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The role of genetic polymorphisms in cytochrome P450 and effects of tuberculosis co-treatment on the predictive value of CYP2B6 SNPs and on efavirenz plasma levels in adult HIV patients

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ABSTRACT

Efavirenz (EFV) exhibits interindividual pharmacokinetic variability caused by differences in cytochrome P450 (CYP) expression. Most tuberculosis (TB) drugs interact with the CYP metabolizing enzymes, while the clinical validity of genotyping in predicting EFV plasma levels in Rwandan subjects is not known. We investigated in patients co-infected with human immunodeficiency virus (HIV) and TB recruited in Rwanda the effects of 10 SNPs in five drug-metabolizing enzymes on EFV plasma levels and treatment response when patients are treated with EFV-containing therapy alone ($n = 28$) and when combined with rifampicin-based TB treatment ($n = 62$), and the validity of genotyping for CYP2B6 single nucleotide polymorphisms in predicting supra-therapeutic EFV levels. There was a significant difference between CYP1A2 −739T/G and T/T genotypes when patients were treated with EFV-containing therapy combined with rifampicin-based TB treatment, but not when EFV-containing therapy was alone. CYP2B6 516T/T genotype was associated with high EFV levels compared to other CYP2B6 516G>T genotypes in the presence and in the absence of rifampicin-based TB treatment. Predictive factors of EFV plasma levels in the presence of rifampicin-based TB treatment were CYP2A6 1093G>A, CYP2B6 516G>T, and CYP2B6 983T>C accounting for 27%, 43%, and 29% of the total variance in EFV levels, respectively. There was a high positive predictive value (PPV) (100%) for CYP2B6 516T/T and 983T/T genotypes in predicting EFV plasma levels above the therapeutic range, but this PPV decreased in the presence of rifampicin-based TB treatment. Rifampicin-based TB treatment was also shown to affect EFV plasma levels significantly, but did not affect the significant reduction of HIV-RNA copies. These results indicate that genotyping for CYP2B6 SNPs could be used as a tool in predicting supra-therapeutic EFV plasma levels, which could minimize adverse drug events.

1. Introduction

Among people infected with human immunodeficiency virus (HIV), tuberculosis (TB) is the most common opportunistic infection, with almost 50% of TB patients in Africa being co-infected (WHO, 2012). Managing HIV and TB infections requires multidrug therapy. The standard regimen for the treatment of TB is a combination of two to four drugs, where a rifamycin agent (usually rifampicin) is combined with non-rifamycin agents (ethambutol, isoniazid, and pyrazinamide). Streptomycin may be indicated when one of these drugs is contraindicated (WHO, 2010). The recommended first line antiretroviral therapy (ART) for the management of HIV/AIDS involves a combination of three drugs consisting of two nucleoside reverse transcriptase inhibitors with a non-nucleoside reverse transcriptase inhibitor (NNRTI). Efavirenz (EFV) is the NNRTI commonly administered as part of ART either in patients infected with HIV alone or co-infected with TB (Pozniak et al., 2011).

EFV plasma levels below 1 μg/ml and above 4 μg/ml have been associated with increased risks of virologic failure and central nervous system side effects, respectively (Marzolini et al., 2001). EFV is mainly metabolized in humans through hydroxylation by cytochrome P450 (CYP) 2B6, and to a lesser degree by CYP1A2, CYP2A6, CYP3A4, and CYP3A5 (Bélanger et al., 2009; di Iulio et al., 2009; Ogburn et al., 2010; Pozniak et al., 2011; Ward et al., 2003). EFV has been shown to exhibit interindividual variability in pharmacokinetics, which is thought to be caused by interindividual differences in CYP activities and expression (Bélanger et al., 2009; di Iulio et al., 2009; Holzinger et al., 2012; King and Aberg, 2008; Sánchez et al., 2011). Thus, factors affecting the metabolism of EFV

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could influence EFV exposure and the treatment response. Genetic influence is the most important among multiple factors affecting the pharmacokinetics of EFV (Sánchez et al., 2011). To our knowledge, there is no study that investigated in Rwandan adult HIV subjects the relationship between EFV exposure and single nucleotide polymorphisms (SNPs) with respect to EFV metabolizing enzymes, and the utility of genotyping as a way of improving the prediction of EFV plasma levels.

Despite the important role of genetics, drug–drug interactions deserve a particular attention because it is difficult to predict the outcome of complex interactions, such as those that might occur when several substrates for same CYP enzymes are used concomitantly, some being inhibitors or inducers of the CYP system (CDC, 2007). Non-rifamycin agents have been reported to induce or inhibit the hepatic CYP enzymes (Court et al., 2013; Rodríguez-Nóvoa et al., 2006; Manzi and Shannon, 2005; Wen et al., 2002) and interactions between rifamycin TB agents and antiretrovirals have been investigated, with focus on the induction effect of rifampicin on CYP2B6 enzyme and its impact on EFV disposition in different populations (Kwara et al., 2011a; Rodríguez-Nóvoa et al., 2006). Thus, the therapeutic management of HIV/TB co-infection may result in complex pharmacokinetic drug–drug interactions taking place collectively between ART components and TB drugs, impacting on blood levels of both HIV and TB drugs. Here we hypothesized that in HIV/TB co-infected patients, the genetic influence on EFV levels when patients are treated with EFV-containing therapy alone may differ from when the patients are treated with EFV-containing therapy combined with rifampicin-based TB treatment.

Therefore, the aim of this study was twofold, first to investigate the effects of 10 SNPs in five drug-metabolizing enzymes on EFV levels and treatment response in adult HIV/TB co-infected patients recruited in Rwanda when they are treated with EFV-containing therapy alone and when combined with rifampicin-based TB treatment, and secondly, to evaluate the effects of rifampicin-based TB treatment including its impact on the validity of genotyping for CYP2B6 SNPs in predicting EFV plasma levels above 4 μg/ml.

2. Methods

2.1. Study design and patients

This study was open-label and observational, and was conducted in Rwanda. Medications were administered in accordance with the Rwandan guidelines for the management of HIV/TB co-infection. Adherence to ART was assessed at each weekly visit by means of patient self-report. The patient had received a diary and was explained how to use it, writing down the date and time of every dose intake. The diary was brought to the clinic every visit, where the investigator asked the patient adherence related questions to assess the accuracy of the diary records; answers were recorded in the patient case report form. Rifampicin-based TB treatment was administered in a directly observed therapy program. Approval for the study protocol was given by the National Ethics Committee of the Ministry of Health in Rwanda on December 9th, 2008 for 12 months, and was renewed on December 12th, 2009 for another 12 months. The recruitment of patients started on August 15th, 2009 and the follow up occasions concluded in November 2010.

