ORIGINAL RESEARCH

Association of *CYP2B6* Genetic Variation with Efavirenz and Nevirapine Drug Resistance in HIV-1 Patients from Botswana

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Purpose: CYP2B6 liver enzyme metabolizes the two non-nucleoside reverse transcriptase inhibitors Efavirenz (EFV) and Nevirapine (NVP) used in the antiretroviral therapy (ART) regimens for HIV-infected individuals. Polymorphisms of the *CYP2B6* gene influence drug levels in plasma and possibly virological outcomes. The aim of this study was to explore the potential impact of *CYP2B6* genotype and haplotype variation on the risk of developing EFV/NVP drug resistance mutations (DRMs) in HIV-1 patients receiving EFV-/NVP-containing regimens in Botswana.

Patients and Methods: Participants were a sub-sample of a larger study (Tshepo study) conducted in Gaborone, Botswana, among HIV-infected individuals taking EFV/NVP containing ART. Study samples were retrieved and assigned to cases (with DRMs) and controls (without DRMs). Four single-nucleotide polymorphisms (SNPs) in the *CYP2B6* gene (−82T>C; 516G>T; 785A>G; 983T>C) were genotyped, the haplotypes reconstructed, and the metabolic score assigned. The possible association between drug resistance and several independent factors (baseline characteristics and *CYP2B6* genotypes) was assessed by Binary Logistic Regression (BLR) analysis. EFV/NVP resistance status and *CYP2B6* haplotypes were also analyzed using Z-test, chi-square and Fisher's exact test statistics.

Results: Two hundred and twenty-seven samples were analysed (40 with DRMs, 187 without DRMs). BLR analysis showed an association between EFV/NVP resistance and *CYP2B6* 516G allele (OR: 2.26; 95% CI: 1.27–4.01; *P*=0.005). Moreover, haplotype analysis revealed that the proportion of EFV/NVP-resistant infections was higher among *CYP2B6* fast than extensive/slow metabolizers (30.8% vs 16.8%; *P*=0.035), with the 516G allele more represented in the haplotypes of fast than extensive/slow metabolizers (100.0% vs 53.8%; *P*<0.001).

Conclusion: We demonstrated that the *CYP2B6* 516G allele, and even more when combined in fast metabolic haplotypes, is associated with the presence of EFV/NVP resistance, strengthening the need to assess the *CYP2B6* genetic profiles in HIV-infected patients in order to improve the virologic outcomes of NNRTI containing ART.

Keywords: ART, *CYP2B6* gene, drug resistance selection, fast metabolizers, HIV

Introduction

Antiretroviral therapy (ART) has significantly reduced HIV-related morbidity and mortality globally. However, the emerging threat of HIV drug resistance may reduce ART efficacy resulting in pronounced negative public health impact, especially in sub-Saharan Africa which account for about 70% of the global HIV epidemic.[1] Scale-up of ART availability has been implemented and, in 2019,

a global median of 67% of those in need (90% in Botswana)[2,3] have had access to ART.[4] Among the antiretroviral drugs (ARVs) used as first-line ART regimen, first-generation Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs), such as Efavirenz (EFV) and Nevirapine (NVP), are still largely used for HIV treatment in sub-Saharan Africa,[5] although since 2017 the World Health Organization (WHO) has been recommending an EFV/NVP-sparing ART regimen in countries in which resistance to NNRTIs exceeds 10%.[6] Despite the introduction of Dolutegravir (DTG)-based first-line ART in Botswana in June 2016, a significant proportion of HIV patients are still on EFV- or NVP-containing ART regimen.[7] A recent (2019) WHO HIV drug resistance report encompassing 18 countries (6 from sub-Saharan Africa) revealed that pre-treatment HIV drug resistance rate to EFV/NVP exceeds 10% amongst adults initiating first-line ART (nearly twice as high in women than men).[5] The rate was even higher (up to 30%) in those previously exposed to ARVs, including women having taken ARVs for the prevention of mother-to-child transmission.[5]

Antiretroviral therapy efficacy largely depends on adequate drug exposure to suppress viral replication and allow the immune system to recover. However, occurrence of drug toxicity, suboptimal patient's compliance, suboptimal virologic responses, incomplete immune reconstitution and/or emergence of drug resistance limit therapeutic outcomes.[8] HIV drug resistance, beside known viral factors, more frequently occurs because of sub-therapeutic ARV drug exposure and/or acquisition of drug-resistant strains. In resource-limited settings such as Botswana, HIV-diagnosed individuals with virologic failure are more likely to stay on virologically failing regimens for prolonged periods, because of lack of adequate virological follow-up. This may result in an ineffective drug exposure potentially causing drug toxicity and a higher risk of selecting and transmitting drug-resistant viruses.[9] Moreover, the presence of HIV drug resistance mutations (DRMs) in minor viral populations is associated with an increased risk of virologic failure, in particular for NNRTI-based ART regimens, regardless of adherence, ethnicity, and immuno-virological basal characteristics of patients.[8]

