping assay mix (Applied Biosystems). The PCR conditions were as follows: an initial step at 60 °C for 30 s, hold-stage at 95 °C for 10 min and PCR stage for 40 cycles step 1 with 95 °C for 15 and step 2 with 60 °C for 1 min and after read-stage with 60 °C for 30 s [23].

Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 26 (IBM® SPSS® Statistics) was used for statistical analyses. Means with one standard deviations (SD) or medians with interquartile range (IQR) for continuous variables, and proportions for categorical variables were used to describe baseline sociodemographic and laboratory parameters. The

association between these variables and incidence of GMD was evaluated using an independent student's t-test for normally distributed continuous variables and chi-square or F-test association analysis for categoric variables.

ATV and RTV plasma concentrations were log-transformed before statistical analysis. The effect of ATV concentration was studied in case and control groups using chi-square association, with geometric mean ATV concentrations of ≥ 850 ng/ml considered supratherapeutic level and levels below that as non-toxic. An independent t-test was used to compare the mean log CP12 of ATV/r between cases, controls, and genetic variants. A univariate followed by a multivariate regression analysis was used to identify predictors of GMD by considering pharmacokinetic, pharmacogenetic, sociodemographic and clinical variables. Variables with $p<0.2$ in univariate analysis were included in the multivariate analysis. The crude odds ratio (COR) and adjusted odds ratio (AOR) from univariate and multivariate logistic regression were calculated. A stepwise linear regression analysis was also performed to identify the corelates of log CP12 ATV and RTV. *p*-values were two-sided in all analyses, and $p<0.05$ were considered statistically significant.

Ethical considerations

The study was performed according to the Declaration of Helsinki and approved by the Institutional Review Board of the College of Health Sciences, Addis Abeba University (Protocol No. 019/19/SoP) and the National Ethical Review Committee of the Ministry of Science and Higher Education of Ethiopia (Ref. No. MoSHE/RD14.1/9324/20). Informed consent was obtained from all study participants involved in the study. Each study participant was given a thorough explanation of the aim and nature of every procedure before providing their written informed consent. All participants were made aware of their

right to discontinue participation in the study at any time. Participants in the study were also informed that the collected data and results would be kept confidential and coded, with no identifiers used in any form or publication. All data were kept secure in a locked cabinet and analyzed anonymously.

Results

A total of 111 subjects, 89 controls and 22 cases, were recruited and plasma samples were collected for determination of mid-dose plasma concentration (CP12) of ATV and RTV. Due to plasma concentrations falling below LLQ or not being picked up by our method, 21 samples were excluded from analysis. Thus, a total of 90 study subjects, 74 controls and 16 cases, on ATV/r-based cART were included (66 females and 24 males) in the statistical analysis.

The sociodemographic characteristics, clinical characteristics, and allele frequencies of the study subjects are shown in **Table 1**. Female and male cases made up about 11.1% and 6.7 % of the overall study subjects, respectively. Out of the control study subjects, 62.2% (56/90) were females and 20.0% (18/90) were males. The average age of study participants in controls and cases was 40.8±1.1(Mean ± SEM=1.1) and 42.4±3.4 (Mean ± SEM=3.4) years, respectively.

There was no significant difference between controls and cases in the number of subjects with detectable (>1000 copies/ml) and undetectable (1000 copies/ml) viral load (p=0.29). Similarly, there was no significant difference in the proportion of participants who adhered or did not adhere to the treatment regimen between the two groups (p=0.45).

Table 1 Sociodemographic, clinical, biochemical and genetic characteristics of study population, *N*=90.

Variables	Categories	GMD		p
		Control (%)	Case (%)	
	Frequency	74 (82.2)	16 (17.8)	
Age (years)	Mean±SEM	40.8±1.1	42.4±3.4	0.57
Age category	18≤45	24 (26.7)	8 (8.9)	0.18
	≥46 years	50 (55.5)	8 (8.9)	
Gender	Female	56 (62.2)	10 (11.1)	0.22
	Male	18 (20.0)	6 (6.7)	
Treatment adherence (3-day test)	Adhered	72 (80.0)	15 (16.7)	0.45
	Non-adhered	2 (2.2)	1 (1.1)	
Viral load status(n=335)	≥1000 copies/ml	17 (18.9)	3 (3.3)	0.71
	<1000 copies/ml	57 (63.3)	13 (14.5)	
Specific ARVs contained in cART	TDF containing	37 (41.1)	9 (10.0)	0.65
	AZT containing	29 (32.2)	6 (6.7)	0.90
	ABC containing	8 (8.9)	1 (1.1)	0.58
Time since HIV confirmed date (months) §		127.7±5.9	117.6±13.5	0.48
Cumulative time on cART (month) §		113.8±5.1	127.3±12.4	0.28
Cumulative time on ATV/r-based (month) §		40.2±4.1	37.1±4.6	0.40
Time on current cART regimen type (month) §		36.4±3.5	38.9±6.4	0.75
Time on prior 1st-line (EFV- or NVP-based) (month) §		95.2±17.4	97.2±12.2	0.96
Weight (Kg)		62.1±1.6	60.7±4.2	0.73
Waist circumference (cm) §		33.4±0.5	34.1±1.0	0.62
Body Mass Index (Kg/m^2)		24.0±0.7	24.6±1.2	0.70
CD4+ (cells/ul) (baseline) §		147.9±21.4	209.0±46.7	0.23
CD4+ (cells/ul) (recent) §		365.5±25.0	286.1±39.6	0.16
Fasting serum glucose		87.8±1.1	109.8±9.5	<0.00
Fasting serum Insulin		6.7±0.4	16.8±1.9	<0.00
HOMA-IR		1.4±0.08	4.1±0.4	<0.00
Total Cholesterol		187.9±5.1	185.1±7.6	0.81
Triglyceride		164.2±7.3	215.2±22.9	0.048
LDL-C		110.0±4.2	98.8±7.5	0.25
HDL-C		46.2±5.2	37.6±1.9	0.46
Creatinine		0.7±0.02	0.7±0.06	0.91
Urea		20.3±0.8	22.7±2.2	0.23
ALP		93.1±3.5	118.8±10.4	0.03
ALT		12.3±0.6	15.4±2.0	0.049
AST		20.5±0.7	26.7±4.9	0.22
Direct Bilirubin		0.4±0.02	0.6±0.1	0.15
Total Bilirubin		2.0±0.14	2.1±0.3	0.77
Log ATV (ng/ml)		3.24±0.04	3.26±0.07	0.83
Log RTV (ng/ml)		2.52±0.06	2.54±0.10	0.87
Genotype	**Minor allele**			
*CYP3A4*1B*	**1B*	46 (51.1)	7 (8.9)	0.37
*CYP3A5*3*	**3*	66 (73.3)	12 (13.3)	0.13
*CYP3A5*6*	**6*	19 (21.1)	5 (5.6)	0.76
UGT2B7 g.-372 G>A	*A*	55 (61.1)	12 (13.3)	0.96
ABCB1B c.3435	*T*	37 (41.1)	6 (6.7)	0.36
ABCB1B c.4036 (rs3842)	*G*	28 (31.1)	3 (3.3)	0.14
*SLCO1B1*1B*	**1B*	60 (66.7)	15 (16.7)	0.29
*SLCO1B*5*	C	21 (23.3)	8 (8.9)	0.09

Chi-square or F test for categorical variables and t-test for continuous variables.

Mid dose ATV and RTV plasma concentration (CP12 ATV and RTV)

The mean log CP12 of ATV was 3.24±0.04 ng/ml in controls and 3.52±0.06 ng/ml in cases (Table 1). On the other hand, the mean log RTV CP12 was 2.26±0.07 ng/ml in controls and 2.54 ±0.1 ng/ml in cases. There was a significant correlation (r^2 = 0.7, *p*<0.000) between the log CP12 of ATV and RTV (**Figure 1)**. About 1.1 % (1/90) of the study participants had CP12 ATV concentrations less than 150 ng/ml (the minimum goal), while 88.9% (80/90) had levels >850 ng/ml. Splitting the data, 89.2% (66/74) of the control and 87.5% (14/16) of the case group had ATV CP12 >850 ng/ml without significant difference (p=0.84). Only about 10% (9/90) of the samples achieved the desired CP12 of ATV (150-850 ng/ml).