One hundred and forty-seven (147) adult HIV/AIDS patients co-infected with TB were screened. One hundred and five (105) patients who met inclusion criteria, including provision of informed consent were recruited. Eighty (80) patients who completed the study were genotyped. Of these, 4 patients who had been treated with nevirapine-containing therapy were excluded from this EFV study; the remaining 76 patients had received EFV-containing therapy and were to be considered in this study. Among the 76 patients, there were naive ones, who initiated rifampicin-based TB treatment, with ART to be initiated after 2–8 weeks if CD4 counts are below 500 cells/μL, in accordance with the local treatment guidelines (Group A; $n = 41$). Others were diagnosed for TB after several months of HIV treatment (Group B; $n = 35$) and had immediately initiated rifampicin-based TB treatment with ART continuing. The day of initiation of TB treatment was the day of enrolment in the study. At enrolment, demographic variables were recorded, and two samples collected for selected clinical chemistry tests and genotyping. After initiation of both HIV and TB treatments, patients were monitored for 6 weeks to collect blood samples for determination of EFV and treatment response data (CD4 cell counts and HIV-RNA copies). Those collected in patients taking HIV treatment and after at least 2 weeks of initiation of rifampicin-based TB treatment were used for determination of EFV in the presence of TB drugs. EFV plasma levels in the absence of TB drugs were quantified in blood samples collected after at least 2 weeks of completion of rifampicin-based TB treatment. For the present study, data were considered as complete for each patient when one of the variables to use (plasma concentration or genotype) is not missing. In each analysis, only patients with complete EFV data were considered, and the results are reported along with the sample size considered in the analysis.

2.2. Pharmacokinetic analysis

The patients studied had taken an EFV dose (600 mg) in the evening as per the current treatment guidelines. Samples were collected in the morning at arrival at the clinic, approximately 13 h after the previous evening EFV dose. We used mid-dose sampling (sampling performed between doses, usually between 8 and 20 h) because it is usually used in clinical studies of EFV disposition for patient convenience, given that EFV dose is invariably taken at bedtime (Kwara et al., 2009a). In addition, mid-dose levels of EFV have been reported to be highly associated with EFV area under the curve values when measured at steady-state (Kwara et al., 2009a, 2008). Blood samples collected at weeks 1–6 were used in the form of mean of levels of each patient to assess associations between genotype groups and EFV levels.

All blood samples were collected into an ethylene diamine tetra-acetic acid (EDTA)-containing tubes (4 ml) and stored at −80 °C until analysis. Samples for determination of EFV were centrifuged (10 min at 10,500*g*) before storage. EFV was quantitated in the Unit for Pharmacokinetics and Drug Metabolism, Department of Pharmacology of University of Gothenburg in Sweden, using a validated high-performance liquid chromatography method with ultraviolet detection described elsewhere (Bienvenu et al., 2013a). Briefly, chromatographic separation was carried out using a C_{18} analytical column equipped with a security guard column. The mobile phase consisted of the mixture acetonitrile–water (75:25% v/v; pH of water adjusted at 3.2 using 0.1% formic acid), and was pumped at a flow rate of 0.3 mL/min. Efavirenz and ritonavir (internal standard) were monitored at 247 nm. Plasma proteins were precipitated by centrifugation. The lower limit of quantitation was set to 0.06 μg/mL with deviation from the nominal concentrations being <20%, in accordance with the bioanalytical method validation guidelines of the The United States Food and Drug Administration. The response was linear with a correlation coefficient of 0.9997, a slope of 0.189 and y intercept of 0.003. The relative standard deviation for the slope was 5.474%. The accuracy ranged between 98% and 115% (intraday) and between 99% and 117% (interday). The precision ranged from 1.670% to 4.087% (intraday) and from 3.447% to 13.347% (interday). Recovery ranged from 98% to 132%. Stability ranged between 99% and 123%. The

selectivity was proven by analysis of drugs used for the management of HIV/AIDS and tuberculosis.

2.3. Genotyping

In this study, we use the term "allele" to indicate a different form of a gene at a particular locus on a chromosome and "allele frequency" refers to the proportion of a particular allele among the chromosomes carried by individuals in a population. "Genotype" refers to the pair of alleles present at a single locus for an individual (genetic makeup) and "haplotype" to a set of closely linked genetic markers (genotypes) present on one chromosome which tend to be inherited together. Thus, genotyping consists of determining the genetic constitution (the genotype) of an individual by examining the individual's deoxyribonucleic acid (DNA) sequence. The genotype defines a given characteristic, condition or disease. Hence, genotyping helps in controlling the spreading of pathogens, by tracing the origin of outbreaks. DNA pattern for an individual is made of many single nucleotides. DNA sequence variations occurs when a single nucleotide in the genome sequence is altered and this refers to single nucleotide polymorphisms (SNP). Variations in the DNA sequences (SNPs) of humans can affect how humans develop diseases, respond to pathogens, drugs, etc. and advance the ability to understand and treat human disease (Gunder and Martin, 2011).

Patients participating in this study were genotyped using a PCR-based technology with respect to different SNPs including the following 10 SNPs analyzed in this study; CYP1A2 (−739T>G, −163C>A, and 2159G>A); CYP2A6 (1436G>T, −48T>G, and 1093G>A); CYP2B6 (516G>T and 983T>C); CYP3A4 (−392A>G) and CYP3A5 (6986A>G) SNPs. The background, genotyping methodology and findings including the frequency of alleles, genotypes, and haplotypes were part of another study described elsewhere (Bienvenu et al., 2013b). In summary, SNPs were selected on the basis of their pharmacogenetic relevance and their prevalence in African populations. The selection was performed based on information from the literature, the Human Cytochrome P450 Allele Nomenclature Database (http://www.cypalleles.ki.se/) and the Database of single nucleotide polymorphisms (dbSNPs) (http://www.ncbi.nlm.nih.gov/snp/). Whole blood sample was collected for genotyping. DNA was extracted at LGC Genomics GmbH (Berlin, Germany) using the patented PLUS XL manual kit (catalog: catalogue number 40801 and 40810).

Selected SNPs were genotyped at LGC Genomics Ltd., (Hoddesdon, UK) using the patented KASP assay (Kompetitive Allele Specific PCR), which is a polymerase chain reaction based, homogeneous, fluorescent, endpoint-genotyping technology. The KASP genotyping system was comprised of the SNP-specific assay (a combination of three unlabelled primers) and the universal reaction mix, which contains all other required components including the universal fluorescent reporting system and a specially-developed Taq polymerase. The Hardy–Weinberg equilibrium (HWE) testing was performed for each of the SNP to validate the quality of genotyping, and all the SNPs conformed to HWE.