Efavirenz and NVP are primarily metabolized in the liver by the cytochrome P450 2B6 enzyme (CYP2B6) with a minor contribution from other cytochromes (i.e. CYP2A6, CYP3A4/5).[10–12] Studies on several populations have shown that Africans display the greatest level of genetic diversity in the *CYP2B6* gene.[13] Cytochrome P450 *2B6* is one of the most polymorphic *CYP450* genes in humans with over 100 described single nucleotide polymorphisms (SNPs), numerous complex haplotypes and distinct ethnic frequencies.[14] Cytochrome P450 *2B6* gene polymorphism has been associated with interindividual differences in drug pharmacokinetics and consequent plasma exposure, with possible consequences on drug efficacy and safety.[14] There are different SNPs in the *CYP2B6* gene that, according to their combination as haplotypes, may lead to different degrees of slow and/or fast EFV/NVP metabolizer phenotypes.[14] Among those SNPs, 516G>T (rs3745274) and 983T>C (rs28399499) are associated with a significant loss of CYP2B6 function, leading to reduced clearance and prolonged half-life both for EFV[15–18] and NVP.[19,20] The 983T>C SNP affects the metabolism of both EFV and NVP; the 516G>T SNP influences mainly the EFV metabolism, while data on its impact on NVP metabolism are less conclusive.[21,22] Conversely, two other SNPs, namely 785A>G (rs2279343) and −82T>C (rs34223104) are associated with a gain of CYP2B6 function, leading to lower drug exposure.[23–25] Indeed, the 785A>G SNP increases EFV[26] and NVP[27] metabolism, whereas there are no studies assessing the clinical/pharmacological impact in vivo of the −82T>C SNP. Nonetheless, *CYP2B6* −82T>C has recently been included in the panel of *CYP2B6* SNPs that should be considered for the evaluation of therapeutic impact by the Clinical Pharmacogenetics Implementation Consortium.[28]

Pharmacogenetic studies of EFV and NVP have mostly been based on the *CYP2B6* 516G>T and 983T>C SNPs, with little or no clear assessment of the impact of the *CYP2B6* 785A>G and −82T>C SNPs.[14] Studies from Botswana on HIV patients taking EFV-based ART showed that the *CYP2B6* 516T allele was protective against 1--year,[29] but not at 6-months,[30] virologic breakthrough. However, no HIV DRMs have been assessed in either study. Similar results on the influence of the *CYP2B6* 516G>T substitution on virologic outcome were observed in studies from the US involving HIV patients of African ancestry[31] and HIV-diagnosed women from a multi-ethnic cohort,[32] whereas other studies did not find any evidence of protection.[33–35] Another work, on 66 HIV-diagnosed women from Kenya taking NVP-based ART, showed no associations of *CYP2B6* 516G>T and 983T>C with virologic response and toxicity at 12 months of follow-up.[20]

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Study results concerning the association of CYP2B6 slow metabolizer profiles (defined by the presence of 516T and/or 983C alleles) and EFV and NVP-related adverse events and/ or toxicity are also conflicting, with some showing an association[15,36–39] and others not.[18–20,30,31,40,41] Notably, due to the complexity of the *CYP2B6* polymorphisms and the highest frequency of slow/intermediate genotypes among individuals of African ancestry, it is likely that haplotypes rather than a single polymorphism are better predictors of EFV/NVP plasma concentrations,[14] as well as of toxicity, in which polymorphisms in genes other than the CYP450 system may also play a role.[42,43]

Efavirenz and NVP have a long half-life (estimated at 40–115 and 25–164hrs, respectively), a low genetic barrier for HIV drug resistance, and complex pharmacogenetics, which raises the possibility of sub-therapeutic drug concentration in plasma, especially among CYP2B6 fast metabolizers, an aspect that has not been fully studied. The CYP2B6 fast metabolizer profile may allow low EFV/NVP plasma exposure, possibly leading to the selection and spread of HIV mutations and consequent viral drug resistance. On the other hand, EFV/NVP CYP2B6 slow metabolizers are exposed to higher drug plasma concentration, leading to potential higher toxicity and consequently reduced patient's adherence and/or loss to care with possibility of sub-therapeutic plasma exposure and higher risk of HIV drug resistance (at least in a long-term perspective). While studies produced conflicting results, a posology adjustment according to the *CYP2B6* polymorphism background has been proposed to address these potential issues in the context of personalized medicine.[44,45]

In summary, different *CYP2B6* genotypes may influence immuno-virological response and/or toxicity by affecting EFV and NVP plasma concentration.[14] We explored the possible impact of *CYP2B6* genetic (and haplotype) variation on the risk of selection, accumulation and spread of HIV DRMs, providing a particular attention to the CYP2B6 fast metabolizer profile. To date, to the best of our knowledge, this aspect has not yet been fully evaluated.

This study was performed in Botswana with the aim to: i) assess *CYP2B6* genotypes (for SNPs −82T>C, 516G>T, 785A>G, 983 T>C) in HIV-diagnosed adults taking EFV or NVP containing ART, and to classify corresponding CYP2B6 phenotypes as very slow, slow, extensive, rapid and ultra-rapid metabolizers; ii) construct haplotypes and apply a metabolic score according to the DRMs profile; iii) determine if there is any association of the *CYP2B6* genotypes and haplotypes with the presence of EFV/NVP-resistant infections.