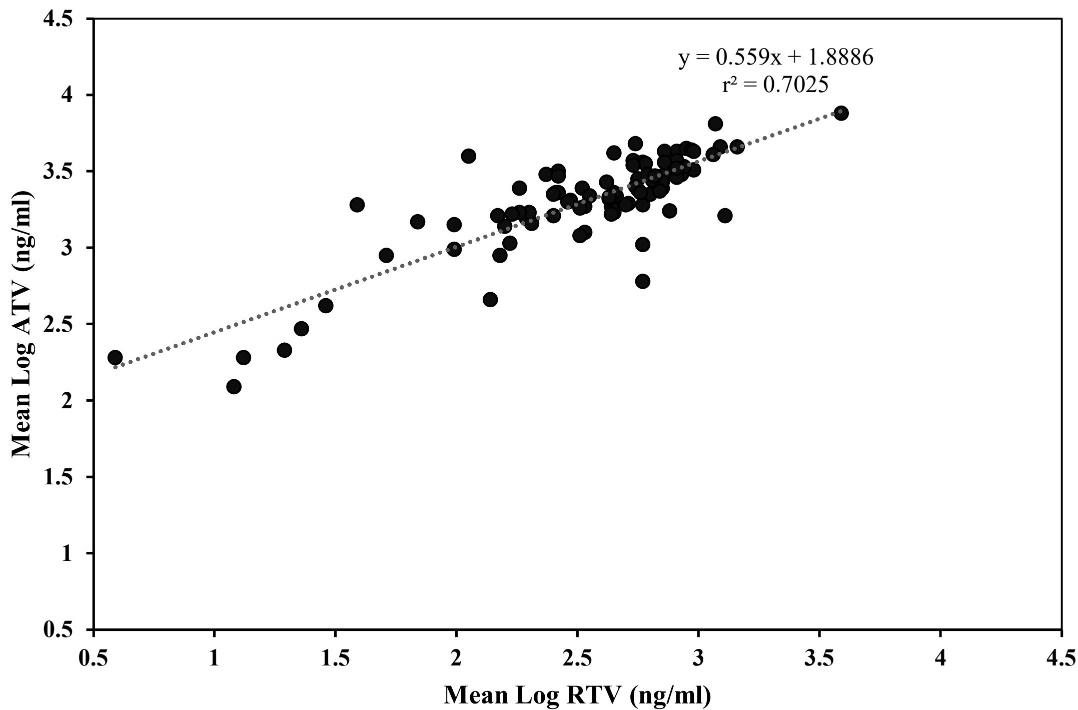

Figure 1. Linear regration analysis of the mean log ATV mid-dose concentration versus the mean log RTV mid-dose concentration. (r^2=0.7025, p<0.000).

Pharmacokinetics and GMDs in ATV/r cART

As shown in **Figure 2**, the log CP12 of both ATV and RTV did not show a statistically significant relationship with any of the GMD types (IFG, IR, DM, and overall GMD). A greater proportion of cases had CP12 of ATV >850 ng/ml than controls, although the difference failed to reach statistical significance (p=0.84) (**Figure 3**). Furthermore, regression analysis (**Table 3**) revealed that CP12 in both ATV and RTV did not predict the occurrence of any type of GMD.

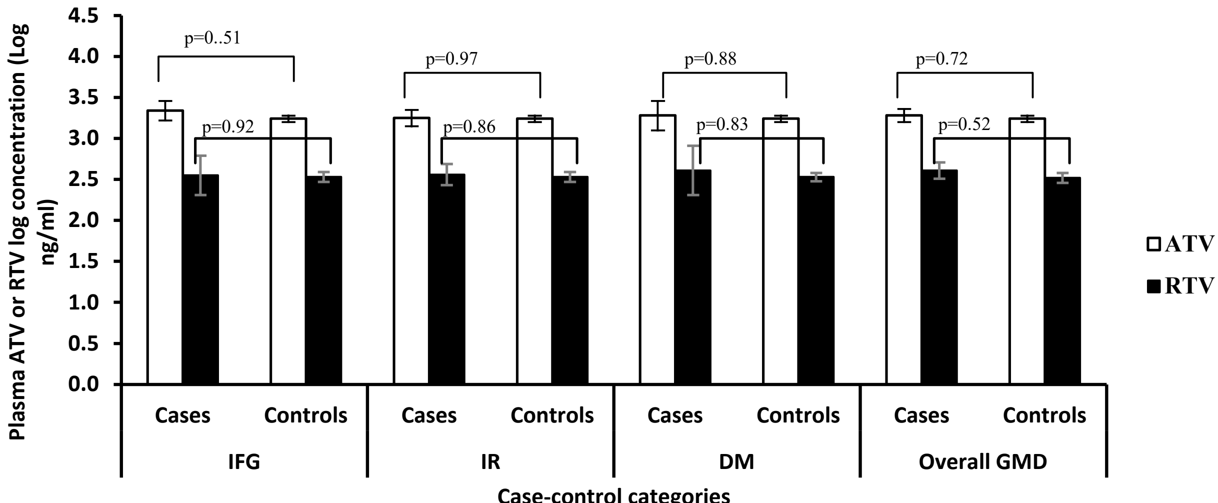

Figure 2 The mean log ATV and RTV mid-dose concentration versus specific forms of glucose metabolism disorders (GMDs). *p*= level of significance between the case and controls; IFG=impaired fasting glycemia; IR=insulin resistance, DM=diabetes mellites; GMD= glucose metabolism disorder.

Unlike plasma concentration, proteins involved in the disposition of the ARTs appeared to influence GMDs. Accordingly, *CYP3A5*3* allele lowered the incidence of overall GMD by 20% (AOR=0.2, 95%CI 0.03-0.9, p=0.04) and IR by 10% (AOR=0.1, 95%CI 0.02-0.9, p=0.04) than the wildtype allele in subjects on ATV/r-based cART (**Table 3**). By contrast, the C allele of *SLCO1B1*5* increased the incidence of overall GMD by about nine-fold than the wildtype allele (AOR= 8.8, 95%CI 1.8-41.7, p=0.006). Furthermore, ALP, ALT,

and direct bilirubin levels significantly predicted the occurrence of GMD. ALP was found to be a common predictor of IFG (p=0.002), IR (p=0.02), and overall GMD (p=0.005), whereas ALT and direct bilirubin levels predicted the incidence of overall GMD (both p=0.03). The incidence of IR was strongly predicted by direct bilirubin level, with a unit increase in bilirubin resulting in a 28.9 times higher risk (p=0.02).

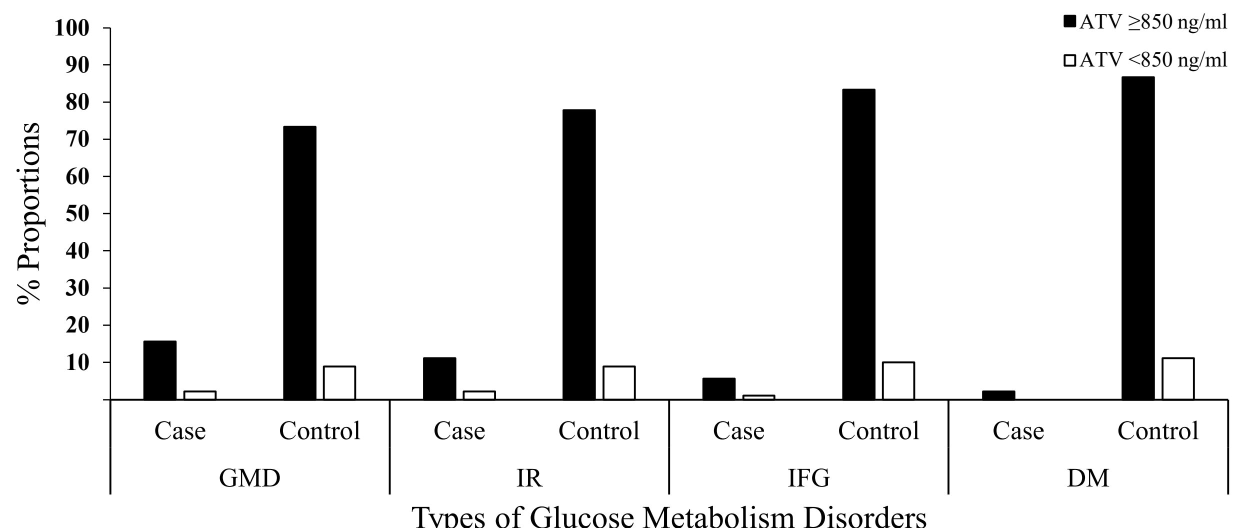

Figure 3 percentage proportions of GMD types based on category of ATV mid-dose concentration <850 or ≥850.DM and IFG exclusively observed among ATV ≥850 ng/ml of plasma concentration (toxic level).