2.4. Statistical analysis

Analyses were carried out using Predictive Analytics SoftWare (PASW) Statistics 18 (Chicago, IL). The data to be investigated in this study (EFV plasma levels, HIV-RNA copies, CD4 cell counts) were tested for normality using the Shapiro–Wilk test in order to be able to decide on the statistical test to use. Normally distributed data were expressed as mean ± SD (range). Non-normally distributed data were described by using median and interquartile range (IQR). A P-value < 0.05 was considered to be statistically significant. For each participating patient, a single plasma EFV level value derived based on the mean of week 1 to week 6 values was used for statistical analyses, to account for reported variability in EFV plasma levels.

2.4.1. Relationship between EFV levels and baseline characteristics, and SNPs

Since EFV plasma levels were not normally distributed, the Spearman's correlation was used to assess relationships between EFV plasma levels and patient baseline characteristics, and the association of genotype groups with EFV levels was performed using Kruskal–Wallis test for groups of three, followed by Mann–Whitney test for pairwise comparisons with Bonferroni correction. Mann–Whitney test was also used where only two genotype groups were present. To better illustrate low EFV levels, EFV levels were plotted in the form of log-transformed (Figs. 2 and 4); consequently one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to compare between haplotypes (Fig. 4) and EFV levels over the 6 weeks of follow up (Fig. 2D and F). Before association analyzes, repeated measures ANOVA was also conducted to investigate any interaction between genes of interest in this study.

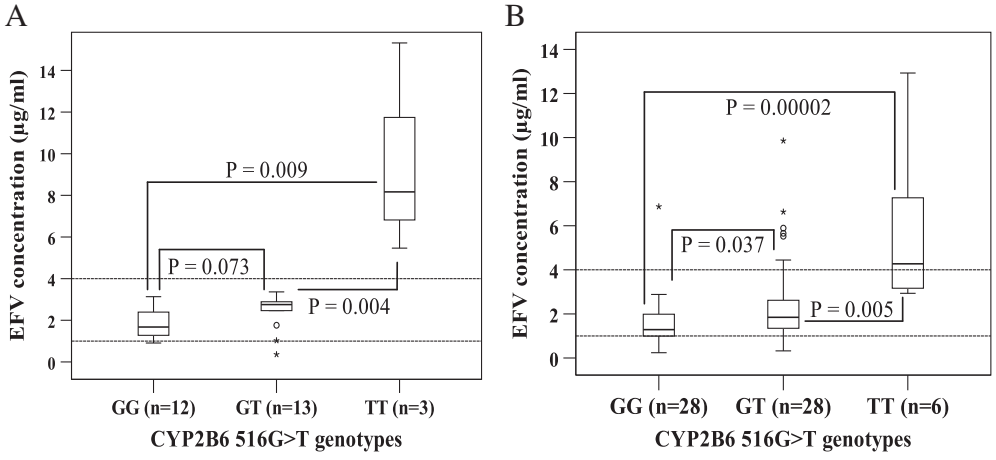

Fig. 1. Effects of CYP2B6 516G>T genotypes on EFV plasma levels when patients were treated with EFV-containing therapy alone (A) and when it was combined with TB treatment (B). Horizontal lines represent median values; bars, interquartile ranges. ○ and ∗ indicate outlier and extreme values, respectively. Dotted lines represent the therapeutic window for EFV (1–4 g/ml). P-values were obtained by pairwise comparison using Mann–Whitney test followed by Bonferroni correction.

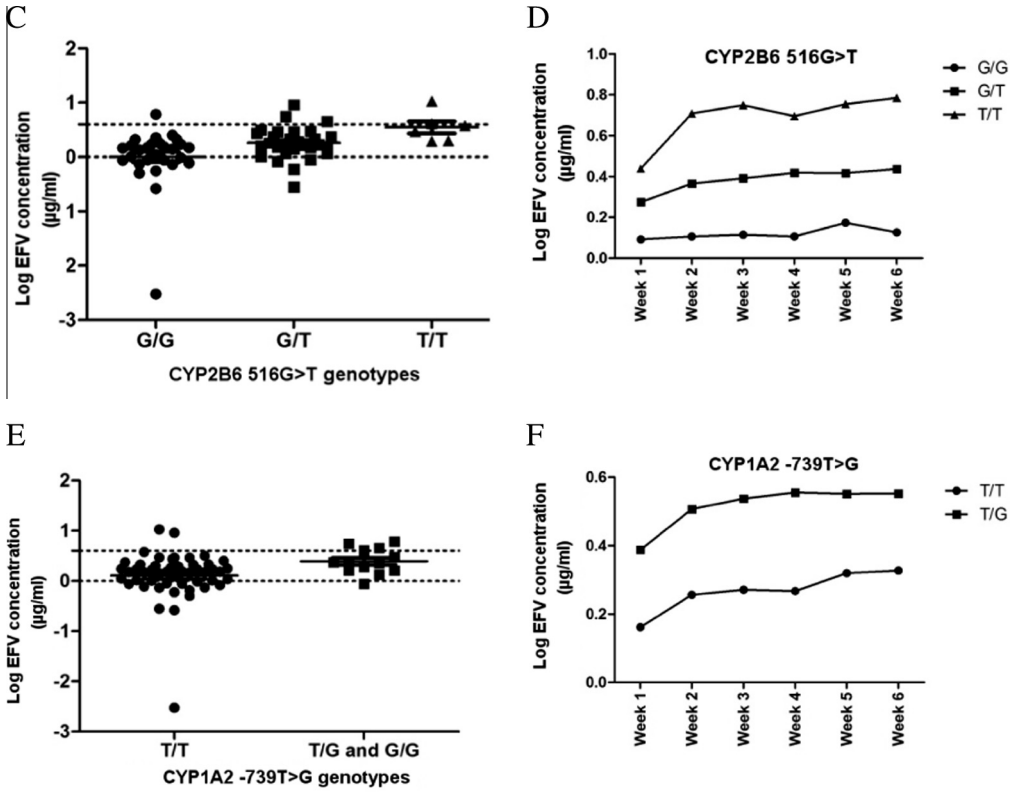

Fig. 2. Distribution of EFV plasma levels by CYP2B6 516G>T (C) and CYP1A2 −739T>G (E) genotypes and comparisons over the 6 weeks of follow up (D, F). The standard deviation of EFV levels at weeks 1 to 6 (D, F) is ± 0.2. Over 6 weeks, CYP2B6 516T/T genotype was significantly different in EFV levels compared to G/T and G/G genotypes (D) ($P = 0.0001$; One-Way ANOVA), and the difference between CYP1A2 −739T/G and T/T genotypes (F) was also significant ($P = 0.0001$; One-Way ANOVA).

