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# *CYP2B6*6* and *CYP2B6*18* Predict Long-Term Efavirenz Exposure Measured in Hair Samples in HIV-Positive South African Women

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## Abstract

Long-term exposure to efavirenz (EFV) measured in hair samples may predict response to antiretroviral treatment (ART). Polymorphisms in *CYP2B6* are known to alter EFV levels. The aim of this study was to assess the relationship between *CYP2B6* genotype, EFV levels measured in hair, and virological outcomes on ART in a real-world setting. We measured EFV levels in hair from HIV-positive South African females who had been receiving EFV-based treatment for at least 3 months from the South African Black (SAB) ($n=81$) and Cape Mixed Ancestry (CMA) ($n=53$) populations. Common genetic variation in *CYP2B6* was determined in 15 individuals from each population using bidirectional Sanger sequencing. Prioritized variants ($n=16$) were subsequently genotyped in the entire patient cohort ($n=134$). The predictive value of EFV levels in hair and selected variants in *CYP2B6* on virological treatment outcomes was assessed. Previously described alleles (*CYP2B6*2*, *CYP2B6*5*, *CYP2B6*6*, *CYP2B6*17*, and *CYP2B6*18*), as well as two novel alleles (*CYP2B6*31* and *CYP2B6*32*), were detected in this study. Compared to noncarriers, individuals homozygous for *CYP2B6*6* had $\sim$109% increased EFV levels in hair ($p=.016$) and *CYP2B6*18* heterozygotes demonstrated 82% higher EFV hair levels ($p=.0006$). This study confirmed that alleles affecting CYP2B6 metabolism and subsequent EFV exposure are present at significant frequencies in both the SAB and CMA populations. Furthermore, this study demonstrated that the use of hair samples for testing EFV concentrations may be a useful tool in determining long-term drug exposure in resource-limited countries.

## Introduction

HIV/AIDS prevalence estimates in South Africa continue to rank among the highest in the world. The cost of failing first-line antiretroviral treatment (ART) in resource-limited settings,[1] where second- and third-line treatment options are restricted,[2] is high. The widely prescribed nonnucleoside reverse transcriptase inhibitor (NNRTI), efavirenz (EFV), in combination with tenofovir disoproxil fumarate (TDF) and lamivudine (3TC), is currently the most efficacious treatment combination in South Africa and a first-line option worldwide.[3] Maintaining therapeutic EFV levels during chronic treatment is crucial for durable and optimal responses, with subtherapeutic exposure putting patients at risk for treatment failure and supratherapeutic concentrations being associated with the central nervous system (CNS) side effects.[4–8]

The incidence of adverse drug reactions (ADRs) on ART is high in South Africa, partially due to concomitant conditions and drug–drug interactions.[9] ADRs may contribute to high ART attrition and failure rates in this setting.[10,11] Owing to the long half-life of EFV and its low genetic barrier to resistance, a single dose of EFV monotherapy can select for NNRTI-resistant virus in patients interrupting treatment.[12–14] Monitoring antiretroviral (ARV) drug exposure in hair may be an inexpensive, noninvasive, objective biomarker of adherence, which is of value in resource-limited countries, such as South Africa.[7,15–20] Drug levels measured in hair provide a

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long-term indicator of exposure and may thus be a more accurate means of measurement than the use of single plasma levels.[16,21] Moreover, genetic testing for traits impacting EFV levels as measured in hair may provide a molecular explanation for varying treatment outcomes and further facilitate optimization of HIV treatments.[21,22]

Average EFV exposure is reported to be higher in Africans compared to non-Africans and higher in African females compared to African males.[23–26] In addition, environmental factors can also contribute to differences in EFV plasma levels and immunologic recovery between African populations.[27] While the effect of gender on EFV levels is yet to be explained,[24] the effect of ethnicity on EFV exposure can partially be attributed to genetic underpinnings. In line with this, it has recently been reported that when comparing different ARVs, EFV metabolism is ranked the highest in terms of heritability, thereby providing a strong rationale for pharmacogenomic studies relating to this drug.[28]

Increased EFV levels in Africans are predominantly accounted for by the high frequency of the nonsynonymous polymorphisms, c.516G>T (rs3745274, Q172H) in exon 4 and c.983T>C (rs28399499, I328T) in exon 7 of *CYP2B6*.[13,22,25,29] These variants, representing the *CYP2B6*6* and *CYP2B6*18* alleles, decrease the activity of CYP2B6,[30,31] which is the major metabolizing enzyme for EFV.[32] Although these variants explain a large proportion of increases in EFV levels, additional polymorphisms in *CYP2B6* and its regulatory regions have been implicated in altering EFV metabolism,[14,33] and sequencing of *CYP2B6* continuously leads to the discovery of novel alleles in different populations that might affect drug concentrations.[25,34,35]