Predictors of Log CP12 of ATV and RTV

Overall study subjects

Based on linear regression prediction model (Table 4), BMI (p=0.036), creatinine serum level (p=0.014), direct bilirubin (p=0.022), HOMA-IR (p=0.026), *CYP3A5*3* (p=0.047), and *ABCB1 c.4036A>G* (p=0.019) alleles were positive predictors of ATV CP12 in the overall study participants. Whereas, the duration on prior 1st line cART (p=0.005), ALT (p=0.007), and fasting serum insulin (0.029) negatively predicted the ATV CP12 among overall study subjects.

In the case of CP12 of RTV, duration on prior 1stline cART (p=0.005) and ALT level (p=0.007) were negative predictors, while the pharmacogenetic factors showed a positive association [*CYP3A5*3* allele (p=0.047), *SLCO1B1B *5*(p=0.013) and **1B*(p=0.045), and *ABCB1 c.4036A>G* (p=0.05)] (Table 4).

Table 3 Pharmacokinetic-pharmacogenetic predictors of glucose metabolism disorders in patients receiving ATV/r-based cART, *N*=90.

Variables		Overall GMD				IFG				IR			
		Univariate		Multivariate		Univariate		Multivariate		Univariate		Multivariate	
		COR (95`%CI)	*p*	AOR (95%CI)	*p*	COR (95%CI)	*P*	AOR (95%CI)	*p*	COR (95%CI)	p	AOR (95%CI)	*p*
Gender	F M	1 1.9 (0.60, 5.9)	0.28			1 0.5 (0.06, 4.8)	0.57			1 1.5 (0.4, 5.3)	0.58		
Age (years)	<46 ≥46	1 2.1 (0.7, 6.2)	0.19			1 0.9 (0.16,5.2)	0.91			1 2.0 (0.6,6.8)	0.27		
BMI		1.0 (0.92, 1.1)	0.70			0.9 (0.8,1.1)	0.56			1.03 (0.9, 1.1)	0.66		
Cumulative time on cART (Months)		1.0 (0.99, 1.0)	0.28			1.0 (0.99, 1.02)	0.84			1.0 (0.99, 1.02)	0.71		
Cumulative time on ATV/r-based (Months)		0.99 (0.98, 1.0)	0.74			1.0 (0.95,1.02)	0.46			1.0 (0.97, 1.02)	0.67		
Time on prior 1st-line (Months)		1.0 (0.98, 1.0)	0.83			1.0 (0.99,1.01)	0.61			1.0 (0.99, 1.0)	0.83		
Creatinine		0.8 (0.05,14.0)	0.91			0.1 (0.0,11.8)	0.34			0.9 (0.04, 19.9)	0.92		
Urea		1.0 (0.97,1.1)	0.23			1.1 (0.97,1.2)	0.15			1.0 (0.9, 1.1)	0.74		
ALP		1.02 (1.01,1.04)	0.01	1.03 (1.01, 1.05)	0.005	1.04 (1.02,1.07)	0.002	1.04(1.02,1.07)	0.002	1.02 (1.0,1.04)	0.02	1.03 (1.0,1.05)	0.02
ALT		1.1 (1.0, 1.2)	0.06	1.1 (1.0, 1.3)	0.03	1.1 (1.0,1.2)	0.24			1.1 (1.0,1.2)	0.047	1.1 (0.98,1.3)	0.09
AST		1.0 (1.0, 1.1)	0.07			1.03 (0.98,1.08)	0.27			1.06 (1.0,1.1)	0.039		
Direct Bilirubin		8.8 (1.02, 75.7)	0.05	31.3 (2.9, 341.9)	0.03	0.9 (0.03, 25.4)	0.94			19.2 (1.5,246.4)	0.02	28.9 (1.7, 484.9)	0.02
Total Bilirubin		1.1 (0.7, 1.7)	0.76			0.9 (0.4,1.7)	0.62			1.1 (0.7,1.8)	0.73		
Log ATV CP12 (ng/ml)		1.2 (0.2, 6.5)	0.83			2.4 (0.1,50.0)	0.57			0.9 (0.2, 5.3)	0.90		
Log RTV CP12 (ng/ml)		1.1 (0.4, 3.3)	0.87			0.9 (0.2, 4.6)	0.92			0.8 (0.3, 2.6)	0.72		
Minor Alleles (included in model 3)													
*CYP3A4*1B*	**1B*	0.6 (0.2,1.8)	0.37			0.3 (0.05,1.8)	0.19			0.9 (0.3,3.2)	0.90		
*CYP3A5*3*	**3*	0.4 (0.1, 1.4)	0.14	0.2 (0.03, 0.9)	0.04	0.8 (0.1,7.1)	0.80			0.2 (0.06,0.9)	0.04	0.1 (0.02,0.9)	0.04
*CYP3A5*6*	**6*	1.3 (0.4, 4.3)	0.65			1.4 (0.2,8.2)	0.70			1.5 (0.4,5.3)	0.58		
UGT2B7 g.-372 G>A	A	1.0 (0.3, 3.6)	0.96			1.8 (0.2,16.0)	0.61			1.0 (0.3,4.2)	0.96		
*SLCO1B1*5 c.521 T>C*	C	2.5 (0.8, 7.6)	0.10	8.8 (1.8, 41.7)	0.006	2.2 (0.4,11.8)	0.34			1.6 (0.5, 5.6)	0.46		
*SLCO1B1*1B c.388 A>G*	G	3.5 (0.4,28.8)	0.24			-				2.4 (0.3,20.2)	0.42		
ABCB1 c.3435 C>T	T	0.6 (0.2,1.8)	0.37			1.1 (0.2,5.8)	0.91			0.5 (0.14,1.80)	0.29		
*ABCB1 c.4036 (c.*193) A>G*	G	0.4 (0.1,1.4)	0.16			0.4 (0.04,3.2)	0.36			0.1 (0.02,1.2)	0.07	0.2 (0.02, 1.6)	0.12

Among cases and controls

Linear regression analysis identified similar predictors for both ATV and RTV CP12 in controls. Duration on prior 1st-line therapy ($p=0.007$ & 0.008) and ALT levels ($p=0.004$ and $p=0.001$) were negative predictors, whereas direct bilirubin ($p=0.037$ and 0.024) and *ABCB1 c.4036A>G* ($p=0.001$ and 0.012) were positive predictors. On the other hand, while creatinine was positively associated with CP12 of ATV ($p=0.002$), it was marginally associated with CP12 of RTV ($p= 0.052$) (Table 4).

In cases, different predictors for the two drugs were identified. Whereas age, body weight, urea, ALP and *SLCO1B1B *5*C allele were identified as a negative predictor ($p<.000$ to $p<0.002$); gender, cumulative time on cART, cumulative time on ATV/r-based cART, fasting glucose level, *CYP3A5*3, CYP3A5*6*, *ABCB1 c.3435C>T* identified as positive predictors ($p<0.05$) of ATV CP12. On the other hand, cumulative time on ATV/r-based cART, lipid parameters (triglyceride and LDL-c), *CYP3A4*1B*, *CYP3A5*3&*6*, *SLCO1B1B *5*, and *ABCB1 c.3435C>T* positively predicted ($p<0.05$); body weight, cumulative time on cART, duration on prior 1st-line therapy, *SLCO1B1B *1B*, and *ABCB1 c.4036A>G* negatively predicted CP12 of RTV (Table 4).

Table 4 Predictors of the log ATV/RTV mid-dose plasma concentration (CP12) in patients with GMD (cases), without GMD (controls), and overall study participants based on linear regression (stepwise) analysis, *N*=90.