Patients and Methods
Study Design and Sample Size
This retrospective case–control study was part of a larger Tshepo study[46] that was conducted at Botswana-Harvard AIDS Institute Partnership (BHP) between 2002 and 2007. The Tshepo study was a 5-years open-label, randomized study with a sample of 650 HIV-1 diagnosed ART naïve Botswana citizens (451 females and 199 males) attending the Infectious Disease Care Clinic (IDCC) in Princess Marina Hospital in Gaborone. The aim of Tshepo study was to evaluate the efficacy, tolerability and occurrence of drug resistance of six (6) different first-line ART regimens, all including an NNRTI either EFV or NVP, during a follow-up period of 156 weeks. For the purpose of the present study, being patient's adherence assessed and comparable results,[46] cases were defined as HIV-diagnosed individuals taking EFV or NVP containing ART and having virological failure (evaluated after at least 4 months of ART) related to DRMs assessed by HIV genotyping, whereas controls were HIV-diagnosed individuals taking EFV or NVP-based ART without virological failure.[46]

Overall, 242 available residual samples were included in the present study. Of them, 40 were available cases that developed NNRTI resistance mutations, and 202 were controls.

DNA Extraction
Genomic DNA was extracted using Qiagen kits manual platform according to the manufacturer's protocol (Qiagen, Hilden, Germany) from about 200μL of peripheral blood mononuclear cells (PMBC's) stored at −80°C.

CYP2B6 Genotyping
CYP2B6 516G>T (rs3745274) detection was carried out using PCR-RFLP technique according to Lavandera et al[47] protocol with minor modifications. *CYP2B6* 983T>C (rs28399499) detection was carried out using a touchdown PCR-RFLP assay published by Paganotti et al.[48] *CYP2B6* 785A>G (rs2279343) detection was done using an in-house optimized RFLP-PCR protocol.[49]

For purposes of this study, we also adopted a new in-house assay for analysis of the *CYP2B6* −82T>C (rs34223104) polymorphism. We designed two (2) primers (forward primer: 5′-CAAGCAGGAAGTCTGGGTTC-3′; reverse primer: 3′-AGTTCCATGGTCCTGGTCT-5′). PCR reaction was conducted in a total volume of 20μL containing 100ng genomic DNA. PCR protocol with the

following conditions was used: 3min of denaturation at 94°C, 30s at 94 °C, 30s at 64 °C and 60s at 72 °C for 35 cycles with a final step of 5min at 72 °C. The PCR product was then digested with *PsiI* restriction enzyme at 37 °C for 90 min. The enzyme cuts the wild-type allele (T) in two fragments of 92 bp and 88bp; while the mutant allele is not cut. The digested fragments were visualized on a 4% metaphor gel stained with ethidium bromide.

Metabolic Score (MS) by Haplotypes and *CYP2B6* Inferred Metabolic Phenotypes

CYP2B6 genotype and haplotype information was translated into a measure of phenotype using the metabolic score (MS) system,[49] already adopted as "activity score" for *CYP2A6*,[50] *CYP2C19*,[51] *CYP2D6*,[52] and also consistent with Vujkovic et al,[41] for *CYP2B6*. The MS translates composite genotype and/or haplotype information into a qualitative measure of phenotype. The scores are based on the algebraic sum of the individual allele values according to an additive model for *CYP2B6*.[49] The MS was set conferring a −1 value for each slow metabolism alleles (516T, 983C) and +1 for rapid metabolism alleles (−82C, 785G), while an extensive metabolism allele was scored 0 (−82T, 516G, 785A, 983T) both for composite *CYP2B6* genotypes and haplotypes.[49]

Statistical Analysis

Several methods were applied. We used the Arlequin software (v3.5.2.2)[53] to test for Hardy–Weinberg Equilibrium (HWE) and the genetic fixation index (F_{ST}) with default settings, while Linkage Disequilibrium (LD) was tested using the Expectation-Maximization (EM) algorithm with 20,000 permutations and three initial conditions. Binary

Logistic Regression (BLR) analysis (run on IBM SPSS statistical package, version 20) was applied to find any association between the dependent variable "drug resistance" with the independent variables (age, BMI, baseline CD4+ T-cell count and viral load, *CYP2B6* genotype). Fisher's exact test, chi-square test and z-statistic were applied for statistical significance where needed.

Results
Baseline Population Demographics

Out of the 242 samples, 15 were excluded (5 due to the lack of complete genotypic information and 10 failed PCR). Thus, 227 samples were retained for analysis, with 40 (18.6%) belonging to the group that developed virological failure with EFV/NVP DRMs. The remaining 187 samples (81.4%) belonged to the group that did not develop virological failure during the follow-up period. Information about gender was available for 223 individuals, 146 (65.5%) being females. The study population characteristics at baseline were available for 225 individuals, being as follows: mean age 33.7 years (range: 20.-4–50.9); mean BMI 21.3 (range: 14.5–34.6); median baseline CD4+ T-cell count 188 cells/μL (IQR: 147–221); median baseline viral load 5.30 Log_{10} copies/mL (IQR: 4.83–5.71). The ART regimen data were available for 225 individuals, with 117 (50.6%) receiving EFV, and 108 (46.8%) receiving NVP containing ART. Table 1 summarises the baseline characteristics of the study population according to the EFV/NVP resistance status.