Novel variation is expected in sub-Saharan African populations as individuals from these populations are more genetically diverse than those from other populations[36,37] and are understudied in genetic/genomic research.[38–40] Two such populations are the South African Black (SAB) and Cape Mixed Ancestry (CMA) populations, both of which are severely affected by the HIV/AIDS pandemic.[41] The SAB is the largest population group (79%) in South Africa.[41] According to first language statistics for the Western Cape Province of the country, where this study was conducted, the majority of SAB individuals in this province speak isiXhosa.[41] The Xhosa population are descendants of the western African Bantu, although linguistic similarities and population genetic data suggest admixture with ancient Khoisan tribes.[36] The CMA population, also known as the Colored population, is genetically heterogeneous, representing recent admixture from Khoisan, Bantu, European, and Asian populations.[42] This population comprises 9% of the entire South African population but represents the largest group (54%) of the population in the Western Cape Province.[41] Sequence analysis of other important cytochrome P450 (*CYP*) genes in these two populations has previously revealed the presence of novel variation potentially affecting the function of protein products,[43–47] emphasizing the importance of the characterization of pharmacogenes, such as *CYP2B6*, in these population groups.

In this study, we comprehensively characterize the genetic variation present in the *CYP2B6* gene in HIV-infected females of SAB and CMA ethnicities. Using this information, associations between 17 genetic variants in the exonic and 5′ upstream regulatory regions of *CYP2B6* and EFV concentrations measured in hair were investigated to determine the effect of these variants on EFV exposure and EFV-based ART outcomes.

## Materials and Methods

### *Study subjects and sample collection*

Unrelated HIV-positive nonpregnant females ($n = 134$) older than 18 years, of self-reported SAB or CMA ethnicity, receiving EFV-based treatment for more than 3 months at an ARV clinic in Paarl, in the Western Cape, South Africa, gave written informed consent for participation in this study. Patients were recruited, and samples were collected from March to July 2010, but some of the patients had already started with ART long before this period, some as early as 2004. It was convenient to use only female patients for the study, as most SAB and CMA men keep their hair very short. Ethical approval was granted by the Committee for Human Research at Stellenbosch University (N08/08/225) and the South African Department of Health, Western Cape, South Africa while the Institutional Review Board at the University of California, San Francisco (UCSF) (10-03898) approved analyses of EFU in hair samples. Patients were recruited regardless of concomitant drug therapy. Clinical data were made available 1 year after sample collection and included treatment history, biannual viral load (VL) measurements, pretreatment CD4 counts, height and weight, the World Health Organization (WHO) clinical AIDS status, and adherence estimates based on percentage of pill counts per month for 6 months before sampling, as recorded by clinical staff (Table 1).

### *Hair specimen processing*

Patients donated a small thatch of ~20 strands of hair, cut as close to the scalp as possible, with the distal portion labeled to indicate directionality. Samples were sent to the Drug Studies Unit at UCSF, where EFV (Stocrin; Merck & Co., Inc., Whitehouse Station, NJ; and Viranz; Aurobindo Pharma, Hyderabad, India) was extracted and measured by liquid chromatography and tandem mass spectrometry (LC/MS/MS) as previously described.[21,48] Assays at UCSF have been developed and validated according to the principles of Good Laboratory Practices and some have undergone peer review and validation by the Division of AIDS Clinical Pharmacology and Quality Assurance (CPQA) program subsequent to the analysis of hair samples in this study.[49] This method has been validated from 0.05 to 20 ng/mg hair for EFV with good linearity ($R^2 > 0.99$) and reproducibility [coefficient of variation (CV) <15%]; interday and intraday assay precision was high (CV <15%), and accuracy ranged from 89% to 110% (M Gandhi and Y Huang, personal communication).

### *Identification, prioritization, and genotyping of CYP2B6 genetic variants*

Genomic DNA (gDNA) was extracted from patient saliva using the Oragene® DNA extraction kit according to the manufacturer's protocol (DNA Genotek, Inc., Kanata, Ontario, Canada). Amplification of the 5′ upstream and exonic regions was performed using the primers (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/aid) and conditions described in the Supplementary Materials. The gDNA from 15 individuals from each population was subjected to bidirectional Sanger

Table 1. Summary of Patient Characteristics [Median (Range) or Count (Percentage)], Stratified by Responder Status and Ethnicity