Variables		***Overall study subjects***				***Controls***				***GMD cases***			
		ATV (r=0.71, p=0.018)		***RTV (r=0.71, p=0.026)***		***ATV (r=0.802, p=0.002)***		***RTV (r=0.779, p=0.014)***		***ATV (r=1.0, p<0.000)***		***RTV (r=1.0, p=0.002)***	
		β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
Age (years)		0.003	0.517	0.006	0.33	0.004	0.386	0.01	0.151	-0.008	0.000		
Gender (male)		0.061	0.617	0.197	0.296	0.013	0.920	0.118	0.580	0.144	0.001		
Body weight (Kg)		-0.01	0.125	-0.011	0.298	-0.01	0.247	-0.013	0.347	-0.012	0.000	-0.007	0.002
BMI (Kg/m^2)		0.037	0.036	0.037	0.161	0.033	0.111	0.039	0.246				
Cumulative time on cART (Months)		0.001	0.832	0.001	0.517	0.001	0.221	0.001	0.484	0.001	0.000	-0.002	0.004
Cumulative time on ATV/r-based (Months)		0.000	0.910	0.001	0.545	-0.001	0.454	-0.002	0.472	0.003	0.000	0.010	0.001
Time on prior 1st-line (Months)		-0.001	0.005	-0.001	0.005	-0.001	0.007	-0.001	0.008			-0.002	0.006
Creatinine		0.558	0.014	0.464	0.172	0.777	0.002	0.762	0.052				
Urea		-0.004	0.499	-0.006	0.477	-0.006	0.344	-0.013	0.237	-0.002	0.002		
ALP		0.001	0.572	0.001	0.597	0.000	0.781	-0.003	0.309	-0.001	0.001		
ALT		-0.029	0.007	-0.056	0.001	-0.033	0.004	-0.068	0.001				
AST		0.002	0.647	0.012	0.151	-0.008	0.309	0.005	0.701				
Direct Bilirubin		0.457	0.022	0.509	0.092	0.638	0.037	1.131	0.024				
Total Bilirubin		0.044	0.251	0.061	0.294	0.025	0.566	-0.007	0.920				
Fasting serum glucose		-0.005	0.147	-0.004	0.366	0.000	0.988	0.006	0.749	0.002	0.000		
Fasting serum Insulin		-0.089	0.029	-0.099	0.110	-0.044	0.468	0.113	0.620				
HOMA-IR		0.400	0.026	0.439	0.106	0.206	0.438	-0.499	0.621				
Total Cholesterol		-0.003	0.275	-0.007	0.152	-0.002	0.461	-0.008	0.154				
Triglyceride		0.000	0.824	0.000	0.775	-0.001	0.502	-0.001	0.638			0.001	0.008
LDL-C		0.005	0.158	0.008	0.156	0.004	0.252	0.009	0.157			0.007	0.003
HDL-C		0.000	0.938	0.000	0.975	0.000	0.811	0.000	0.972				
*CYP3A4*1B*	**1B*	-0.033	0.699	0.074	0.576	-0.044	0.631	0.159	0.298			0.48	0.005
*CYP3A5*3*	**3*	0.242	0.047	0.414	0.027	0.181	0.193	0.428	0.068	0.007	0.012	0.321	0.009
*CYP3A5*6*	**6*	-0.005	0.958	-0.096	0.486	-0.044	0.666	-0.188	0.260	0.274	0.000	0.234	0.003
UGT2B7 g.-372 G>A	*A*	-0.070	0.473	-0.171	0.256	-0.011	0.911	-0.164	0.347				
*SLCO1B1*5 c.521 T>C*	*C*	-0.092	0.296	-0.346	0.013	0.010	0.918	-0.228	0.133	-0.342	0.000	0.030	0.061
*SLCO1B1*1B c.388 A>G*	*G*	0.139	0.187	0.326	0.045	0.038	0.726	0.124	0.493			-0.517	0.006
ABCB1 c.3435 C>T	*T*	0.002	0.979	0.051	0.663	-0.089	0.313	-0.064	0.648	0.263	0.000	0.981	0.002
*ABCB1 c.4036 (c.*193) A>G*	*G*	0.193	0.019	0.243	0.050	0.307	0.001	0.356	0.012			-0.188	0.015

Discussion

The aim of this study was to identify pharmacokinetic and pharmacogenetic factors associated with the development of GMD in patients receiving ATV/r-based second-line cART. To the best of our knowledge, this was the first study to directly relate either plasma concentrations of ATV and RTV or the important gene variants of metabolizing enzyme and drug-transporters of ATV and RTV with the incidence of GMDs in PLWH. The minimum effective concentration of 150 ng/ml for ATV has been proposed based on virologic suppression [24,25]. Based on the concentration of ATV that is more likely to be associated with hyperbilirubinemia, >850 ng/ml, has been considered as supratherapeutic range of ATV [24,25]. Thus, the recommended plasma concentration range for ATV is 150 to 850 ng/m [25].

In this study, about 88.9% of all study subjects had ATV CP12 levels above the recommended plasma level, >850 ng/ml, which might indicate a higher boosting effect of RTV. The observed proportions of sub-therapeutic, therapeutic, and supratherapeutic ranges of ATV based on CP12 in our study was relatively similar to a Kenyan study when the proportion at the C_{max} was taken for comparison [2]. However, Gervasoni *et al.* [14] reported a higher proportion of subtherapeutic ATV concentration (11.6%) than the present study (1.1%). In fact, our study's proportions contrasted with those of Colombo *et al.* [25]and Boffito*et al*. [26], as there were no patients with levels below the recommended 150 ng/ml. Unlike to the UK cohort study, which reported 64% for ATV/r [24], our study found only 10% of study subjects who attained the recommended therapeutic range (150-850 ng/ml). Such differences in the studies might be attributed to difference in sample

collection time, study design (e.g., 24-72 h and 17 samples in Boffito*et al.* [26], and study population differences. Moreover, the cut-off therapeutic ranges (e.g. Gervasoni *et al.,* categorized 150 to 800 ng/ml as target and >800 as supratherapeutic) could also be another factor [14]. On the other hand, a strong correlation (r^2=0.7, p<0.000) between the CP12 of RTV and ATV in our study imply the significant booster effect of RTV, which have a positive relationship with higher ATV concentration as observed among the majority of study participants.

A number of ART-related adverse effects have been linked to elevated plasma concentrations [2,24,27]. One of the best examples is hyperbilirubinemia, which has been linked to higher ATV levels [28], due to a competitive inhibition of the bilirubin-conjugating enzyme, uridine-diphosphate glucuronosyl transferease-1A1 (UGT1A1) by ATV [3]. Although ATV induces hyperbilirubinemia, which in turn is clinically believed to predict IR and GMDs, the present study revealed that neither the log ATV nor the log RTV CP12 levels predicted the occurrence of GMDs. The supratherapeutic range of ATV had no association with GMD, as similar proportion of controls (89.2%) and cases (87.5%) had CP12 of ATV falling in this range. This appears to be in contradistinction with the notion that associates incidence of hyperbilirubinemia with elevated ATV concentrations. In fact, published reports in the literature indicate that ATV is considered to have safe metabolic profile with fewer effect on lipids and glucose [29], reinforcing our finding that ATV had no clinically significant effect on glucose metabolism and GMD incidence.

Our study also demonstrated that *CYP3A5*3* bought about a 20% protection than the wildtype genotype. Only a few studies implicated *CYP* genotypes with the incidence of glucose related disorders among general population. Our study, however, assessed the

association between genetic variants of drug metabolizing and transporter genotypes and incidence of GMD among PLWH on specific cART. Despite these differences, two studies reported the association of CYP genotypes with the incidence of T2DM. A Japanese study identified the association between CYP3A4 13989 A>G polymorphism and the prevalence of T2DM, with the heterozygous genotype being protective against this disorder [30]. A study on cohort of French general population revealed that CYP11B2 variants, -344T>C and 3097 G>A, are associated with T2DM in men participants [31]. In difference with these studies, our study found that *CYP3A5*3* variants had a protective role among patients on ATV/r-based cARTs.

In our study, the *SLCO1B1*5* allele increased the GMD occurrence by about nine-fold than the respective wildtypes. Due to the lack of data in the literature, this finding could not be adequately compared with other studies. However, an animal study revealed the significance of OATP1B (a protein encoded by SLCO1B1) in the control of hepatic function, particularly in the maintenance of glucose and cholesterol homeostasis [32]. According to this experiment, *Slco1b2* knockout mice were unable to lower glucose level after oral glucose tolerance tests and had a trend of increased fasting glucose level [32]. Aside from the role of *SLCO1B1* gene polymorphisms in the therapeutic effects of oral anti-diabetic and lipid-lowering agents [33,34], no study has examined this polymorphism as a risk factor for GMDs to yet.

ALP, ALT, and direct bilirubin levels independently predicted the incidence of IR and GMDs from clinical parameters, while ALP also predicted IFG. Chung *et al.* [35] found ALT, but not ALP, as a predictor of insulin resistance, specifically HOMA-IR, fasting glucose, and fasting insulin levels, after multivariate analysis in HIV-associated

lipodystrophy. Because these measurements were used to define IR and GMDs, our findings on ALT are consistent with their findings regardless of antiretroviral treatment status of the study subjects in their study. Regarding direct bilirubin, latest evidence supports bilirubin concentrations, including the total bilirubin level, as a protective (inverse) predictor of metabolic disorders [36–38]. In contrast, our study and other studies did not find significant association between total bilirubin and T2DM (GMD in our case) but with direct bilirubin [39,40]. Literature suggested age and race might contribute to such inconsistent findings [40]. Moreover, the difference in methodology and study subjects, for instance PLWH on ATV/r in our case, might also be a factor for such discrepancy [35].