Virologic Failure and EFV/NVP Resistance

This study was based on a subsample of the Tshepo study[46] conducted in Botswana on HIV-diagnosed

Table 1 Baseline Characteristics of the Study Population

Characteristics	Overall	EFV/NVP-Resistant	EFV/NVP-Susceptible
Subjects, n (%)	227 (100.0%)	40 (17.6%)	187 (82.4%)
Females, n (%)	146 (65.5%)*,[a]	27 (67.5%)*	119 (65.0%)*,[a]
Males, n (%)	77 (34.5%)*,[a]	13 (32.5%)*	64 (35.0%)*,[a]
Mean age, years (range)	33.7 (20.4–50.9)	34.7 (20.4–50.9)	33.5 (22.9–49.6)
Mean BMI (range)	21.3 (14.5–34.6)	21.8 (16.3–34.6)	21.1 (14.5–31.8)
Median T-CD4, cells/μL (IQR)	188 (147–221)	194 (97.5–241.5)	187 (152.2–219.0)
Median Viral Load, Log_{10} copies/mL (IQR)	5.30 (4.83–5.71)	5.41 (4.91–5.75)	5.27 (4.82–5.66)
EFV-based ART, n (%)	115 (50.7%)[§]	16 (13.9%)[§]	99 (86.1%)[§]
NVP-based ART, n (%)	107 (47.1%)[§]	24 (22.4%)[§]	83 (77.6%)[§]
Unspecified EFV/NVP-based ART, n (%)	5 (2.2%)[§]	0 (0.0%)[§]	5 (100.0%)[§]

Notes: *Proportions calculated for columns; [§]proportions calculated for rows; [a]gender data were not available for 4 individuals (all NNRTI-susceptible).
Abbreviations: ART, antiretroviral therapy; NVP, nevirapine; EFV, efavirenz; BMI, body mass index; IQR, interquartile range.

individuals taking EFV/NVP-based ART regimen and followed up to 3-years (156 weeks) after the treatment's start. All cases of virologic failure reported in the Tshepo study underwent HIV genotyping[46] and EFV/NVP DRMs were detected in all the 40 cases used in the present study (Supplementary Table 1). Thus, in the present study, virologic failure and EFV/NVP DRMs coincide.[46] The median interval between start of ART and appearance of virologic failure was 72 weeks (IQR: 45.5–104).

CYP2B6 Genotype and Allele Frequencies

The *CYP2B6* genotype distribution and allele frequencies of the four SNPs (−82T>C, 516G>T, 785A>G, 983T>C) are summarized in Table 2. The comparisons of the three genotypes distribution between the subjects with and those without HIV DRM for each single SNP were all not statistically different but for the *CYP2B6* 516G>T polymorphism (chi-square = 8.121; $P = 0.017$) (Figure 1), with the wild-type extensive metaboliser 516G allele at higher frequency among resistant than sensitive infections (70% vs 54%, calculated from Table 2).

Hardy–Weinberg Equilibrium and Linkage Disequilibrium Analysis

Hardy–Weinberg Equilibrium analysis showed that *CYP2B6* 983 locus displayed significant deviations from HWE in EFV/NVP-resistant, EFV/NVP-susceptible and combined (overall) samples ($P = 0.001$; $P < 0.001$; $P < 0.001$, respectively). A reason for this deviation may be due to a defect in heterozygous samples. Furthermore, *CYP2B6* −82 and *CYP2B6* 516 did not show a statistically significant deviation from HWE in the EFV/NVP-resistant HIV infections ($P = 0.449$ and $P = 0.230$, respectively), whereas a statistically significant deviation from HWE was noted in the EFV/NVP-susceptible ($P = 0.039$ and $P = 0.031$, respectively) and all the samples combined ($P = 0.030$ and $P = 0.010$, respectively), with an excess of heterozygotes in both groups. *CYP2B6* 785 genotypes were in equilibrium in all the groups analysed (EFV/NVP-resistant, EFV/NVP-susceptible and both combined).