| *Characteristic* | *Responders,*[a] n = *104* | | *Nonresponders,*[a] n = *16* | |
|---|---|---|---|---|
| *Ethnicity* | *CMA,* n = *35 (34%)* | *SAB,* n = *69 (66%)* | *CMA,* n = *8 (50%)* | *SAB,* n = *8 (50%)* |
| EFV, ng/mg hair | 5.9 (0.9–20.9) | 5.2 (0.5–27.0) | 5.5 (1.2–10.2) | 8.2 (1.1–9.9) |
| VL, copies/ml[b] | Lower than detectable limit | Lower than detectable limit | 980 (140–23,000) | 2,750 (51–79,432) |
| CD4, cells/ml | 381 (83–824) | 469 (124–1,465) | 300 (135–446) | 446 (411–762) |
| Pretreatment CD4, cells/ml | 148 (12–391) | 159 (3–720) | 108 (74–198) | 151 (26–246) |
| Age collected, years | 43 (34–69) | 39 (26–63) | 41 (33–44) | 36 (25–45) |
| Pretreatment weight, kg | 57 (37–115) | 68 (39–109) | 57 (50–70) | 73 (64–95) |
| Time on ART, years | 3.0 (0.0–6.0) | 4.0 (1.0–7.0) | 3.5 (1.0–6.0) | 4.5 (1.0–6.0) |
| Adherence,[c] *n* (%) | | | | |
| Good | 30 (85.7) | 56 (81.2) | 5 (62.5) | 7 (87.5) |
| Acceptable | 5 (14.3) | 11 (15.9) | 3 (37.5) | 1 (12.5) |
| Inadequate | 0 (0.0) | 2 (2.9) | 0 (0.0) | 0 (0.0) |
| WHO stage, *n* (%) | | | | |
| 1 | 2 (5.7) | 3 (4.3) | 0 (0.0) | 0 (0.0) |
| 2 | 9 (25.7) | 12 (17.4) | 0 (0.0) | 2 (25.0) |
| 3 | 14 (40.0) | 33 (47.8) | 5 (62.5) | 5 (62.5) |
| 4 | 10 (28.6) | 18 (26.1) | 2 (25.0) | 1 (12.5) |
| Missing data | 0 (0.0) | 3 (4.3) | 1 (12.5) | 0 (0.0) |
| Metabolizer | | | | |
| EM | 18 (51.4) | 25 (36.2) | 2 (25.0) | 2 (25.0) |
| IM | 14 (40.0) | 36 (52.2) | 5 (62.5) | 3 (37.5) |
| PM | 3 (8.6) | 8 (11.6) | 1 (12.5) | 3 (37.5) |

[a]Patients with biannual VL measurements <50 copies/ml were classified as responders and those with VL measurements >50 copies/ml were classified as nonresponders.

[b]VL measurements as recorded on the closest date before hair sample collection were used. VL measurement and hair sampling could therefore differ substantially between patients and be up to, but not more than, 6 months.

[c]Adherence levels were classified as good, acceptable, and inadequate if 70%–100%, 50%–70%, and 0%–50% of pills, respectively, were taken.

ART, antiretroviral treatment; CMA, Cape Mixed Ancestry; EFV, efavirenz; EM, extensive metabolizer; IM, intermediate metabolizer; PM, poor metabolizer; SAB, South African Black; VL, viral load.

sequencing to assess common *CYP2B6* genetic variation compared to the reference sequence (Ensembl transcript ID: ENST00000324071). To select those variants most likely to affect EFV clearance and exposure, variants with a known effect on enzymatic activity, expression, and/or association with altered EFV levels were prioritized for genotyping in the entire cohort.[14,21,33,50] In cases where no information was available for a variant, *in silico* analyses were performed, making use of the publicly available bioinformatic tools, Alibaba v2.1,[51] P-Match v1.0,[52] SIFT v.4.0.3b,[53] PolyPhen,[54] m-Fold v3.2,[55] and GeneSplicer,[56] using default settings. In addition, all novel variants were prioritized for genotyping. To further characterize novel nonsynonymous variants for submission to the *CYP* allele Web site,[50] bidirectional Sanger sequencing was performed for the entire gene for all individuals carrying the novel variants. With the exception of *CYP2B6*17* (rs33980385) and g.15582C>T (rs4803419), genotype and allele frequencies of all prioritized variants were subsequently determined in the entire patient cohort by polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) analyses (Supplementary Table S2). In the case of *CYP2B6*17*, carriers of this allele were identified by screening the cohort for c.83A>G (rs33980385) and prioritizing carriers of this variant for sequencing of exon 1 to determine the presence of the remaining *CYP2B6*17* allele-defining variants. In the case of g.15582C>T (rs4803419), due to the difficulty in designing a PCR–RFLP assay, Sanger sequencing of exon 4 was performed to detect the presence of this variant. All variants were genotyped with 100% success rate, with the exception of rs4802101 (99% success rate), rs4803419 (98% success rate), rs193922917 (98% success rate), and rs3211371 (99% success rate) (refer to Table 2 for more details).

### *Statistical methods*

Genotype distributions were tested for deviations from Hardy–Weinberg equilibrium (HWE) in the SAB and CMA separately using Tools for Population Genetic Analysis (TFPGA) software v1.3.[57] Maximum likelihood haplotypes were assessed in Haploview v3.31,[58] and logarithm of the odds (LOD) scores ≥2 and $r^2$ measures >0.6 were regarded as an indication of significant linkage disequilibrium (LD). Association analyses were subsequently performed in the entire cohort, as well as in the CMA and SAB populations separately, for all variants that were prioritized for genotyping in the entire cohort. All potential confounders were assessed, and those confounders that were found to correlate significantly with EFV levels or treatment response were adjusted for in the association analyses. Subsequent association analyses were