Our study also identified pharmacogenetic covariates correlated with CP12 of ATV and RTV among the overall study participant, cases and controls. In the linear regression analysis of the entire study participants, the *CYP3A5*3* and *ABCB1B c. 4036* minor alleles were found to be significantly correlated with the CP12 of both ATV and RTV. Also, in cases, the *CYP3A5*3*, the *CYP3A5*6*, and the *ABCB1B c.3435C>T* minor alleles showed a significant positive correlation with ATV and RTV CP12. Comparing expressers against non-expressors, Anderson *et. al.* [35] demonstrated the association of ATV and RTV pharmacokinetics with *CYP3A5* and *ABCB1* haplotypes, which is consistent with our finding that a slow clearance was observed among non-expressors than expressors. Furthermore, the *CYP3A4*1B* affects ATV and RTV pharmacokinetics by altering metabolism [3], but our study found significant correlation only with CP12 of RTV. Consistent with the accumulating data, our study suggested that the *CYP3A5* and

*CYP3A4*1B* genotype had effect on ATV and RTV metabolism and variation in plasma concentrations [3,17,41].

Strikingly, another study pointed out that *ABCB1 c. 3435* was inversely associated with ATV concentration [42], unlike that of our study in which the c. 4036 was positively corelated with ATV CP12 among the overall study participants and the controls. Yet, among the cases, the ABCB1 c.3435 was positively associated with ATV CP12, which is in opposite direction of the finding of the above stated study [42]. *ABCB1* encodes for p-gp transporter that mediates the efflux of different substrates and has important role in drug pharmacokinetics, including PIs. Thus, genetic polymorphism may affect efflux transporter function that directly alter pharmacokinetic and plasma concentrations. Therefore, this may explain the observed correlation of ATV and RTV concentration in this study.

Besides that, *SLCO1B*1B* and *SLCO1B*5* minor alleles displayed a significant positive and inverse correlation, respectively, with the CP12 of RTV in the overall study subjects. Our study contradicts with a study that displayed no association of **5* (521T>C) and **1* (388A>G) with trough concentration of RTV [43]. Studies, however, reported the association of the **5* variant with an increasing trough concentration of lopinavir [44,45]. Comparing lopinavir in these studies with PIs of our study, the same trend was observed in our study with *5 variant and RTV CP12, but inversely with ATV CP12, among the cases. The OATP1B1 transporters, encoded by SLCO1B1, mediate the hepatic uptake of a diverse range of amphipathic organic compounds, including PIs [34]. SNPs of the genes of these transporters have been consistently associated with altered transport activity, which alters plasma concentrations of drugs, like PIs [34]. Therefore, a similar mechanism may account for positive or inverse association of ATV and RTV CP12 observed in our study.

The study has some limitation that the relatively small number of study subjects, but with approximate the cases: control ratio of 1:4. The time considered for the last dose was based on individual participants report. However, our study come up with a finding that assessed the pharmacokinetic and pharmacogenetic factors and the link with GMD among PLWH on ATV/r-based cART, which is the first at least in Ethiopia. Furthermore, our study generated data on the correlation between mid-dose ATV/RTV concentration and various sociodemographic, clinical, pharmacokinetic and pharmacogenetic factors among the overall, control and GMD case groups.

Conclusion

Overwhelming majority of the study participants had ATV CP12 levels above the recommended 850 ng/ml. Nevertheless, neither the log ATV nor the log RTV CP12 level predicted the occurrence of GMDs in patients receiving ATV/r-based cART. Whereas, the *CYP3A5*3* allele demonstrated a protective association while the *SLCO1B1*5* increased the risk of GMD than their respective wildtype alleles. On the other hand, the *CYP3A5*3* and *ABCB1B c. 4036* minor alleles were found to be correlated with the CP12 of both ATV and RTV, while the *SLCO1B*5* and *SLCO1B*1* minor alleles were found to be correlated specifically with RTV CP12. Our findings warrant further research with a larger sample size.

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Author's contribution

Conceptualization, W.T.T., W.A.D., E.E., and E.A.; methodology, W.T.T., E.M.M., N.F.T., W.A.D., E.E., and E.A.; software, W.T.T., E.M.M., and E.A.; validation, W.T.T., N.F.T., W.A.D., E.E., and E.A.; formal analysis, W.T.T., E.M.M., and E.A.; investigation, W.T.T., E.M.M., N.F.T., W.S., W.A.D., E.E., and E.A.; resources, W.T.T., W.S., W.A.D., E.E., and E.A.; data curation, W.T.T., E.M.M., N.F.T., and E.A.; writing—original draft preparation, W.T.T.; writing—review and editing, W.T.T., E.M.M., N.F.T., W.S., W.A.D., E.E., and E.A.; visualization, W.T.T., N.F.T., E.E., and E.A.; supervision, W.A.D., E.E., and E.A.; project administration, W.T.T., W.A.D., and E.A.; funding acquisition, W.S., E.E., and E.A. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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ANNEXES

Annex I: Confidential Medical Record Data Collection Form

Form completed date:	Visit No:	Study site: TASH- ART clinic (Adult)

1. Patient identification (Name and Address)

Patient's Chart/card Number | Phone No:

Sample Code No.

Current Address: Region: | Sub-city | Kebele: | House No:

2. Fasting status | Overnight Fasting ☐ | Non-Fasting ☐

3. Measure of level of Adherence, last dosing time and sample collection time

Number of missed doses over the last 3 days ______________________	**Any remark/ note**
Time of last dose of EFV or ATV/r last night? __________	
Sample collection time: _____________________________	

4. Demographic Information

Diagnostic status
- ☐ IHIV infection (Not AIDS)
- ☐ AIDS state
- ☐ HIV-DM comorbid
- ☐ HIV-HCB coinfection
- ☐ HIV-TB coinfection
- ☐ HIV-TB-DM comorbidity

Age:
Sex:
☐ Male ☐ Female

For females only (current state)
☐ pregnant ☐ non-pregnant

Religious Belief
- ☐ Orthodox
- ☐ Protestant
- ☐ Catholic
- ☐ Muslim
- ☐ Other --------------------
- ☐

Marital status
- ☐ Single
- ☐ Married
- ☐ Widowed
- ☐ Divorced

Level of Education
- ☐ Illiterate
- ☐ Able to read and write
- ☐ Primary school
- ☐ Secondary school
- ☐ Tertiary

5. Patient characteristics

Date of HIV diagnosis	**WHO clinical stage before ART start**	**Current WHO clinical stage**
Date / Month / Year	☐ Stage 1 Stage 3 Stage 2 Stage 4	Stage 1 Stage 3 Stage 2 Stage 4

CD4 count	**Viral Load**
Baseline ART initiation _________ Recorded Date______________	Baseline ART initiation __________ Recorded Date _________
Current ________________ Recorded Date _______________	Current ____________________ Recorded Date ____________

Physical examination of Vital Signs

BP= __________mmHg Pulse= _________________ RR = _____________________	Weight _________________________ Height __________________________ Waist circumference: _______________	**BMI** (will be calculated later) _______________________

6. Medication History

Initial ART regimens (if there were switches)

	Regimens	Start date	Reason for switch
Initial			
1st Switch			
2nd Switch			
3rd Switch			

Current ART regimens	
Regimens	Start date

Co-administered medications currently used (including antidiabetics) other than antivirals

	Drug	Dose	Indication	Start Date	End date
1					
2					
3					
4					
5					

7. Herbal Use Along with ARV Agents

Herbal medication used by the patient currently	**1.**	**2.**

8. Adverse effect identified

ADRs	**No**	**Yes**	**Responsible drug with dose**	**Recorded Date**	**ADRs**	**No**	**Yes**	**Responsible drug with dose**	**Recorded Date**
Fever	☐	☐			Mood problems	☐	☐		
Weight gain	☐	☐			Delusions	☐	☐		
Nausea & Vomiting	☐	☐			Hallucinations	☐	☐		
Loss of appetite	☐	☐			Suicidal ideation	☐	☐		
Diarrhea	☐	☐			Skin rash	☐	☐		
Polyphagia	☐	☐			Drowsiness	☐	☐		
Polyuria	☐	☐			Headache	☐	☐		
Polydipsia	☐	☐			Dizziness	☐	☐		
Night mare	☐	☐			Fatigue	☐	☐		
Lack of concentration	☐	☐			Lactic acidosis	☐	☐		
Depression	☐	☐			Cough	☐	☐		
Insomnia	☐	☐			Dyspepsia	☐	☐		
Arthralgia /myalgia	☐	☐			Allergy/hypersen sitivity	☐	☐		
Anemia	☐	☐			Peripheral neuropathy	☐	☐		