Linkage disequilibrium was observed between *CYP2B6* −82 and *CYP2B6* 516 in all groups (Table 3). Linkage disequilibrium was also observed between *CYP2B6* −82 and *CYP2B6* 983, as well as between *CYP2B6* 516 and *CYP2B6* 983, in the EFV/NVP-susceptible and overall samples, but not in the EFV/NVP-resistant group (Table 3). Finally, *CYP2B6* 785 did not

Table 2 *CYP2B6* Genotype and Allele Frequencies of the Four SNPs

	−82 T>C				516 G>T				785 A>G				983 T>C			
	TT (%)	TC (%)	CC (%)	f(C)	GG (%)	GT (%)	TT (%)	f(T)	AA (%)	AG (%)	GG (%)	f(G)	TT (%)	TC (%)	CC (%)	f(C)
EFV/NVP-S[a] (n=187)	176 (94.1)	11 (5.9)	0 (0.0)	2.94	47 (25.1)	108 (57.8)	32 (17.1)	45.99	62 (33.2)	104 (55.6)	21 (11.2)	39.03	138 (73.8)	32 (17.1)	17 (9.1)	17.64
EFV/NVP-R[b] (n=40)	36 (90.0)	3 (7.5)	1 (2.5)	6.25	18 (45.0)	20 (50.0)	2 (5.0)	30.00	19 (47.5)	20 (50.0)	1 (2.5)	27.50	32 (80.0)	4 (10.0)	4 (10.0)	15.00
Overall (n=227)	212 (93.4)	14 (6.2)	1 (0.4)	3.52	65 (28.6)	128 (56.4)	34 (15.0)	43.17	81 (35.7)	124 (54.6)	22 (9.7)	32.60	170 (74.9)	36 (15.9)	21 (9.2)	17.18

Notes: [a]EFV/NVP-Susceptible; [b]EFV/NVP-Resistant.
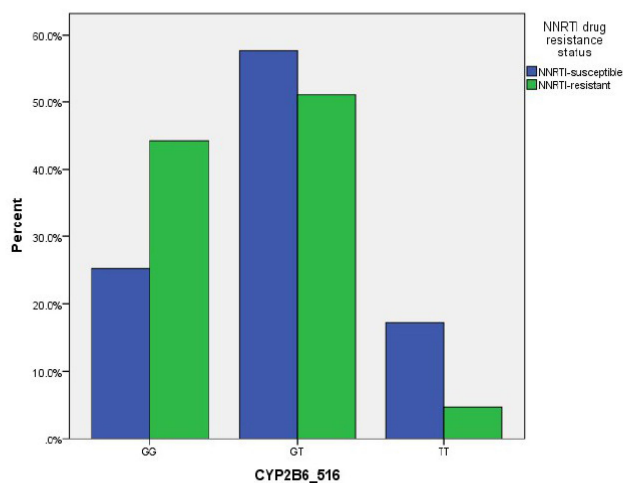
Figure 1 Distribution of *CYP2B6-516* genotypes according to NNRTI-resistance status. Chi-square associated *P*-value is 0.017.

show strong association with the other three loci, thus justifying the respect of HWE in all the three groups (EFV/NVP-resistant, EFV/NVP-susceptible, overall samples) (Table 3).

Population Differentiation Tests

The fixation index (F_{ST}) between EFV/NVP-resistant and susceptible samples gave a distance of 0.023 ($P = 0.008 \pm$

Table 3 Pairwise Linkage Disequilibrium (LD) Analysis for the Four Polymorphic Loci (*CYP2B6* −82, 516, 785, 983)

Phenotype	Comparison	*P*-value
EFV/NVP-Resistant	785 vs −82	0.054
EFV/NVP-Resistant	785 vs 516	0.882
EFV/NVP-Resistant	785 vs 983	0.195
EFV/NVP-Resistant	**−82 vs 516**	**0.001**
EFV/NVP-Resistant	−82 vs 983	0.078
EFV/NVP-Resistant	516 vs 983	0.118
EFV/NVP-Susceptible	785 vs −82	0.513
EFV/NVP-Susceptible	785 vs 516	0.095
EFV/NVP-Susceptible	785 vs 983	0.685
EFV/NVP-Susceptible	**−82 vs 516**	**<0.001**
EFV/NVP-Susceptible	**−82 vs 983**	**0.001**
EFV/NVP-Susceptible	**516 vs 983**	**0.023**
Overall	785 vs −82	0.221
Overall	785 vs 516	0.254
Overall	785 vs 983	0.324
Overall	**−82 vs 516**	**<0.001**
Overall	**−82 vs 983**	**<0.001**
Overall	**516 vs 983**	**0.006**

Notes: *P*-values for LD analysis were obtained using Arlequin. Significant comparisons are highlighted in bold.
Abbreviations: EFV, efavirenz; NVP, nevirapine.

0.003, 1023 permutations). While significant, this difference is consistent with panmixia between the two groups. Furthermore, an exact test of population differentiation revealed no statistical difference between the two groups ($P = 0.060 \pm 0.008$; 100,000 Markov chain steps).

Binary Logistic Regression Analysis and EFV/NVP Resistance According to *CYP2B6* Genotypes

Binary Logistic Regression analysis was set with the dependent dichotomic variable assuming two values according to the presence of EFV/NVP-susceptible or EFV/NVP-resistant HIV infections. Factors tested were age, BMI, baseline lymphocytes T-CD4+, viral load, genotypes for *CYP2B6* −82, *CYP2B6* 516, *CYP2B6* 785, *CYP2B6* 983 (see Supplementary Table 2). First step results showed only one significant factor (*CYP2B6* 516G). After removal of the non-significant factors, the BLR was repeated with only one (significant) factor in the model (*CYP2B6* 516G>T). This second step analysis revealed a significant statistical association between presence of *CYP2B6* 516G and DRMs status (OR: 2.26; 95% CI:1.27–4.01; *P* = 0.005).