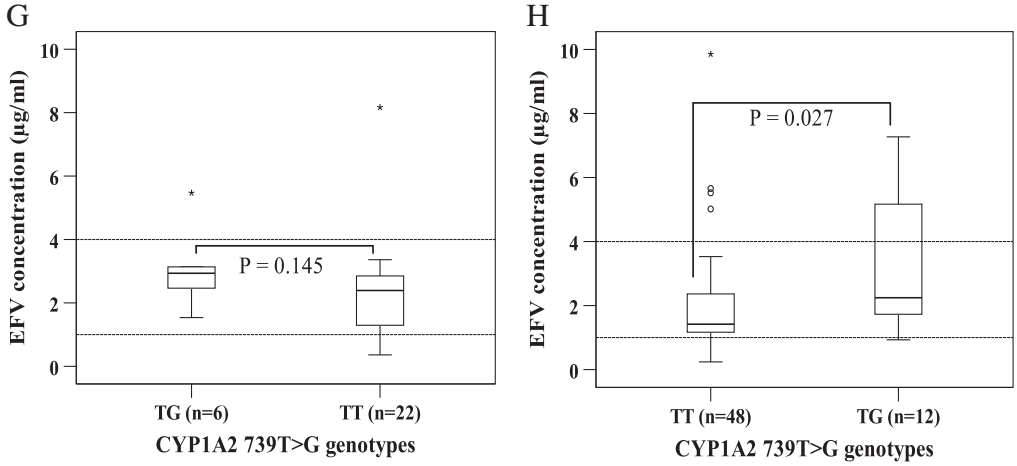

Fig. 3. Effects of CYP1A2 739T>G genotypes on EFV plasma levels when patients were treated with EFV-containing therapy alone (G) and when it was combined with TB treatment (H). Horizontal lines represent median values; bars, interquartile ranges. ○ and ∗ indicate outlier and extreme values, respectively. Dotted lines represent the therapeutic window for EFV (1–4 g/ml). *P*-values were obtained by pairwise comparison using Mann–Whitney test followed by Bonferroni correction.

2.4.2. Regression analysis

Genotypes were dichotomized according to the dominant genetic model (wild-type = 0; heterozygote/homozygote variants = 1) (Lewis and Knight, 2012), as shown in Table 2. Univariate analyses of the effects of patients' baseline characteristics and CYP enzyme genotypes on $\log_{10}$ EFV levels (dependant variable) were assessed by linear regression. The percentage change in EFV plasma levels, with the 95% confidence interval (CI), was calculated as 100 × regression coefficient. Multivariate regression analysis, using a stepwise backward elimination method, was conducted by including variables that achieved statistical significance in the univariate analyses. To avoid confounding effects on the relation between selected genotypes and the end-points, covariates that had a *P*-value of less than 0.20 in univariate

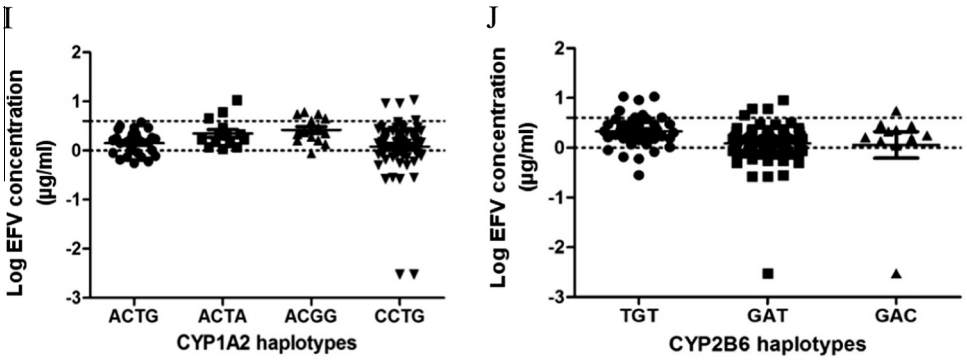

Fig. 4. Effects of CYP1A2 (I) and CYP2B6 (J) haplotypes on EFV plasma levels. The difference between CYP1A2 CCTG and ACGG haplotypes (I) and between CYP2B6 TGT and GAT haplotypes (J) was statistically significant ($P = 0.0001$; One-Way ANOVA).

analyses were included in the multivariate analyses. Plasma levels of EFV were log-transformed in order to achieve data normality for the linear regression analysis and equal variance.

2.4.3. Effects of rifampicin-based TB treatment on EFV plasma levels and clinical response

Within-patient comparisons of EFV levels and HIV-RNA copies were made using Wilcoxon signed-ranks test (the data were not normally distributed), and CD4 cell counts (normally distributed) using paired sample *t*-test.

2.4.4. Assessment of prognostic values

Prognostic values (clinical sensitivity, specificity, negative (NPV) and positive (PPV) predictive values) were assessed for evaluation of the validity of CYP2B6 genotyping in predicting EFV plasma levels above the upper limit of the therapeutic range (4 µg/ml), and were expressed in percentages. The PPV is the proportion of patients with positive test result who actually have the characteristic (presenting with above 4 µg/ml of EFV in the context of our analysis), whereas the NPV is the proportion of patients with a negative test result who do not have the characteristic (Parikh et al., 2008; Swart et al., 2013). The PPV and the NPV were calculated using the methods previously described by Parikh et al. (2008) as:

$$\text{PPV} = \frac{\text{number of TPs}}{\text{number of TPs} + \text{number of FPs}}$$

where TP refers to a true positive result (in the event that the patient has the characteristic and tests positive for it), and FP is a false positive result (the patient does not have the characteristic but tests positive for it);

$$\text{NPV} = \frac{\text{number of TNs}}{\text{number of TNs} + \text{number of FNs}}$$

where TN is a true negative result (the patient does not have the characteristic and tests negative for it), and FN a false negative (the patient has the characteristic but tests negative for it). The PPV indicates the chance of having the characteristic among those that test positive, while the NPV indicate the chance of not having the characteristic among those that test negative. The clinical sensitivity (proportion of patients that are known to have the characteristic who test positive for it) and the clinical specificity (proportion of patients that are known not to have the characteristic who test negative for it) were calculated using the following formula previously described by Parikh et al. (2008):

$$\text{SENSITIVITY} = \frac{\text{number of TPs}}{\text{number of TPs} + \text{number of FNs}}$$

$$\text{SPECIFICITY} = \frac{\text{number of TNs}}{\text{number of TNs} + \text{number of FPs}}$$

3. Results

3.1. Population characteristics

The gender ratio (male:female) was 1.05 (39:37) and the mean ± SD (range) age was 38.0 ± 7.8 (21–57) years. There were no statistically significant associations ($P > 0.05$) when baseline characteristics (patient gender, age, body weight, alanine transaminase ALAT, aspartate transaminase ASAT, total bilirubin and baseline CD4 cell counts and viral loads) were compared to EFV plasma levels, whether when with or without concomitant rifampicin-based TB treatment (results not shown).