9. Patient history of diseases since ART start

Past medical problem identified/treated	**No**	**Yes**	**Not recorded**	**Specific status**	**Recorded Date**
HBV infection	☐	☐	☐		
TB infection	☐	☐	☐		
Uncontrolled HTN	☐	☐	☐		
Dyslipidemia	☐	☐	☐		
Diabetes Mellitus	☐	☐	☐		
Kidney Diseases	☐	☐	☐		

Liver Diseases	☐	☐	☐		
Thyroid abnormalities	☐	☐	☐		
Malignancy requiring treatment	☐	☐	☐		
Cardiovascular diseases	☐	☐	☐		
Neuropsychiatric problems	☐	☐	☐		
Epilepsy or convulsion	☐	☐	☐		
AIDS defining condition	☐	☐	☐		
Other previous diseases	______________________ ______________________			________________________ ________________________	

10. Abuse of substances	
Alcohol intake	☐ More than daily; ________Number of Drinks/days ☐ Daily ☐ 3-4 times per week ☐ Once/week ☐ Once last 30 days ☐ Past use ☐ Never =0
Regular *khat* chewer	☐ More than daily; ___________number/days ☐ Daily ☐ 3-4 times per week ☐ Once/week ☐ Once/last 30 days ☐ Past use ☐ Never
Smoking	☐ Current smoker ☐ Previous smokers ☐ Never smoked Number of cigarettes/days= _______ Number of times smoked= _______

11. Retrospective/Prospective Record of Laboratory Results

Type of Lab	Results from Study Samples	1st nearest record Date: ________	2nd nearest record Date: ________	3rd nearest record Date: ________	4th nearest record Date: ________	5th nearest record Date: ________	6th nearest record Date: ________	7th nearest record Date: ________	Initial record (baseline) Date: ________
FBS									
Insulin									
HbA1c									
T. Chole.									
TG									
HDL									
LDL									

HOMA									
AST(SGOT)									
ALT(SGPT)									
ALP									
Bil (T)									
Bil (D)									
T. Protein									
Albumin									
α-1 acid glycoprotein									
Urea									
Creatinine									
HCG									
CD4									
HIV RNA									

Annex II: Participants Information Sheet

Addis Ababa University

School of Pharmacy

Participants Information Sheet

Dear Participant,

Before you decide to take part in this study, we encourage you to read or listen carefully when it's read. Please, feel free to ask any question related to the study.

My name is Wondmagegn Tamiru. I am PhD student at School of Pharmacy, Addis Ababa University. You are kindly asked to take part in a study entitled 'Effect of Efavirenz, Dolutegravir- or Ritonavir Boosted Atazanavir-Based Combination Antiretroviral Therapy on Glucose Metabolism or Drug Induced Adverse Effects among Treatment Experienced HIV Patients: Pharmacokinetic and Pharmacogenetic Study'. Stated below are some descriptions about the research.

Purpose of Study: The purpose of this research is to investigate the effect of long-term anti-HIV therapy on blood sugar level and related disorder. Besides, it assesses the relationship between elevated blood sugar level and associated disorders relative to factors affecting blood drug level and genetic factors related to drug metabolizing enzymes.

Procedures: If you agree to be in this study, we would like to draw two teaspoons full (10ml) venous blood samples to screen fasting plasma glucose levels, lipid levels as well as genetic factors related to drug level in the blood and drug metabolizing enzymes. The extracted data from these studies will be corelated with your medical information collected from your medical records at the clinic.

Sample storage and shipment: blood samples collected will be stored at -80°C at our laboratory until all research samples are collected, for maximum of 6 months locally, and will be transported to the Divisions of Clinical Pharmacology and Clinical Chemistry, Karolinska Institute, Stockholm (Sweden) for genotyping analysis and determination of pharmacokinetics of various drugs and their markers where samples will be stored for a

maximum of 3 months there. After all laboratory procedures are done, all ruminant samples will be destroyed and will not be stored for any other kind of research purpose or procedure.

Confidentiality: All information provided for this research will be maintained confidentially. Information related to your participation including your personal identity and medical information will also be maintained confidentially. No information that discloses your identity will be used in any reports about the study. Only identification code will be used at all levels of the study to record and document all information associated with the study. All information associated with this study will be kept in locked cabinets or computer files will be secured with passwords protection. Your doctor, the responsible researcher and responsible institutional ethical review board members may have the access to your medical records. However, no records that identify you will be allowed to leave the trial center.

Risks/Discomforts of being in this Study: there is only minimal risk from participating in this study. Little discomfort may occur during blood collection just similar to the regular medical procedure during medical follow-up. However, in the rare occurrences of pain, bleeding or other reactions appropriate treatment will be provided as a result of blood collection procedure.

Benefits: - There are no direct benefits/remuneration to you for participating in this research study. The results of the study will help identify the possible association of glucose metabolism disorders with antiretroviral therapy that such information may optimize drug selection and minimize glucose related adverse effects based on possible clinical adjustments made after the generation of evidences based on such studies. This study may help improve drug selection and dosage or avoid unnecessary combinations to minimize glucose related abnormalities during long-term antiretroviral therapy.

Right to withdraw the study: If you are not comfortable, you have the right to withdraw from the study at any level. Please feel free to stop participation when you don’t want to at any of the study. Some question may be personal and some may be difficult to answer. You are free to refuse to answer any question for any reason and withdraw from the study at any time without a problem to you.

Right to Ask Questions and Report Concerns: You have the right to ask any questions about this research study or report any concern related the research conduct. If you have any further questions or want to file a complaint about the research at any time, please feel free to contact:

1. The IRB office: telephone no. +251118961396
2. Wondmagegn Tamiru Tadesse: mobile Phone: +251911637599, e-mail: mail2wondm@gmail.com or wondmagegn.tamiru@aau.edu.et

I appreciate your cooperation to a great extent.

_______________________ ____________ ____/____/___

Name of study participant Signature Day/month/year

_________________ ___________ ____/____/___

Witness (Illiterate) Signature Day/month/year

_______________________ _________________ ____/____/___

Name of the researcher Signature Day/month/year

Annex III: Amharic version of the information sheet

ቅፅ 3፡ - ለተሳታፊው አጠቃላይ መረጃ ለመስጠት የተዘጋጀ ቅፅ (አማረኛ ትርጉም)

አዲስ አበባ ዩኒቨርሰቲ

የፋርማሲ ት/ቤት

ውድ ተሳታፊውች

በጥናቱ ለመሳተፍ ከመወሰኖ በፊት ይህንን ቅፅ በትክክል እንዲያነቡ ወይም ሲነበብልዎ በጥሞና እንዲያዳምጡ እንዲሁም ግልፅ ያልሆኖሎትን ነገር ቢኖር በነፃነት እንዲጠይቁ እናበርታታለን፡፡

ወንድማገኝ ታምሩ እባላለሁ:: የአ.አ.ዩ. የፋርማሲ ት/ቤት በፋርማኮሎጅ ትምህርት ክፍል የፒ ኤች ዲ ዲግሪ ተማሪ ነኝ:: በዚሁ ወቅት የኤች. አይ. ቪ.. ህክምናና የደም ስኳር ተያያዥ የሆኑ የጤና ችግሮች ያላቸውን ቁርኝት እንዲሁም መዲኃኒትን የሚያብላሉ ኢንዛይሞች ከሰው ሰው ያላቸውን የዘረመል ልዩነት እና የደም ስኳር ተያያዥ የሆኑ የጤና ችግሮች ያላቸውን ቁርኝት ና ከስኳር ዝውውር ጋር ተያያዥነት ያላቸው የጎንዮሽ ጉዳቶችን ቁርኝት በዚህ ጥናት ይካሄዳል፡፡

የጥናቱ ዋና አላማ፡ የኤች. አይ. ቪ.. ህክምና የሚሰጡ መዲኃኒቶች ና የደም ስኳር ጋር ተያያዥ የሆኑ የጤና ችግሮች ያላቸውን ቁርኝት እንዲሁም መድኃኒት አብላይ ኢንዛይሞች ያላቸውን የዘረመል ልዩነት ከተጠቀሰው የጤና ችግር ጋር ያላቸውን ግንኙነት ያጠናል።