Finally, the *CYP2B6* 516T allele presence (GT and TT genotypes) was tested for its possible protection against virologic failure (corresponding to EFV/NVP resistance in the present study as stated in the Methods section): no association was found at 6 months, nor beyond six months (up to 3 years) (Fisher's exact test, *P* > 0.05).

CYP2B6 Haplotype Frequency Estimation in EFV/NVP Susceptible and Resistant Groups

Haplotype frequencies for *CYP2B6* were estimated using the EM algorithm in Arlequin (Table 4). For all the samples, based on the four SNPs of *CYP2B6* analysed (−82T>C, 516G>T, 785A>G, 983T>C), the TGAT haplotype was the most common, whereas the CGAC, CTAT and CGGT haplotypes the rarest (Table 4). The haplotype TGAT was also the most abundant haplotype when EFV/NVP-resistant and susceptible samples were analysed separately, whereas no CGGT haplotypes were estimated in the EFV/NVP-susceptible group, and no TGGC, CGAC and CTAT haplotypes were estimated in the EFV/NVP-resistant group (Table 4).

Metabolic Score by *CYP2B6* Haplotype and EFV/NVP Resistance Status

The inferred MS was calculated for the haplotypes and they are shown in Table 4. In order to assess the possible risk of

Table 4 Estimated and Maximum-Likelihood (ML) Haplotype Frequencies by Phenotype and for All the Samples Combined. Maximum-Likelihood Haplotype Frequencies are Shown in Parenthesis with Their Standard Deviations (SD). The Order of Nucleotides in the Reconstructed Haplotypes is Made According to the SNP Position in the *CYP2B6* Gene (−82T>C, 516G>T, 785A>G, 983T>C)

Haplotype	MS	Phenotype	Estimated and ML Frequencies ± SD		
			Overall*	EFV/NVP- Resistant	EFV/NVP-Susceptible
TGAT	0	Extensive	201 (0.441 ± 0.023)	43 (0.526 ± 0.065)	162 (0.430 ± 0.029)
TGAC	−1	Slow	24 (0.054 ± 0.013)	5 (0.065 ± 0.035)	19 (0.052 ± 0.015)
CGAT	1	Rapid	12 (0.019 ± 0.008)	3 (0.039 ± 0.024)	5 (0.008 ± 0.006)
CGGT	2	Ultra-rapid	2 (0.004 ± 0.003)	2 (0.024 ± 0.018)	0 (0.000 ± 0.000)
TTAT	−1	Slow	37 (0.078 ± 0.015)	6 (0.082 ± 0.036)	27 (0.073 ± 0.017)
TTAC	−2	Very slow	10 (0.026 ± 0.010)	1 (0.013 ± 0.017)	9 (0.030 ± 0.011)
TGGT	1	Rapid	16 (0.041 ± 0.011)	3 (0.046 ± 0.023)	13 (0.041 ± 0.011)
TTGC	−1	Slow	41 (0.083 ± 0.015)	6 (0.073 ± 0.032)	35 (0.085 ± 0.016)
TTGT	0	Extensive	107 (0.236 ± 0.020)	11 (0.132 ± 0.046)	96 (0.252 ± 0.026)
TGGC	0	Extensive	2 (0.006 ± 0.005)	0 (0.000 ± 0.000)	2 (0.006 ± 0.006)
CGAC	0	Extensive	1 (0.002 ± 0.003)	0 (0.000 ± 0.000)	1 (0.003 ± 0.003)
CTAT	0	Extensive	1 (0.009 ± 0.009)	0 (0.000 ± 0.000)	5 (0.013 ± 0.006)
CTGT	1	Rapid	0 (0.000 ± 0.000)	0 (0.000 ± 0.000)	0 (0.005 ± 0.005)

Notes: *The combination of susceptible and resistant dataset does not necessarily result in a "sum" of the two haplotype counts estimated separately. Different sample counts in each group have an effect on the accuracy of the estimates.
Abbreviations: MS, metabolic score; N/A, not applicable; NC, not calculable (because of lack of estimated haplotype frequency).

carrying EFV/NVP-resistant HIV infections according to the CYP2B6 fast metabolizer profile, the metabolic phenotypes were classified as follows: MS≤0, this including extensive, slow and very slow inferred metabolic phenotypes; MS≥1, this including rapid and ultra-rapid inferred metabolic phenotypes. The breakdown of metabolic phenotypes between groups according to the EFV/NVP resistance status are shown in Table 5. The comparison between EFV/NVP-resistant and susceptible HIV infections by MS was associated to a *z*-statistic of 1.812 ($P = 0.035$), therefore showing that the rate of EFV/NV resistance was significantly higher among fast metabolizers haplotypes compared to the other group (30.8% vs 16.8%, respectively).