3.2. Efavirenz plasma levels and patients' genotypes

The categorization of measured EFV plasma levels from patients of our cohort showed that 32% of the patients had plasma EFV levels outside the expected therapeutic range (1–4 µg/ml) with 14% of these having levels above 4 µg/ml and 18% having levels below 1 µg/ml, whereas 68% presented with plasma EFV levels within the expected therapeutic range. The coefficients of variation (±SD) within and between subject plasma levels were 28% (±16.9) and 88% (±2.1), respectively. Among the patients considered for the present investigation, the median EFV plasma levels in patients who were not taking rifampicin-based TB treatment was 2.5 µg/ml (1.5–3.0) with individual values ranging from a minimum of 1.0 µg/ml to a maximum of 15.3 µg/ml. Corresponding median EFV plasma levels for patients on concomitant treatment of HIV and TB were 1.7 µg/ml (1.2–2.6) with individual values ranging from a minimum of 0.2 µg/ml to a maximum of 12.9 µg/ml. Genotyping was conducted with the aim of investigating the possible association and effects of genotypes on EFV plasma levels. Table 1 shows the stratification of EFV plasma levels (µg/ml) by genotype with respect to CYP1A2, CYP2A6, CYP2B6, CYP3A4, and CYP3A5 enzymes.

3.3. Association between the genotypic variants and EFV plasma levels

There were statistically significant differences between the three CYP2B6 516G>T genotype groups when patients were treated with EFV-containing therapy alone ($P = 0.006$) and when combined with rifampicin-based TB treatment ($P = 0.0005$). Pairwise comparisons revealed that CYP2B6 T/T genotype was associated with high EFV plasma levels compared to G/G ($P = 0.009$) and G/T ($P = 0.004$) genotypes when EFV-containing therapy was given alone (Fig. 1A), and when it was given concurrently with TB drugs ($P = 0.00002$ and 0.005, respectively) (Fig. 1B). The analysis showed a statistically significant difference between CYP2B6 516G/T and G/G genotypes when patients were treated with EFV-containing therapy

Table 1
EFV plasma levels stratified by genotype in the presence and in the absence of TB treatment.

Genotype by SNP	In the presence of TB treatment		In the absence of TB treatment	
	n (%)	Median (IQR) EFV concentrations (μg/ml)	Median (IQR) EFV concentrations (μg/ml)	*n* (%)
CYP1A2 −739T>G				
T/T	48 (79%)	1.4 (1.2–2.4)	2.4 (1.3–2.9)	22 (79%)
T/G	12 (20%)	2.2 (1.7–5.5)	2.9 (2.2–3.7)	6 (21%)
G/G	1 (1%)	6.6		
CYP1A2 −163C>A				
C/C	18 (31%)	1.3 (1.1–1.8)	2.6 (1.5–3.1)	6 (24%)
C/A	29 (49%)	1.9 (1.3–3.0)	2.5 (1.4–4.3)	13 (52%)
A/A	12 (20%)	1.6 (1.2–3.8)	2.6 (1.2–3.0)	6 (24%)
CYP1A2 −2159G>A				
G/G	12 (79%)	1.5 (1.1–2.5)	2.4 (1.4–3.0)	24 (86%)
G/A	12 (19%)	2.0 (1.5–4.1)	2.9 (2.7–12.2)	4 (14%)
A/A	1 (2%)	1.2		
CYP2A6 1436G>T				
G/G	40 (66%)	1.5 (1.1–2.3)	2.4 (1.5–2.9)	20 (71%)
G/T	17 (28%)	2.2 (1.3–5.0)	2.8 (1.3–3.1)	7 (25%)
T/T	4 (6%)	1.5 (1.0–4.2)	8.2	1 (4%)
CYP2A6 1093G>A				
G/G	60 (97%)	1.8 (1.2–2.6)	2.5 (1.5–3.0)	28 (100%)
G/A	2 (3%)	0.6 (0.3–0.9)		
CYP2A6 −48T>G				
T/T	49 (82%)	1.6 (1.2–2.5)	2.5 (1.5–2.9)	24 (89%)
T/G	10 (17%)	2.0 (1.3–3.6)	2.1 (1.2–3.4)	3 (11%)
G/G	1 (1%)	0.7		
CYP2B6 516G>T				
G/G	28 (45%)	1.3 (1.0–2.0)	1.7 (1.3–2.4)	12 (43%)
G/T	28 (45%)	1.8 (1.3–2.6)	2.8 (2.1–2.9)	13 (46%)
T/T	6 (10%)	4.3 (3.1–8.7)	8.2 (5.5–15.3)	3 (10%)
CYP2B6 983T>C				
T/T	50 (83%)	1.5 (1.1–2.4)	2.5 (1.3–3.0)	23 (85%)
T/C	10 (17%)	2.3 (1.6–5.6)	2.1 (1.6–2.9)	4 (15%)
CYP3A4 −392A>G				
A/A	14 (23%)	1.9 (1.4–5.9)	2.8 (0.9–3.0)	7 (25%)
A/G	34 (54%)	1.4 (1.0–2.3)	2.2 (1.4–2.8)	16 (57%)
G/G	14 (23%)	1.9 (1.2–2.6)	2.9 (2.7–3.2)	5 (18%)
CYP3A5 6986A>G				
A/A	28 (46%)	1.4 (1.2–3.4)	2.8 (2.3–3.1)	11 (39%)
A/G	28 (46%)	1.9 (1.2–2.6)	1.8 (1.3–2.9)	15 (54%)
G/G	5 (8%)	0.9 (0.7–1.9)	2.8 (2.7–2.9	2 (7%)

combined with rifampicin-based TB treatment ($P = 0.037$) (Fig. 1B), but not when it was given alone ($P = 0.073$) (Fig. 1A). When comparing follow up occasions during concomitant HIV and TB treatment, a significant difference in EFV plasma levels was observed between CYP2B6 516T/T genotype and G/T and G/G genotypes ($P = 0.0001$) over the 6 weeks of follow up (Fig. 2C and D).

Pairwise analysis indicated a significant difference between CYP1A2 −739T/G and T/T genotypes when patients were treated with EFV-containing therapy combined with rifampicin-based TB treatment ($P = 0.027$) (Fig. 3H), but not when EFV-containing therapy was alone ($P = 0.145$) (Fig. 3G). The difference in EFV levels between CYP1A2 −739T/G and T/T genotypes was also significant over the 6 weeks of follow up ($P = 0.0001$) (Fig. 2E and F). A statistically significant difference ($P = 0.0001$) was observed between CYP1A2 CCTG and ACGG haplotypes with CCTG being associated with low EFV levels (Fig. 4I) and between CYP2B6 TGT and GAT haplotypes (Fig. 4J) in patients taking both HIV and TB treatment. No statistically significant associations ($P > 0.05$) were found between EFV plasma levels and CYP1A2 (−163C>A and 2159G>A); CYP2A6 (1436G>T and −48T>G); CYP2B6 (983T>C); CYP3A4 (−392A>G) and CYP3A5 (6986A>G) genotypes whether when with or without concomitant rifampicin-based TB treatment. Furthermore, no significant interactions ($P > 0.05$) were observed between genotypes in genes considered in the present study and this was consistent with a previous report on Ghanaian patients (Kwara et al., 2009b).