የጥናቱ ሂደት፡ - እርስዎ በጥናቱ ለመሳተፍ ፈቃደኛ ከሆኑ ሁለት የሻይ ማንኪያ (10 ሚሊ ሊትር) የሚሆን ደም ከደም-መልስ ቧንቧ ጥናት ለሚያከዉኑ ባለሙያ ናሙና ይሰጣሉ፡፡ ይህም ናሙና የደም ስኳር መጠን፤ የስብ መጠን እንዲሁም ከመድኃኒት የሙብላላት ሂደት ጋር ተዛምዶ ያላቸው ኢንዛይሞች ዘረመል ና በደም ውስጥ የሚገኝ የመድኃኒት መጠን በተለያዩ መሳሪያዎች ጥናት ይደረግበታል፡፡ በተጨማሪም ከታካሚው ሰነድ ላይ የተለያየ ተያያዥ መረጃ በመልቀም ጥናት ይደረግበታላ፡፡

የናሙና አቀማመጥ ና ማጓጓዝ፡ የተሰበሰቡ ናሙናዎች ከዜሮ በታች እስከ -80 °C በሚደርስ ቅዝቃዜ በ ማቀዝቀዣ ውስጥ፤ እጅግ ቢዘገይ ለ 6 ወራት በሀገር ውስጥ፤ በማቆየት የናሙና መሰብሰብ ሂደት ሲጠናቀቅ ለምርምራ ስዊድን በሚገኝ ካሮልኒስካ ኢንስቲትውት ይጓጓዛል። እዚያም አስፈላጊው ምርመራ እስኪጠናቀቅ ቢበዛ ለ 3 ወራት ይቆያል። ምርመራው ሲጠናቀቅ የተረፉ ናሙናዎች እንዲወገዱ ይደረጋል።

የጥናቱ ሚስጢራዊነቱ፡ - የሚሰጡት መረጃዎች ሚስጥራዊነታቸው የተጠበቁ ናቸው፡፡ የታካሚው ስም አይጻፋም እንዲሁም የጥናት ሠነዶች ተቆልፎ የሚቀመጡ ሲሆን የተፈቀደለት ሰው ብቻ ፋይሉን ማየት ይችላል፡፡ ከዚህ ጥናት በሚወጡ ዘገባዎች ወይም የህትመት ውጤቶች ላይ ስም ወይም ሌላ የእርስዎን ማንነት የሚገልጽ መረጃ አይኖርም፤ አይገለጽም፡፡ ከምርመራ የሚገኘውም ውጤት ወይም ሌላ መረጃ ለሚመለከታቸው አካላት ለምሳሌ፤ እርስዎን የሚንከባከቡ የህክምና ባለሙዎች እና ጥናቱን ለሚያካሄዱት ባለሙያዎች እንዲሁም ጥናቱ ስነምግባርን ጠብቆ

መከናወኑን ለሚከተሉት የኮሚቴ አባላት ብቻ ይገለፃል፡፡ ኮምፒውተር ላይ ያሉ መርጃዎች ምስጢራዊነታቸው የተጠበቀ ሲሆን በወረቀት ያሉ መረጃዎችም ደህንነቱ በሚጠበቅ ቦታ የሚቆለፉና የተፈቀደለት ሰው ብቻ ሊያያቸው እንዲችል ተደርጎ ይጠበቃሉ፡፡

ሊከሰቱ ስለሚችሉ ስጋቶችና የምችት መጓደሎች፡ - ለጥናቱ በሚወሰደዉ ናሙና ምክንያት የተለየ ችግር አይከሰትም፡ ፡ የሚያሰጋ ምንም ነገር የለዉም ምክንያቱም የጥናቱ ናሙና አወሳሰድ ከወትሮዉ በሽተኛዉ ለህክምና ብሎ ከሚሰጠዉ የተለየ አይደለም፡፡ ናሙና በሚወሰድበት ሂደት ከትንሽ የህመም ስሜት ውጪ ይሄ ነው የሚባል ችግር የሚያስከትል ወይም የሚያሰጋ አይደለም፡፡ ቢሆንም ከደም ናሙና አወሳሰድ ጋር ተያይዞ ሊከሰቱ የሚችሉ ችግሮች ማለትም ህመም፤ መድማት ና ሌሎች ችግሮች ቢከሰቱ አስፈላጊው ህክምና እንዲሰጥ ይደረጋል።

የሚስገኘው ጥቅም፡ - በጥናቱ በመሳተፍዎ ምንም አይነት ክፍያ አይጠየቁም ወይም የሚያገኙት ገንዘብ አይኖርም፡፡ ከጥናቱ በሚገኘው እውቀት በኤች. አይ. ቪ. ህክምና ሊከሰት የሚችል የስኳር ና ተያያዥ የሆኑ የጤና ችግሮችን ለመከላከል ና የተሻለ የህክምና ስራአት ና ተገቢ የሆነውን መድሃኒት ለተገቢው ታካሚ በትክክለኛ መጠን ለመምረጥ እንዲሁም የዘረ-መል ልዩነቶች ያማከለ ህክምና እንዲፈጠር ይረዳል፡፡

ከጥናቱ ስለማቋረጥ፡- በጥናቱ የሚሳተፉት ፈቃደኛ ከሆኑ ብቻ ነው፡፡ ስለዚህ መሳተፍ ከጀመሩ በኋላ ማቋረጥ ፤ አለመሳተፍ ወይም መመለስ የማይፈልጉት ጥያቄ ቢኖር ይለፈኝ የማለት ሙሉ መብትዎ የተጠበቀ ነው፡፡ በጥናቱ መሳተፍ ወይም አለመሳተፍ አገልግሎት ላይ ምንም አይነት ጥቅምም ሆነ ጉዳት አይኖረውም፡፡ ጊዜዎትን መስዕዋት አድርገው ሰለተባበሩኝ ከልብ አመሰግናለሁ፡፡

ስለ ጥናቱ ጥያቄ ቢኖርዎ ወይም ለመጠቆም ቢፈለጉ፡ - ይህንን ጥናት አስመልክቶ ጥያቄ ካለዎት ወይም የጥናቱ የመጨረሻ ዉጤት ምን እንደሆነ ለማዉቅ ከፈለጉ በሚከተለዉ አድራሻ ሊያገኙን ይችላሉ፡፡

1. አይ. አር. ቢ. ጽ/ቤት፡ ስልክ ቁ. +251118961396
2. ወንድማገኝ ታምሩ ታደሰ፡ ሞባይል ስልክ ቁ. +251911637599፣

   ኢሜል፡ mail2wondm@gmial.com or wondmagegn.tamiru@aau.edu.et

ስለ ትብብርዎ በጣም እናመሰግናለን።

______________________	______________________	___/____/___
የተሳታፊው ሥም	ፊርማ	ቀን /ወር/ዓ.ም
______________________	______________________	____/____/___
ምስክር (ማንበብና መፃፍ ለማይችሉ)	የምስክር ፊርማ	ቀን /ወር/ዓ.ም
________________	_________________	___/____/___
የተመራማሪው ስም	ፊርማ	ቀን /ወር/ዓ.ም

Annex IV: Institutional Review Board Ethical approval

ADDIS ABABA UNIVERSITY, COLLEGE OF HEALTH SCIENCES (IRB)

አዲስ አበባ ዩኒቨርስቲ ጤና ሳይንስ ኮሌጅ

Institutional Review Board

ANNEX 3

Form AAUMF 03-008

IRB's Decision

Meeting No: 03/2019 Date: 20/03/2019

Protocol number: 019/19/sop

Protocol Title: Effect of efavirenz/Dolutegravir or Ritonavir boosted Atazanavir based Combination Antiretroviral therapy on Glucose metabolism or drug induced adverse effects among Treatment experienced HIV patients: Pharmacokinetics and Pharmacogenetics study

Principal Investigator:	Wondmagegn Tamiru
Institute:	College of Health Sciences, AAU
Elements Reviewed (AAUMF 01-008)	☑ Attached ☐ Not attached
Review of Revised Application ☐ Yes ☐ No	Date of Previous review:
Decision of the meeting:	☑ Approved ☐ Approved with Recommendation ☐ Resubmission ☐ Disapproved

I. Elements approved-
1. Protocol Version No: 03
2. Protocol Version Date:
3. Informed consent Version No: 03
4. Informed Consent Version Date:

II. Obligations of the PI-
1. Should comply with the standard international & national scientific and ethical guidelines
2. All amendments and changes made in protocol and consent form needs IRB approval
3. The PI should report SAE within 10 days of the event
4. End of the study, including manuscripts and thesis works should be reported to the IRB
5. The PI should report non-compliance and unanticipated events

III. TO NERC ☒

Institution Review Board (IRB) Approval: Period from: April 29, 2019 to April 28, 2020