Table 2. MAF for Variants Genotyped in the SAB and CMA Populations

| *Region* | *Allele* | *Variant (rs number)* | *Amino acid change* | *MAF SAB,* n = *81*[a] | *MAF CMA,* n = *53*[a] |
|---|---|---|---|---|---|
| 5′ | NA | g.-8427T>C (rs8105382) | NA | 0.321 (*n* = 81) | 0.245 (*n* = 53) |
| 5′ | NA | g.-8372G>A (rs193922919) | NA | 0.006 (*n* = 81) | 0.000 (*n* = 53) |
| 5′ | NA | g.-2320T>C (rs7254579) | NA | 0.204 (*n* = 81) | 0.321 (*n* = 53) |
| 5′ | NA | g.-1578C>T (rs12721652) | NA | 0.222 (*n* = 81) | 0.170 (*n* = 53) |
| 5′ | NA | g.-1456T>C (rs2054675) | NA | 0.481 (*n* = 81) | 0.321 (*n* = 53) |
| 5′ | NA | g.-750T>C (rs4802101) | NA | 0.963 (*n* = 81) | 0.885 (*n* = 52) |
| Exon 1 | *CYP2B6*2* | c.64C>T (rs8192709) | R22C | 0.031 (*n* = 81) | 0.019 (*n* = 53) |
| Exon 1 | *CYP2B6*17* | c.83A>G (rs33980385)[b] | T26S, D28G, R29S | 0.019 (*n* = 81) | 0.019 (*n* = 53) |
| Intron 3 | NA | g. 15582 C > T (rs4803419) | NA | 0.039 (*n* = 78) | 0.161 (*n* = 53) |
| Exon 4 | *CYP2B6*6* | c.516G>T (rs3745274) | Q172H | 0.352 (*n* = 81) | 0.283 (*n* = 53) |
| Exon 5 | *CYP2B6*6* | c.785A>G (rs2279343) | K262A | 0.352 (*n* = 81) | 0.283 (*n* = 53) |
| Intron 5 | NA | g.18627G>A (rs12721649) | NA | 0.21 (*n* = 81) | 0.179 (*n* = 53) |
| Exon 6 | NA | c.18742G>T (rs112848152) | T292T | 0.08 (*n* = 81) | 0.009 (*n* = 53) |
| Exon 6 | *CYP2B6*31* | c.937C>A (rs193922917) | L313I | 0.006 (*n* = 79) | 0.000 (*n* = 52) |
| Exon 7 | *CYP2B6*18* | c.983C>T (rs28399499) | I328T | 0.037 (*n* = 81) | 0.038 (*n* = 53) |
| Exon 8 | *CYP2B6*32* | c.1219G>A (rs193922918) | A407T | 0.019 (*n* = 81) | 0.000 (*n* = 53) |
| Exon 9 | *CYP2B6*5* | c.1459C>T (rs3211371) | R487C | 0.00 (*n* = 80) | 0.047 (*n* = 53) |

[a]*n*: Although the sample size in total consisted of SAB, *n* = 81, and CMA, *n* = 53, the sample size for a specific amplicon varied because some samples failed to amplify.

[b]All individuals who were carriers of g.83A>G (rs33980385; p.D28G) were also carriers of g.76A>T (rs33973337; p.T26S) and g.85C>A (rs33926104; p.R29S), and thus, the allele frequencies of these variants are identical.

MAF, minor allele frequencies.

adjusted for (1) *CYP2B6*6* alone and (2) *CYP2B6*6* and *CYP2B6*18* to identify independent signals. Linear models were used to estimate the association of the natural log of EFV levels in hair with single *CYP2B6* variants, using additive allelelic models (each variant coded 0, 1, or 2; rare alleles and *CYP2B6* alleles were each coded in the same additive way, and association tested separately for each), and metabolizer phenotypes. The additive model was used due to the fact that this is the most commonly used model in genetic case–control studies when the mode of inheritance is unknown.[59] Statistically significant variant effects were further analyzed to determine their penetrance (additive, dominant, or recessive), and results are based on the model with the smallest *p*-value. Subsequently, biannual VL measurements as recorded on the closest date before hair sample collection were dichotomized by classifying patients as ''responders'' (VL <50 copies/ml) and ''nonresponders'' (VL >50 copies/ml). Logistic regression models were used to investigate the association of dichotomous VL treatment outcomes with time on EFV treatment, EFV levels in hair, and genetic factors. A *p*-value <.05 was regarded as statistically significant in all analyses, with the exception of the HWE analyses, where a *p*-value <.01 was regarded as statistically significant. R,[60] the language and environment for statistical computing, was used for all statistical analyses, unless otherwise stated above.

## Results

Of 134 patients for whom genotype information was available, the majority received EFV in combination with stavudine and 3TC (*n* = 80), with few patients receiving EFV with zidovudine and 3TC (*n* = 31) and EFV with TDF and 3TC (*n* = 22). Duration of EFV treatment varied greatly among patients. A few patients received concomitant medication, such as enalapril, amitriptyline, metformin, warfarin, and gliclazide, and 15 of the patients received tuberculosis treatment (rifampicin) during the time of sample collection. Due to small sample sizes and an increasing number of studies indicating that plasma EFV levels may not be influenced by rifampicin cotreatment,[61,62] we have not investigated any drug–drug interactions in this study. For 1 patient, no clinical data were available, so EFV levels in hair were analyzed in 133 patients. Thirteen more patients were excluded from VL treatment outcome analysis because these patients were not yet eligible for a VL measurement, leaving a smaller final patient cohort (*n* = 120) for analyses. The patient characteristics of these 120 patients are summarized in Table 1.