3.4. Association between genotypic variants and treatment response

In our analysis, we found no statistically significant associations ($P > 0.05$) between HIV-RNA copies and CD4 cell counts, and CYP1A2, CYP2A6, CYP2B6, CYP3A4, and CYP3A5 genotypes and haplotypes.

3.5. Predictive factors of EFV plasma levels

Univariate regression analysis was conducted in order to determine the effects of patients' baseline characteristics (gender, age, body weight, ALAT, ASAT, baseline CD4 cell counts, baseline viral loads, and total bilirubin), and the genotypes with respect to the 10 SNPs, CYP1A2 (−739T>G, −163C>A, and 2159G>A), CYP2A6 (1436G>T, 1093G>A and −48T>G), CYP2B6 (516G>T and 983T>C), CYP3A4 (−392A>G), and CYP3A5 (6986A>G) as independent variables on $\log_{10}$ EFV plasma levels (dependant variable) collected from patients on concurrent HIV and TB treatment. Total bilirubin and the genotypes of CYP1A2 −739T>G, CYP2A6 1436G>T, CYP2A6 1093G>A, CYP2B6 516G>T, and CYP3A4 −392A>G SNPs statistically significantly predicted EFV levels (Table 2). Thus, they were

Table 2
Regression analysis of association between EFV plasma levels and influential factors.

Independent variable	% Log_{10} EFV (95%CI)	*P*-value	R^2
Univariate			
Gender	−5.4 (−20.6 to 13.5)	0.679	0.003
Age (years)	−2.6 (−1.2 to 1.0)	0.843	0.001
Body weight (kg)	8.8 (−0.6 to 1.3)	0.495	0.008
Baseline CD4* (cells/μL)	10.7 (−0.03 to 0.1)	0.429	0.011
Baseline log_{10} HIV-RNA* (copies/mL)	15.1 (−2.6 to 8.7)	0.289	0.023
Alanine transaminase (U/ml)	−2.7 (−0.4 to 0.3)	0.836	0.001
Aspartate transaminase (U/ml)	−4.1 (−0.3 to 0.2)	0.749	0.002
Total bilirubin (mg/dl)	25.8 (0.3 to 15.6)	0.043	0.067
CYP1A2			
−739T>G	30.5 (4.6 to 44.7)	0.017	0.093
−163C>A	17.5 (−6.3 to 31.9)	0.186	0.031
2159G>A	16.8 (−7.9 to 36.5)	0.203	0.028
CYP2A6			
1436G>T	27.6 (1.8 to 36.8)	0.031	0.076
1093G>A	-28.8 (−100.1 to −7.5)	0.023	0.083
−48T>G	3.3 (−19.5 to 25.1)	0.805	0.001
CYP2B6			
516G>T	37.1 (8.6 to 40.6)	0.003	0.138
983T>C	22.1 (−3.2 to 42.8)	0.090	0.049
CYP3A4			
−392A>G	−28.2 (−41.9 to −2.7)	0.026	0.079
CYP3A5			
6986A>G	−8.9 (−23.3 to 11.4)	0.494	0.008
Multivariate		0.0002	0.302
CYP2A6			
1093G>A	−26.5 (−90.3 to −7.0)	0.023	
CYP2B6			
516G>T	43.3 (13.5 to 44.4)	0.0004	
983T>C	28.7 (4.9 to 46.1)	0.016	

Genotypes were dichotomized and organized as follows: TT vs TG/GG for CYP1A2 −739T>G; CC vs CA/AA for CYP1A2 −163C>A; GG vs GA/AA for CYP1A2 2159G>A; GG vs GT/TT for CYP2A6 1436G>T; GG vs GA/AA for CYP2A6 1093G>A; TT vs TG/GG for CYP2A6 −48T>G; GG vs GT/TT for CYP2B6 516G>T; TT vs TC/CC for CYP2B6 983T>C; AA vs AG/GG for CYP3A4 −392A>G and for CYP3A5 6986A>G.
* Refer to baseline before any HIV treatment, after enrolment.

included in the multivariate analysis. The covariates that had a *P*-value of less than 0.20 (CYP1A2 −163C>A and CYP2B6 983T>C) were also included to avoid confounding effects on the relation between selected genotypes and the end-points. Multivariate analysis was then performed to identify independent predictors of EFV plasma levels and evaluate their contribution to overall variability in EFV plasma levels. Three independent variables with $P < 0.05$ remained in the final model, including CYP2A6 1093G>A ($P = 0.023$), CYP2B6 516G>T (P = 0.0004), and CYP2B6 983T>C ($P = 0.016$) (Table 2). The coefficient of determination (R^2) for the regression was 0.302 ($P = 0.0002$), indicating that the model explained 30% of the variability in EFV plasma levels. The standardized regression coefficients indicated that CYP2A6 1093G>A, CYP2B6 516G>T, and CYP2B6 983T>C accounted for 27%, 43%, and 29% of the total variance in EFV plasma levels, respectively. These results for CYP2B6 516G>T are congruent with its association with EFV plasma levels shown in Fig. 1. *For* CYP2B6 983T>C, there was no statistically significant difference between CYP2B6 983T>C genotypes ($P > 0.05$); over the 6 weeks however, the CYP2B6 983T/T genotype was associated with significantly higher EFV levels ($P = 0.0035$) compared to T/C genotype. CYP2A6 1093G>A was not investigated for the association with EFV levels because of small numbers of heterozygous G/A (Table 1), while the patients of our cohort had only G/G and G/A genotypes.

3.6. Prognostic values of CYP2B6 genotyping in predicting supra-therapeutic EFV plasma levels

Sensitivity, specificity, PPV, and NPV were evaluated to determine whether CYP2B6 SNPs could be used in predicting EFV plasma levels above 4 μg/ml, which is the upper limit of the therapeutic range. The results are presented in Table 3. Overall, the predictive values for CYP2B6 SNPs were shown to change in the presence of rifampicin-based TB treatment. Of note in this analysis was the higher PPV percentage (100%) for CYP2B6 516T/T and 983T/T genotypes in the absence of rifampicin-based TB treatment, but which decreased in the presence of rifampicin-based TB treatment by 3.3-folds for CYP2B6 516T/T genotype and by 1.4-folds for CYP2B6 983T/T genotype. The latter genotype also had high specificity in the absence (100%) and in the presence (93%) of rifampicin-based TB treatment.