Follow up report expected in: 3 Months 6 Months X___ 9 Months ___One year____

Chairperson, IRB
Dr. Adamu Addissie

Signature

Director of Research & Technology Transfer, CHS
Dr. Wondwossen Amogne

Signature

ADDIS ABABA UNIVERSITY, COLLEGE OF HEALTH SCIENCES (IRB)

አዲስ አበባ ዩኒቨርስቲ ጤና ሳይንስ ኮሌጅ

Institutional Review Board

ANNEX 3

Form AAUMF 03-008

IRB's Decision

Meeting No: 03/2019 Date: 20/03/ 2019

Protocol number: 019/19/SoP

Protocol Title: Effects of Efavirenz, Dolutegravir or Ritonavir boosted Atazanavir- based combination antiretroviral on Glucose metabolism or Drug induced adverse drug effects among treatment experienced HIV/AIDS patients: Pharmacokinetic and Pharmacogenetic study"	
Principal Investigator:	Wondmagegn Tamiru
Institute:	College of Health Sciences, AAU
Elements Reviewed (AAUMF 01-008)	☑ Attached ☐ Not attached
Review of Revised Application ☐ Yes ☐ No	Date of Previous review:
Decision of the meeting:	☑ Approved ☐ Approved with Recommendation ☐ Resubmission ☐ Disapproved

I. Elements approved-
1. Protocol Version No: 03
2. Protocol Version Date:
3. Informed consent Version No: 03
4. Informed Consent Version Date:

II. Obligations of the PI-
1. Should comply with the standard international & national scientific and ethical guidelines
2. All amendments and changes made in protocol and consent form needs IRB approval
3. The PI should report SAE within 10 days of the event
4. End of the study, including manuscripts and thesis works should be reported to the IRB
5. The PI should report non-compliance and unanticipated events

III. TO NERC ☒

Institution Review Board (IRB) Approval: Period from: April 29, 2020 to, April 28, 2021

Follow up report expected in: 3 Months___ 6 Months__X__ 9 Months __One year____

Chairperson, IRB
Dr. Adamu Addissie

Signature ____________
Date: 14/05/2020

Director of Research & Technology Transfer, CHS
Dr. Wondwossen Amogne

Signature ____________
Date 5/19/2020

Annex V: Approval by National Research Ethics Review Committee

Ref.No. MoSHE//RD/14.1/9524/20
Date: 27 JAN 2020

Addis Ababa University, College of Health Science
Ehiopia

Subject: *Letter of Approval*

The National Research Ethics Review Committee (NRERC) has reviewed "**Effect of Efavirenz-, Dolutegravir- or Ritonavir Boosted Atazanavir-Based Combination Antiretroviral Therapy on Glucose Metabolism or Drug Induced Adverse Effects among Treatment Experienced HIV Patients: Pharmacokinetic and Pharmacogenetic Study**"project protocol in an expedited manner. We are writing to advise you that NRERC has granted full Approval to the above named project, for a period of **one year (January 27, 2020- January 26, 2021)**.

All your most recently submitted documentshave been approved for use in this study. The study should comply with the standard international and national scientific and ethical guidelines. Any change to the approved protocol or consent material must be reviewed and approved through the amendment process prior to its implementation. In addition, any adverse or unanticipated events should be reported within 24-48 hours to the NRERC. Please ensure that you submit biannual progress report once in six months and annual renewal application 30 days prior to the expiry date.

We, therefore, request you as PI and your esteemed organization to ensure the commencement and conduct of the study accordingly and wish for the successful completion of the project.

With regards

Solomon Benor Belay (PhD)
Director General for Science and Research Affairs

Cc.

- Office of HE the State Minister
- DG for Science and Research Affairs
- Research Ethics Directorate/NRERC Secretareiate MoSHE
- Mr.Wondmagegn Tamiru (PI) AAUCHS

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Annex VI: Material Transfer Agreement

Ministry of Science and Higher Education, Federal Democratic Republic of Ethiopia

National Research Ethics Review Committee

Address. Tel. +251011 1 674353 P.O. Box 2390 Fax +251 011 1 669211 Fax +251 011 1 669211

E-mail:nrerc2015@gmail.com Addis Ababa Ethiopia

Material Transfer Agreement

This Material Transfer Agreement (MTA) has been prepared for use by Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences, Addis Ababa University and Division of Clinical Pharmacology, Department of Laboratory Medicine, Karolinska Institute, Stockholm, Sweden in all transfer of research material (samples, derivatives, and specimens) related to the protocol.

Provider: Department of Pharmacology and clinical pharmacy, College of Health Sciences, Addis Ababa University
P.O Box 1176, Addis Ababa, Ethiopia

Recipient: Department of Laboratory Medicine, Karolinska Institute, Stockholm Sweden

1. Provider agrees to transfer to recipient's designated (Karolinska Institute) the following research materials /specimen

blood/serum samples, and/or DNA extracts derived from HIV patient for pharmacokinetic analysis, Molecular characterization and genotypic sequencing.

The research material will only be used for research purposes as described in the protocol by recipient's investigator in designated laboratory for the research project described below, under suitable containment conditions. This research material will not be used for commercial purposes such as screening, production or sale for which a commercialization license may be required. Recipient agrees to comply with all National and International guidelines rules and regulations applicable to the Research Project and the handling of the Research Material.

a) Are the research materials of human origin? Yes ☑ No ☐

b) If yes, are they collected according to the details in the protocol and in adherence to National Research Ethics Review Committee (NRERC) and College of Health Sciences Ethics Review Committee, CHS, AAU recommendations and their approval?

Yes ☑ No ☐

2. This research material and its derivatives will be used by recipient's investigator solely in connection with the following research project ("Research Project") described with specificity as follows

'Effect of Efavirenz/Dolutegravir or Ritonavir Boosted Atazanavir Based Combination Antiretroviral Therapy on Glucose Metabolism or drug induced adverse effects among treatment experienced HIV Patients: Pharmacokinetics and Pharmacogenetics Study'

3. In all presentations or written publications concerning the research project, recipient will seek agreement of provider and acknowledge provider's contribution of this research material unless requested otherwise.

4. This research material represents a significant contribution on the part of provider and is considered proprietary to provider. Recipient therefore agrees to retain control over this research Material and further agrees not to transfer the research material to other people not under her/his direct supervision without advance written approval of provider. The research material will be disposed of as agreed upon per protocol at the end of completion of the project.

5. The provider does not take any responsibility for loss, damage, wastage or spoilage of the research material during or after shipment to the address provided by the recipient under conditions agreed to in the protocol on shipment of the samples. This research material is provided as a service to the research community. IT IS BEING SUPPLIED TO RECIPIANT WITH NO WARRANTIES, EXPRESS OR IMPLIED, INCLUDING ANY WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. Provider makes no representations that the use of the research material will not infringe any patent or proprietary right of third parties.

6. The recipient shall notify the provider in writing of any intention, improvement, modification discovery or development to the material or the information made by recipient or parties, collaborating with recipient, here in after referred to as "invention". Nothing in this agreement shall, however, be construed as conveying to the provider any rights under any patents or other intellectual property to such invention, other than as explicitly provided herein. At its option the provider shall be entitled to receive sample of any materials derived from the Materials for its own research and evaluation purposes only.

7. The under- signed provider and recipient expressly certify and affirm that the contents of any statements made here in are truthful and accurate.

8. Any additional terms (use an attached page if necessary)

9. The provider maintains, ownership right of the research material and its derivatives unless stated otherwise.

The provider will retain a copy (aliquot) of every sample sent abroad as much as possible for local research needs.

Material Transfer Agreement Signature page

The under-signed PROVIDER and RECIPIENT expressly certify and affirm that the contents of any statements made herein are truthful and accurate.

For Recipient:

Recipient's Investigator: Prof. Eleni Aklillu
Division of Clinical Pharmacology, Department of Laboratory Medicine,
Karolinska Institute, Stockholm, Sweden

Signature — 19 April 2019 Date

Authorized Signatory (at RECIPIENT's Institution)

Signature — 19 April 2019 Date

Provider:
Wondmagegn Tamiru Tadesse
Department of Pharmacology and Clinical Pharmacy
School of Pharmacy, College of Health Sciences,
Addis Ababa University
P.O.Box 1176, Addis Ababa, Ethiopia

Signature — 19 April 2019 Date

Authorized Signatory (at PROVIDER's Institution)
Chairman, Department of Pharmacology and Clinical Pharmacy,
School of Pharmacy, College of Health Sciences
P.O.Box 1176, Addis Ababa, Ethiopia

Signature — 22/04/2019 Date