### *Genetic variation of CYP2B6 in the SAB and CMA populations*

A total of 45 genetic variants were identified in the cohort of sequenced samples from CMA and SAB participants (Fig. 1), of which 17 variants were selected for genotyping. Five previously identified *CYP2B6* alleles were detected, namely *CYP2B6*2* (rs8192709), *CYP2B6*5* (rs3211371), *CYP2B6*6* (rs3745274 and rs2279343), *CYP2B6*17* (rs33980385), and *CYP2B6*18* (rs28399499). Furthermore, two novel nonsynonymous variants were detected in this study, *CYP2B6*31* (c.937C>A, [L313I], rs193922917 in exon 6) and *CYP2B6*32* (c.1219G>A, [A407T], rs193922918 in exon 8) (Supplementary Tables S3 and S4, respectively). Although both these amino acid changes were predicted to be ''benign'' and ''tolerated'' by SIFT and PolyPhen, respectively, and c.937C>A (*CYP2B6*31*) was predicted to cause a change in mRNA folding and the measure of free energy (ΔG) in m-Fold, suggesting a thermodynamically less stable structure (Supplementary Fig. S1).

A third novel variant, also submitted to the NCBI dbSNP database, was detected in the 5′ upstream region g.-8372G>A (rs193922919) and was predicted by Alibaba to

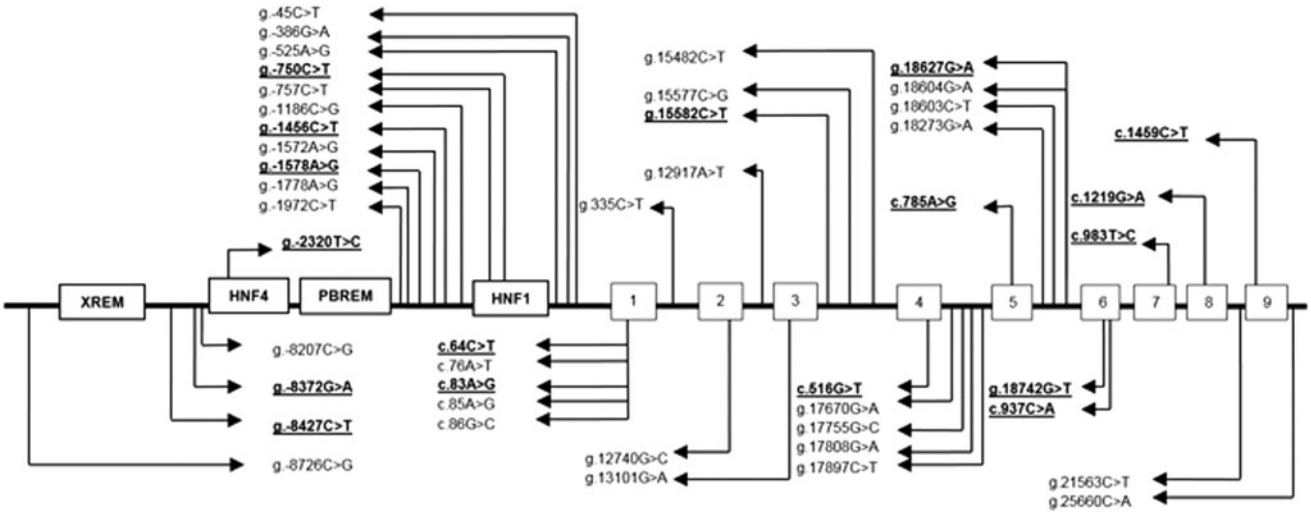


**FIG. 1.** Approximate location of variants identified in and around the *CYP2B6* gene in the South African Black and Cape Mixed Ancestry populations. Exons 1–9 and response elements and nuclear factor–binding sites in the 5′ region are indicated. Variants selected for genotyping are *bold* and *underlined* (Lamba *et al.*[63]). HNF, putative hepatic nuclear factor–binding sites; PBREM, phenobarbital response element; XREM, xenobiotic response element.

abolish the activator protein 1 (AP-1) [TGAmTCA(s/A)nn] and octamer-binding protein (Oct-1) [yrAATnw(G/A)CA] transcription factor sites (Table 2). In addition, a further eight variants were selected for genotyping (Fig. 1 and Table 2) by PCR–RFLP because they have previously been associated with EFV pharmacokinetic parameters[14,21,33] and/or were shown to have a putative functional effect as predicted by *in silico* analyses (Table 2). The variant g.-8427T>C (rs8105382) is predicted to eliminate a glucocorticoid receptor–binding site (T/Cgtgtc)[63] and was, in addition to variants g.-1578C>T (rs12721652), g.-1456T>C (rs2054675), and g.18627G>A (rs12721649), associated with differential EFV pharmacokinetics.[14,33] Both g.-2320T>C (rs7254579) and g.-750T>C (rs4802101) abolish a putative hepatic nuclear factor (HNF)–binding site (Fig. 1) and have an effect on enzyme expression.[63,64] The variant in intron 3, g.15582C>T (rs4803419), was previously shown to be associated with increased plasma EFV levels in a cohort from the United States comprising Caucasian, Black, and Hispanic patients[65] and subsequently repeated in a Black South African cohort.[66] The synonymous variant, g.18742G>T (rs112848152), in exon 6 was previously only identified in an individual from the Khoisan population[37] and was predicted to induce a change in mRNA folding using m-Fold (Supplementary Fig. S1).