3.7. Effects of rifampicin-based TB treatment on EFV plasma levels and clinical response

The effect of rifampicin-based TB treatment on EFV plasma levels was assessed to ascertain whether variability in EFV plasma levels in this cohort is due to genetic polymorphism and rifampicin-based TB treatment, and to assess its impact on the clinical response. The effect of rifampicin-based TB treatment on EFV plasma levels was evaluated by comparing EFV levels measured from samples collected during concomitant HIV and TB treatments with those collected from the same patients after completion of rifampicin-based TB treatment ($n = 21$). The effect of rifampicin-based TB treatment on clinical response was evaluated by comparing HIV-RNA copies measured from group A patients (naive) at baseline and after 6 weeks of initiation of ART in the presence of rifampicin-based TB treatment. Overall, compared to when EFV was alone, EFV plasma levels were lowered during concomitant HIV and rifampicin-based TB treatment for 81% of patients (17/21). The medians (IQR) EFV plasma levels were 2.7 μg/ml (1.5–3.1) and 1.8 μg/ml (1.4–2.3) in the absence and in the presence of rifampicin-based TB

Table 3
Sensitivity, specificity and predictive values for CYP2B6 SNPs on EFV plasma levels.

Genotype	*n*	Sensitivity (%)	Specificity (%)	PPV [b](%)	NPV [b](%)
In the absence of TB treatment					
CYP2B6 516G>T					
G/G	12	0.00	80.00	0.00	48.00
G/T	13	0.00	81.25	0.00	52.00
T/T	3	10.71	–	100.00	0.00
CYP2B6 983T>C					
T/T	23	42.86	100.00	100.00	83.33
T/C	4	0.00	57.14	0.00	16.67
In the presence of TB treatment					
CYP2B6 516G>T					
G/G	28	3.85	75.00	10.00	51.92
G/T	28	16.67	84.62	60.00	42.31
T/T	6	5.77	30.00	30.00	5.77
CYP2B6 983T>C					
T/T	50	50.00	93.48	70.00	86.00
T/C	10	6.52	50.00	30.00	14.00

[b] Negative (NPV) and positive (PPV) predictive values.

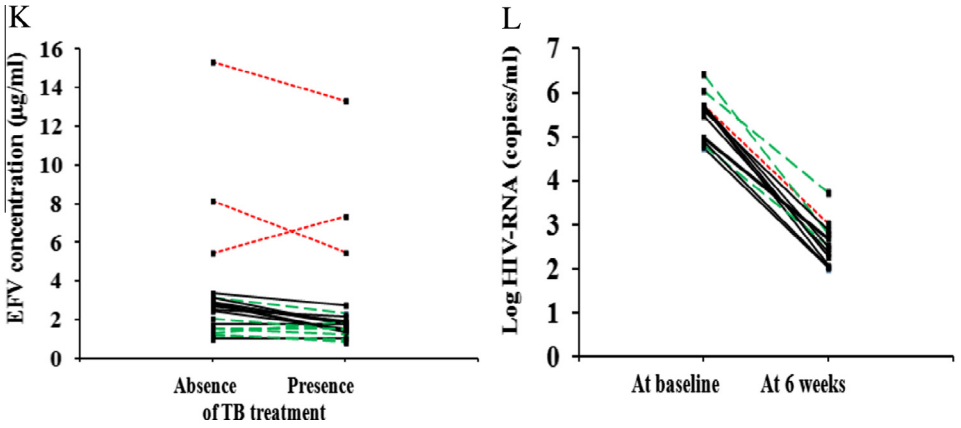

Fig. 5. Changes in EFV plasma levels (g/ml) (K) and HIV-RNA (copies/mL) (L) after 6 weeks of initiation of antiretroviral therapy in the presence of TB treatment. In the presence of TB treatment, EFV plasma levels were statistically significantly lowered ($P = 0.004$; Wilcoxon signed-ranks test) ($n = 21$) and HIV-RNA copies were reduced significantly ($P = 0.002$; Wilcoxon signed-ranks test) ($n = 12$). Square dot, long dash and solid lines show carriers of CYP2B6 516T/T, G/G, and G/T genotypes, respectively.

treatment respectively, corresponding to a statistically significant decrease ($P = 0.004$) of EFV plasma levels by 1.5-folds, following concomitant use of rifampicin-based TB treatment (Fig. 5K). The same comparison performed within each group resulted in statistically significant differences in patients of group A ($P = 0.036$) and in patients of group B ($P = 0.018$). In view of these results, we further explored whether this statistically significant decrease of EFV levels in the presence of rifampicin-based TB treatment had an impact on the treatment response. Wilcoxon signed-ranks test revealed a statistically significant ($P = 0.002$) reduction in HIV-RNA copies (median; IQR) from baseline (446407.0 copies/mL; 83513.8-538809.3) to 6 weeks of initiation of ART (328.0 copies/mL; 140.8–7403) in the presence of rifampicin-based TB treatment (Fig. 5L), indicating no effect of the latter on the clinical response. The change in CD4 cell counts was both ways and not statistically significant ($P > 0.05$). For the comparisons using treatment response data, we only used naive patients (Group A) to avoid bias that could be caused by patients enrolled in the study while they were previously on ART (Group B).

4. Discussion

Our data indicate a difference in association with exposure to EFV between CYP1A2 −739T/G and T/T genotypes during concomitant HIV and rifampicin-based TB treatment, but not during HIV treatment alone. Our regression model indicates that CYP2B6 516G>T, CYP2A6 1093G>A, and CYP2B6 983T>C were independent predictors for EFV plasma levels in the presence of rifampicin-based TB treatment, with CYP2B6 516T/T genotype being associated with high EFV plasma levels in the absence and presence of rifampicin-based TB treatment. There was a higher PPV percentage (100%) for CYP2B6 516T/T and 983T/T genotypes in predicting EFV plasma levels above 4 µg/ml in the absence of rifampicin-based TB treatment. This TB treatment was also shown to affect the PPV of CYP2B6 SNPs, and to lower EFV plasma levels significantly, but did not affect the significant reduction of HIV-RNA copies.