In line with the BLR results, the *CYP2B6* 516G allele was present in 100% (n=30/30) of rapid (CGAT and

TGGT) and ultra-rapid (CGGT) haplotypes, whereas it was only present in 53.8% (n=228/424) of extensive/slow metabolizers haplotypes (Fisher's exact test, P < 0.001) (see Table 4, "overall" column).

Discussion

Understanding the factors that modulate the selection of DRMs associated with HIV-1 infection is essential to design efficient control strategies. Drug resistance usually emerges rapidly when ARV drugs are administered as monotherapy or in the presence of incomplete viral suppression, suggesting that resistance is caused by the selection of mutant viruses within the host.[54] Besides known viral factors (HIV diversity, HIV replication, drug selection pressure and fitness of drug-resistant viral subpopulations) and patient ART adherence, human genetic background is a possible further, not yet fully understood, co-factor affecting HIV drug resistance selection. In this study, we addressed the hypothesis that human pharmacogenetics can drive the selection of HIV drug resistance. We therefore found a statistically significant association between EFV/NVP-resistant HIV infections and *CYP2B6* 516G allele presence (OR: 2.26; 95% CI:1.27–4.01; $P = 0.005$). In fact, EFV/NVP resistant infections had higher 516G allele frequency (Table 2 and Figure 1). Further information comes from the haplotype reconstruction where 100% of rapid (CGAT and TGGT) and ultra-rapid

Table 5 EFV/NVP Resistance by *CYP2B6* Metabolic Phenotype

EFV/NVP Resistance Status	EFV/NVP Metabolic Phenotype	
	MS ≤ 0[a]	MS ≥ 1[b]
Resistant, n (%)	72 (16.8%)	8 (30.8%)
Susceptible, n (%)	356 (83.2%)	18 (69.2%)
Total	428 (100%)	26 (100%)

Notes: Resistance and susceptible haplotypes were counted according to MS as from Table 4. The *z* statistic is 1.812. The one-tailed *P*-value is 0.035. [a]MS ≤ 0: extensive, slow and very slow EFV/NVP metabolizers. [b]MS ≥ 1: rapid and ultra-rapid EFV/NVP metabolizers.

Table 6 *CYP2B6* SNPs (−82T>C, 516G>T, 785A>G, 983T>C) Frequency in Sub-Saharan Africa

Geographic Region/Ethnic Populations	Number of Participants	CYP2B6 SNPs Frequency (%)				References
		−82C	516T	785G	983C	
Southern Africa						
Botswana	101	–	36.6	–	–	[57]
Botswana	1101	–	37.6	–	–	[29]
Botswana	731	–	–	6.0	11.0	[39]
Botswana	570	–	38.1	33.0	13.5	[49]
Malawi	150	–	40.5	37.1	8.6	[19]
Mozambique	105	–	34.7	44.2	8.6	[39]
Mozambique	360	–	42.6	40.9	–	[58]
South Africa	122	–	32.0	–	–	[59]
South Africa	80	–	43.1	–	–	[37]
South Africa	160	–	36.2	36.2	2.5	[60,61]
South Africa	295	–	41.1	41.1	7.1	[61]
South Africa	113	–	36.0	–	7.0	[62]
South Africa	81	–	35.2	35.2	3.7	[63]
South Africa	60	–	41.0	40.8	11.0	[64]
Zimbabwe	36	–	51.4	52.8	11.1	[65]
Zimbabwe	71	–	48.6	–	–	[66]
Zimbabwe	49	–	41.8	41.8	9.1	[67]
Zimbabwe	185	–	43.8	–	15.9	[68]
West-Central Africa						
Cameroon	69	–	36.9	32.6	–	[60]
Cameroon	168	–	44.3	–—	12.8	[48]
Cameroon	122	–	59.4	–—	8.6	[69]
Ghana	40	1.2	48.8	47.5	6.6	[70]
Ghana	42	–	54.0	46.0	7.6	[71,72]
Ghana	705	–	48.0	–	4.0	[18]
Ghana	94	–	–	–	4.2	[42]
Ghana	74	–	44.6	–	4.6	[73]
Guinea	21	–	50.0	48.0	1.6	[71,72]
Ivory Coast	41	–	40.0	38.0	5.5	[71,72]
Nigeria	300	–	36.5	–	–	[74]
Nigeria	77	–	43.7	–	13.2	[75]
Republic of Congo	288	–	55.0	–	–	[76]
Sierra Leone	52	–	47.0	36.0	3.8	[71,72]
East Africa						
Burundi	202	–	31.6	–	6.9	[77]
Ethiopia	163	–	29.7	–	–	[78]
Ethiopia	245	–	31.4	–	–	[79]
Ethiopia	264	–	31.4	–	–	[80]
Ethiopia	298	–	29.2	–	–	[81]
Kenya	66	–	32.6	–	9.8	[20]
Rwanda	80	–	31.9	32.5	9.2	[17]
Rwanda	39	6.4	–	–	–	[82]
Rwanda	90	–	32.8	–	8.0	[83]
Tanzania	183	–	41.8	–	–	[80]
Tanzania	242	–	36.0	–	–	[84]
Tanzania	251	–	35.6	–	19.8	[85]

(*Continued*)

Table 6 (Continued).