Minor allele frequencies (MAF) of the 17 variants, which were genotyped in the entire patient cohort, are shown in Table 2, and LD patterns of these single nucleotide polymorphisms (SNPs) in both populations are shown in Supplementary Fig. S2. The MAF of the remaining variants detected in the SAB ($n = 15$) and CMA ($n = 15$) individuals by bidirectional sequencing analyses are shown in Supplementary Table S5. All variants met the requirements for HWE.

### *Predictors of long-term EFV concentrations*

Median EFV levels in hair were 5.60 ng/mg of hair (IQR 3.93–7.06 ng/mg). One patient was classified as an outlier, with excessively high EFV levels (126 ng/mg), and was excluded from subsequent analyses of EFV levels in hair. None of the demographic or treatment differences between patients were significantly associated with median EFV levels in this cross-sectional analysis. Statistical analyses identified significant associations with *CYP2B6*6* and *CYP2B6*18* in the combined and SAB cohorts, respectively, but not in the CMA population (Table 3 and Supplementary Table S6). In addition, *CYP2B6*17* (rs33980385) was significantly associated with decreased EFV levels in the SAB cohort only ($p = .03$). In the combined cohort, the *CYP2B6*6* allele was associated with increased EFV levels in a recessive manner, as described previously,[61,67,68] with homozygotes demonstrating an estimated 109% (95% CI 70%–158%, $p = .016$) higher EFV levels than noncarriers. *CYP2B6*18* was associated with an estimated 82% (95% CI 12%–195%, $p = .02$) increase in EFV levels. No *CYP2B6*18* homozygotes were detected, but composite carriers of both *CYP2B6*6* and *CYP2B6*18* had the highest median EFV level (16.1 ng/mg, $n = 3$), and their combined estimated effect resulted in EFV exposure 173% (95% CI 83%–308%) higher than in wild-type *CYP2B6*1/*1* homozygotes ($p = .013$). Figure 2 shows the observed distribution of EFV levels in hair samples for selected genotypes in the combined cohort (refer to Supplementary Fig. S3 for these distributions in the two populations separately).

Adjusting the association analyses on *CYP2B6*6* (rs3745274) strengthened the association observed with *CYP2B6*18* (rs28399499) ($p = .0045$) but did not identify further associations in the combined cohort. Similarly, no additional independent associations were identified when the analyses were adjusted on both *CYP2B6*6* (rs3745274) and *CYP2B6*18* (rs28399499) (Table 3) in the combined cohort. Interestingly, correction for *CYP2B6*6* in the SAB cohort identified a significant association with g.-1456T>C (rs2054675) ($p = .03$).

As none of the alleles *CYP2B6*2*, *CYP2B6*3*, *CYP2B6*5*, *CYP2B6*17*, *CYP2B6*31*, and *CYP2B6*32* showed an independent statistically significant effect on long-term EFV levels in hair in the combined cohort, they were categorized

Table 3. *p* Values, Effect Sizes, and 95% CIs for Variants that Were Significantly Associated with EFV Hair Levels

| *Variant* | *Population* | *Adjusted for* | p | *Change in EFV levels (95% CI) per allele* |
|---|---|---|---|---|
| g.-1456T>C (rs2054675) | SAB | *CYP2B6*6* | .0249 | 40% (95% CI 4% to 86%) |
| c.83A>G (rs33980385)[a] (*CYP2B6*17*)[a] | SAB | None | .0251 | −59% (95% CI −81% to −11%) |
| | SAB | *CYP2B6*6* | .0401 | −55% (95% CI −79% to −4%) |
| | SAB | *CYP2B6*6* and *CYP2B6*18* | .0469 | −53% (95% CI −77% to −1%) |
| c.516G>T (rs3745274)[b] (*CYP2B6*6*) | Combined | None | .0289 | 24% (95% CI 2% to 50%) |
| | Combined | Ethnicity | .0331 | 24% (95% CI 2% to 50%) |
| | SAB | None | .0320 | 29% (95% CI 2% to 63%) |
| c.983C>T (rs28399499) (*CYP2B6*18*) | Combined | None | .0152 | 82% (95% CI 12% to 195%) |
| | Combined | Ethnicity | .0155 | 82% (95% CI 12% to 196%) |
| | Combined | *CYP2B6*6* | .0045 | 98% (95% CI 23% to 219%) |
| | SAB | None | .0078 | 129% (95% CI 25% to 318%) |
| | SAB | *CYP2B6*6* | .0048 | 135% (95% CI 31% to 323%) |

[a]All individuals who were carriers of g.83A>G (rs33980385; p.D28G) were also carriers of g.76A>T (rs33973337; p.T26S) and g.85C>A (rs33926104; p.R29S).
[b]All individuals who were carriers of c.516G>T (rs3745274) were also carriers of c.785A>G (rs22793430).
CI, confidence interval.

as ''normal'' with regard to EFV metabolism, and only *CYP2B6*6* and *CYP2B6*18* allelic statuses were used to classify CYP2B6 metabolizer phenotypes (Supplementary Table S7), as in previous studies.[14,30] Metabolizer phenotype predicted EFV exposure ($p = .0002$), with both extensive metabolizers and intermediate metabolizers on average demonstrating an estimated 57% (95% CI 35%–71%) and 55% (33%–69%) lower EFV concentrations in hair, respectively, than poor metabolizers (PMs).