We had hypothesized that in HIV/TB co-infected patients, the genetic influence on EFV plasma levels when patients are treated with EFV-containing therapy alone may differ from when the patients are treated with EFV-containing therapy combined with rifampicin-based TB treatment. Our data shows that in the presence of rifampicin-based TB treatment when genotypes were compared for their association with EFV plasma levels, differences between CYP1A2 −739T/G and T/T genotypes were significant. Given the paucity of the data on relationships between EFV and SNPs in CYP enzymes other than CYP2B6, we could not compare our findings on CYP1A2 and CYP2A6 with the data from other cohorts. CYP1A2 enzyme and CYP2A6 are part of the accessory pathways for EFV metabolism (Mutlib et al., 1999; Ogburn et al., 2010; Ward et al., 2003). CYP1A2 participates in 8-hydroxylation of EFV together with other CYP enzymes with the rank order of CYP2B6 > CYP1A2 > CYP3A5 > CYP3A4 (di Iulio et al., 2009) and

CYP2A6 gets involved in 7-hydroxylation of EFV with the rank order of CYP2B6 > CYP2A6 > CYP3A5 > CYP3A4 (Mutlib et al., 1999; Ogburn et al., 2010; Ward et al., 2003). di Iulio et al. (2009) reported a contribution of CYP2A6 alleles among individuals characterized as CYP2B6 slow metabolizers either when CYP2A6 genotypes were associated with EFV plasma levels or when *in vivo* metabolites were analyzed. In the same perspective, our findings indicating that the difference in association with EFV plasma levels between CYP1A2 −739T/G and T/T genotypes becomes significant during rifampicin-based TB treatment hypothetically suggest that 8-hydroxylation of EFV may be redirected to CYP1A2 accessory pathway in individual with highly impaired CYP2B6 function or in slow CYP2B6 metabolizers. The same applies for 7-hydroxylation of EFV which could be redirected to CYP2A6 given that our regression analysis indicated that CYP2A6 1093G>A could predict EFV plasma levels (Table 2) and that 1093G/A and G/G genotypes were significant different during concomitant HIV and rifampicin-based TB treatments when compared for their association with EFV plasma levels. It would be interesting to study the importance of these observations in larger groups.

Our data indicating that CYP2B6 516T/T genotype were associated with higher EFV levels are comparable to what has been reported among Thai (Sukasem et al., 2012; Uttayamakul et al., 2010) and Caucasian patients on HIV/AIDS treatment (Cabrera et al., 2010). Based on this and the higher PPV observed in our analyses for CYP2B6 516T/T and 983T/T genotypes for the prediction of supra-therapeutic EFV plasma levels, we are thus suggesting the genotyping assay for CYP2B6 SNPs when deciding on EFV dosages is required.

It is worth noting that observed decrease of EFV plasma levels could partly be attributed to the reported induction effect on EFV metabolizing enzymes, such as CYP2B6 and CYP3A4 by rifampicin (Burman et al., 1999; Cohen et al., 2009; Gengiah et al., 2012; Kwara et al., 2011a,b; Li and Chiang, 2006; Manzi and Shannon, 2005; Ramachandran et al., 2009; Rodríguez-Nóvoa et al., 2006; Shapiro and Shear, 2002; Szalat et al., 2007; Uttayamakul et al., 2010), which was one of the TB drugs used by the patients studied, but also to overlapping EFV auto-induction, which however, could have not contributed significantly. In fact, based on the data from his study when HIV and TB are treated concomitantly, Ngaimisi et al. (2011) demonstrated that EFV auto-induction does not exhibit significant additive or synergistic effects over and above ongoing rifampicin-based TB therapy. This implies that a decrease of EFV levels observed in both groups of this cohort during HIV and TB co-treatment could be attributed mainly to rifampicin-based TB treatment effects and to a non significant extent to EFV auto-induction. It is also clinically valuable mentioning that 2 carriers of CYP2B6 515T/T and G/G genotypes had unexpectedly a 1.3-fold and 1.5-fold increase in EFV levels in the presence of rifampicin-based TB treatment (Fig. 5K). Similar trends, however were reported by Gengiah et al. (2012) in South Africans and Luetkemeyer et al. (2013) in Black patients participating in his study, tough the underlying mechanism remains unclear. In line with our objective to ascertain whether both genetic polymorphism and rifampicin-based TB treatment contribute to the variability in EFV plasma levels, we suggest that deciding on EFV dosages for HIV/TB co-infected patients should take into accounts both CYP2B6 polymorphisms and rifampicin-based TB treatment effects.

A limitation of this study is that patients were studied for 6 weeks while they were on concomitant HIV and TB treatments. It would be interesting to conduct similar investigations through a long term follow up and larger groups of patients especially to be able to study sufficiently the treatment outcome. Notwithstanding, our observation that viral loads decreased significantly in the presence of rifampicin-based TB treatment is congruent with reports compiled by Avihingsanon et al. (2009) on lack of association between virological failure and decrease of EFV levels following concomitant use of rifampicin-based TB treatment.

5. Conclusion

This study indicated an association with EFV plasma levels for CYP1A2 −739T/G genotype only during concomitant HIV and TB treatment, and validated the findings on the effects of CYP2B6 516G>T polymorphism on EFV plasma levels in the Rwanda population, particularly the association of CYP2B6 516T/T genotype with elevated EFV plasma levels, with an added value to demonstrate the same effects not only during HIV treatment alone, but also during concomitant HIV and TB treatment. In addition to CYP2B6 516G>T, CYP2A6 1093G>A and CYP2B6 983T>C were shown to be independent predictors for EFV plasma levels in the presence of rifampicin-based TB treatment. This indicates that genotypic data for these SNPs should be taken into consideration when estimating the appropriate dose of EFV. The observed high positive predictive value for CYP2B6 516T/T and 983T/T genotypes in predicting supra-therapeutic EFV plasma levels indicated that genotyping for CYP2B6 516G>T SNPs could serve as a tool to identify patients who could be at risk of EFV-related neurotoxicity and need adjusted EFV dose. Given the suggested possible redirection of EFV metabolism to the accessory pathways, in particular those catalyzed by CYP1A2 and CYP2A6 enzymes, caution should be exercised when administering EFV-containing regimens concomitantly with rifampicin-based TB treatment to slow CYP2B6 metabolizers. The data of this study showed that rifampicin-based TB treatment affects not only prognostic values of EFV plasma levels, but also EFV plasma levels itself in the population studied. This indicated that observed variability in EFV plasma levels in the population studied is due to both CYP2B6 polymorphisms and rifampicin-based TB treatment. The fact that rifampicin-based TB treatment co-administration did not affect the significant reduction of HIV-RNA copies following initiation of ART suggests that taking into account rifampicin-based TB treatment effects during dose adjustment could be made rather at individual than at population level. Specifically, the impact of rifampicin-based TB therapy on EFV plasma levels could be assessed in patients suspected being at risk of sub-therapeutic levels and dose adjustment could only be done when the clinical response is affected. Lastly, even though it is clear from this investigation and various other published studies that specific CYP genotypes and concomitant medications do have a definite effect on EFV levels causing its variation, this however does not seem to influence the efficacy of the EFV-containing regimens in general.

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Disclaimer

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