Geographic Region/Ethnic Populations	Number of Participants	CYP2B6 SNPs Frequency (%)				References
		−82C	516T	785G	983C	
Tanzania	91	–	33.5	–	9.3	[86]
Tanzania	37	–	33.8	–	–	[87]
Uganda	23	–	30.4	–	–	[88]
Uganda	187	–	31.8	–	–	[89]
Uganda	74	–	29.1	32.4	5.4	[65]
Uganda	166	–	39.4	–	–	[90]
Uganda	57	–	33.3	–	8.8	[91]

(CGGT) *CYP2B6* haplotypes carried the 516G allele (Table 4), whereas only 53.8% of the extensive, slow (TGAC, TTAT and TTGC) and very slow (TTAC) haplotypes carried it. Moreover,

CYP2B6 rapid and ultra-rapid metabolizers showed a significantly higher frequency of EFV/NVP-resistant HIV infections than extensive and slow metabolizers (30.8% vs 16.8%; z-statistic = 1.812; P = 0.035), based on their haplotype reconstruction (Table 5). Nevertheless, it is important to note that the rate of rapid and ultra-rapid haplotypes is less than 6% (26/454) of the total haplotypes.

A possible interpretation of these results is that EFV/NVP resistance tends to accumulate based on the presence of the *CYP2B6* 516G allele (GG>GT>TT), and that in rapid and ultra-rapid metabolisers might happen at a higher rate. This could be an important model for drug resistance selection that may be verified on a larger longitudinal cohort and tested on different pathogens, different antimicrobial drugs and epidemiological contexts.

These results are partially in line with similar findings on malaria drug resistance where it has been demonstrated that the *CYP2C8* slow metabolizer phenotype is associated with the risk of carrying chloroquine- and amodiaquine-resistant parasites.[55,56]

We also found that LD into the ≃ 1000 bp region analysed (spanning from *CYP2B6* −82 to 983 nucleotide positions) was strong *(P < 0.001)* and departures from the HWE were coherent with the physical and genetic linkage between SNPs. Genotype and allele frequencies for the four *CYP2B6* SNPs analysed in this study were in line with literature, including most if not all studies from Botswana (Table 6). The only discrepancy was represented by a study[41] conducted in the same area as the present research that indicated an allele frequency for *CYP2B6* 785G of 6%, well outside the accepted range of

35.2–52.8% for Southern Africa (Table 6). Finally, the two groups (with and without EFV/NVP-resistant HIV infections) into which we subdivided our study population appeared to be in panmixia, therefore offering an ideal situation for the comparison.

In summary, subjects carrying the *CYP2B6* 516G allele were more likely to carry HIV drug-resistant infections. Coherently with our hypothesis, the 516G allele is always present in the rapid and ultra-rapid haplotypes, confirming the possibility that rug resistance selection is enhanced when drug metabolism is faster. However, the rate of fast metabolisers was not high in this cohort, therefore reducing its possible impact.

The present study has few limitations: i) the sample size was relatively small, therefore haplotypes reconstruction provided higher statistical power in the comparisons, ii) it would have been more powerful to use matched-case control, to avoid any confounding factors, and iii) the lack of data on EFV/NVP plasma exposure hampered the quantitative confirmation of CYP2B6 metabolic phenotypes. Nonetheless, our findings suggest a trend towards a role for the genetic background of patients affecting drug therapy outcomes, and warrants further studies.

Conclusion

In conclusion, this work indicates that the *CYP2B6* 516G allele, and its combination into rapid and ultra-rapid metabolizer profiles, as defined by the correspondent haplotypes, is directly associated with the risk of development of drug resistance in HIV-diagnosed individuals receiving EFV- or NVP-containing ART in Botswana. However, larger studies will be needed to confirm this association. In general, our findings support the hypothesis that pharmacogenetics may play a significant role in HIV therapy outcomes. Besides the known possible

impact of slow EFV/NVP metabolism on ART toxicity and compliance, fast EFV/NVP metabolism may also affect ART outcomes. A deeper knowledge of the genetic background at an individual level could thus be highly beneficial in personalising ART therapies and improving their efficacy, especially in patients who show poor response following initiation of treatment.

Ethics Approval and Informed Consent

This was a retrospective case–control study approved by Health Research Division Office (HRDC) of the Botswana Ministry of Health and Wellness. The approval was done in accordance with the amendments made to the initial permit of "The host genetics of HIV-1 subtype C infection progression and treatment in Africa/Gwas on determinants of HIV-1 subtype C infection" [Reference No: HPDME 13/18/1 X1 (163)]. For the purpose of this study signed informed consent was sought from the participants. In addition, Botswana-Harvard AIDS Institute Partnership, as the database owner authorized by HRDC, gave permission to use its data and samples for the current study. This study was conducted in accordance with the Declaration of Helsinki.

Disclosure

The authors report no conflicts of interest in this work.

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