### *Predictors of virological treatment outcome*

Of 120 patients, 104 patients were classified as virological ''responders'' (VL <50 copies/ml) and 16 patients were grouped as ''nonresponders'' (VL >50 copies/ml)[15] (Table 1). None of the demographic or treatment differences between patients were statistically significantly associated with virological treatment outcome in this cross-sectional analysis. Investigation of the effects of time on treatment and EFV levels measured in hair revealed that neither of these clinical variables were statistically significantly associated with virological response. Associations with genetic variants, alleles, and metabolizer phenotypes did not identify any significant associations (Supplementary Table S8).

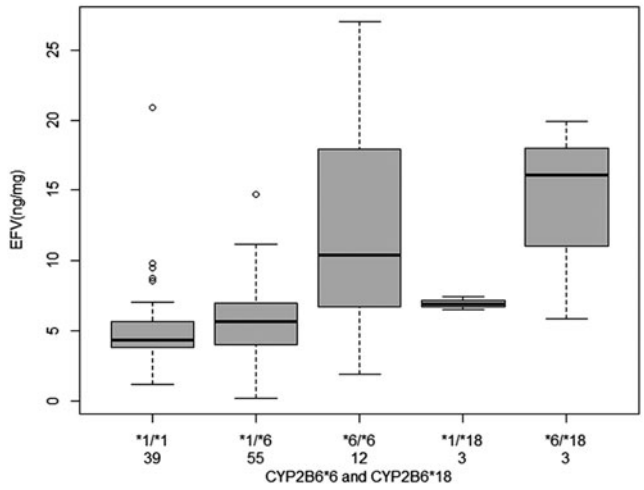


**FIG. 2.** Box plots showing the distribution of EFV levels in hair according to *CYP2B6*6*, *CYP2B6*18*, and *CYP2B6*1* genotype combinations (number of individuals for each genotype combination are given below the genotype combination names). Individuals carrying the *CYP2B6*2*, *CYP2B6*5*, *CYP2B6*6*, *CYP2B6*17*, *CYP2B6*18*, *CYP2B6*31*, and *CYP2B6*32* alleles were excluded from these analyses. EFV, efavirenz.

## Discussion

### *CYP2B6 genetic variation in two South African populations*

This study performed a comprehensive analysis of the influence of the genetic variation present in *CYP2B6* in two South African populations. These analyses highlighted the relatively high frequency of the *CYP2B6*6* and the occurrence of *CYP2B6*18* alleles[39] (Table 2) compared to the scarcity of other variants directly influencing EFV levels. This is of particular interest for pharmacogenetic applications[69] in HIV treatment monitoring and could justify screening for *CYP2B6*6* and *CYP2B6*18* to predict EFV exposure and metabolizer phenotype.

Although the difference was not statistically significant, the frequency of predicted PMs is higher in the SAB (PM = 14%) than in the CMA population (PM = 8%), suggesting that the SAB may be more genetically vulnerable to the clinical consequences of higher EFV levels, such as CNS side effects,[6,8,70,71] and the association results between *CYP2B6*6*/*CYP2B6*18* and EFV hair levels obtained in this study provide further substantiation for these findings. Attrition rates are highest in the first 6 months of EFV-based treatment, coinciding with the time frame during which EFV-induced ADRs occur.[10,11] A recent study in a diverse population showed noninferiority of the 400-mg dose of EFV compared to the typical 600-mg dose in terms of virological outcomes, paving the way for dose adjustment of this

medication when optimal treatment responses are predicted.[72,73] Therefore, the feasibility of improving clinical outcome through early *CYP2B6* genotype-driven dose adjustments, in combination with exposure monitoring via hair levels, should be explored in these two populations of African descent.

Further comparison of these two populations revealed that the *CYP2B6*5* allele, which occurs at low frequencies in Europeans (MAF $\sim$0.12),[25] was completely absent in the SAB cohort but was found at a relatively low frequency in the CMA population (MAF = 0.04), testifying to recent European admixture in this population. In contrast, *CYP2B6*2*, *CYP2B6*6*, *CYP2B6*17*, and *CYP2B6*18* are more frequent, even exclusive, to African populations.[13,25–27,29,31,39] In accordance with this, these alleles were identified in both the SAB (MAF = 0.031, 0.352, 0.019, and 0.037, respectively) and CMA (MAF = 0.019, 0.283, 0.019, and 0.038, respectively) populations in our analysis.

In terms of the spectrum of genetic variation detected in this study, 45 variants were identified in total, of which three were novel (MAF <0.05). This large number of variants, in combination with the presence of rare novel alleles, reflects the genetic diversity of South African populations. This study highlights that rare variants, such as the novel *CYP2B6*31* and *CYP2B6*32* alleles, are likely to be present in African individuals. However, the scarcity of these variants and the lack of information pertaining to the effect that these variants have on CYP2B6 activity may undermine the current feasibility of genotyping such variants in resource-limited settings. Thus, as mentioned above, initial pharmacogenetic tests should screen for *CYP2B6*6* and *CYP2B6*18*.

### *Predictors of long-term EFV levels in hair and virological response*

In accordance with other studies across populations, using plasma[12,21,25,29,74–76] or hair[21] concentrations, both the *CYP2B6*6* and *CYP2B6*18* alleles were associated with increased EFV exposure (Fig. 2) in the combined and SAB cohorts. The lack of statistically significant results observed in the CMA population may be related to the small sample size, in combination with the lower allele frequency of these alleles in this population group. Metabolizer phenotype, classified according to combined presence or absence of these two alleles, was also a statistically significant predictor of EFV hair levels in our study. Interestingly, investigation of the SAB cohort revealed an additional association with *CYP2B6*17* and decreased EFV levels, which may point to a protective effect of this variant. However, as both *in silico* and *in vitro* analyses have not shown this allele to affect CYP2B6 activity,[31] further studies using larger cohorts of well-characterized SAB patients are required to independently replicate these findings.

To further investigate the role of additional variants, association analyses, including *CYP2B6*6* and *CYP2B6*18* as covariates, were performed. Although the inclusion of *CYP2B6*6* strengthened the association of *CYP2B6*18* with EFV, no additional variants were identified in the combined cohort. However, after correcting for *CYP2B6*6* in the SAB cohort, an association with g.-1456T>C (rs2054675) and increased EFV levels were observed. This variant has previously been reported to be associated with EFV levels in an African American and a Haitian population.[14,33]

Investigation of virological outcomes revealed that there were no differences in virological response rates between SAB or CMA patients. Examination of the relevance of genetic variants, or the combinations of genetic variants, on virological treatment response did not detect any significant associations. The lack of significant findings may be attributed to the heterogeneity of the examined cohort and a lack of power to detect associations due to a relatively small sample size. The lack of associations observed for variants that have previously been associated with EFV levels, such as rs8105382, rs12721652, rs12721649,[14,33] and rs4803419,[65,66] may also be attributed to these limitations and may account for the inability of other studies to replicate these subtle findings.[77] With improving sequencing technologies to detect the full spectrum of genetic variation in all relevant genes, the development of more accurate prediction and treatment algorithms that take into account the effect of rare variants or variants with small effect sizes may be a future possibility.[78] Nonetheless, the study has shown that genetic components, particularly *CYP2B6*6* and *CYP2B6*18*, are durable and discerning determinants of exposure.[15,21]

In terms of the collection of hair samples for the investigation of EFV levels, although cultural concerns and beliefs need to be taken into account when collecting hair in different settings, hair specimens were regarded as a feasible and culturally acceptable option for research purposes in this South African cohort[17] as all the women in the current study cohort were willing to donate hair samples. Although we did not collect data on cosmetic hair treatments in this population, work in a large domestic U.S.-based cohort of women has not shown significant differences in ARV hair levels among women whose hair has undergone chemical treatments (M Gandhi, personal communication). Furthermore, hair samples are easy and inexpensive to collect and store, requiring no special skills or phlebotomy, highlighting their value for the measurement of drug levels in resource-limited settings.[20]

### *Limitations of the study*

A significant limitation of this study was the variable timing of the collection of hair samples relative to HIV VLs and the cross-sectional nature of the analysis. VL measurements were taken only at 6-month intervals in this resource-limited setting, so the duration between VL measurement and hair sampling could differ substantially between patients and be up to, but not more than, 6 months. EFV levels in hair in our population, therefore, may not correlate with exposure during the period most relevant to the HIV VL test, limiting our ability to assess the association between hair levels and response. Other limitations of this study include (1) differences in the demographics and treatment histories of the patients, (2) the small number of nonresponders observed (resulting in lower precision of estimates or association), (3) the low frequency of some genetic variants, especially the novel variants, prevented estimation of associations with the current sample size of the cohort, and (4) missing data on baseline VLs for our population, preventing us from including this variable in our models of virological outcome. Furthermore, a *p*-value of .05 was regarded as significant, also

when multiple tests were performed. Use of a smaller value would decrease the probability of detection of false positives but would increase the probability of missing a true effect. In this study, we chose to err on the side of not missing a possible significant effect. Nonetheless, the nonuniformity of the patient cohort shows that even in real-world settings, the effect of genetic modifiers of EFV levels is substantial. Although other variants and genes besides *CYP2B6* are involved in the metabolic pathway of EFV,[21] their effect on long-term EFV exposure was beyond the scope of this study. This study demonstrates that measuring drug levels in hair and genetic testing are potentially feasible in South Africa from a cultural and scientific perspective and may be of use in optimizing treatment outcomes.

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### Author Disclosure Statement

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