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ORIGINAL ARTICLE

Sub- and supratherapeutic efavirenz plasma concentrations with risk for HIV therapy failure are mainly genetically explained in Ugandan children: The prospective GENEFA cohort study

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Aims: Interindividual variations in efavirenz (EFV) plasma concentrations are extensive, but paediatric data on its consequences for viral control are scarce. The aim of this study was to explore the role of genetic variation in achieving therapeutic efavirenz plasma concentrations in a cohort of Ugandan children and the linkage between genetic CYP2B6 variants, EFV plasma variability, viral resistance and viral outcome.

Methods: Ninety-nine treatment-naïve children, aged 3–12 years and living with HIV, were followed for 24 weeks after ART initiation assessing mid-dose efavirenz plasma concentrations, HIV RNA, HIV drug resistance and adherence. Polymorphisms in genes coding for drug-metabolizing enzymes were genotyped. Efavirenz concentrations were determined by liquid chromatography coupled with high-resolution tandem mass spectrometry. Metabolizer phenotype was predicted from composite genotypes of CYP2B6 (c.516G>T and c.983 T>C). A mixed effects restricted maximum likelihood regression model was used to identify important factors for efavirenz exposure.

Results: Efavirenz plasma concentrations were below the therapeutic interval (1000–4000 mg/mL) in 12–17% and above in 21–24% of measurements. Eight children had persisting subtherapeutic concentrations, five of which failed virologically and three acquired at least one new resistant mutation. Multivariate modelling explained 70% of interindividual variation in plasma concentration, with treatment duration, adherence, CYP2B6c.136A>G, and metabolizer phenotype as independent predictors of EFV concentration. In univariate analysis, metabolizer phenotype explained 50% of interindividual variation.

The authors confirm that the Principal Investigator for this observational study is Lars Navér and that he had responsibility for the study patients.

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Conclusions: Metabolizer phenotype explained 50% of interindividual variation in efavirenz plasma concentration. Autoinduction was not confirmed and >33% of the concentrations were outside the therapeutic interval. Subtherapeutic concentrations worsened virological resistance and outcomes. Genotype-based dosing may help avert both sub- and supratherapeutic efavirenz plasma concentrations in Ugandan children.

KEYWORDS
antiretrovirals, children, cytochrome P450 enzymes, genetic polymorphism, HIV/AIDS

1 | INTRODUCTION

Worldwide, approximately 1.5 million children aged 0–14 years lived with HIV in 2022.[1] The number is decreasing due to wider access to antiretroviral therapy (ART) and mother-to-child transmission prevention programmes. The estimated number of children living with HIV (CLWHIV) in Uganda decreased from 100 000 to 88 000 between 2019 and 2021.[2] The non-nucleoside reverse transcriptase inhibitor (NNRTI) **efavirenz** (EFV) combined with two nucleoside reverse transcriptase inhibitors (NRTIs) has been the first-line ART in children and adults, globally recommended by the World Health Organization (WHO).[3] Since 2019, first-line recommendation has been **dolutegravir** together with two NRTIs from 6 years of age.[3] Many low- and middle-income countries (LMIC) have already implemented dolutegravir as first-line therapy. EFV is still an alternative for children above 3 years in Uganda[4] and other LMICs.[5]

The appropriate EFV dosage schedule in adults and children is questioned.[6–12] In adults, attention has been paid to the risk of overdosing[10,11] and a low dose regimen (400 mg day^{-1})[10] was adopted by the WHO as an alternative first-line regimen to reduce the risk for adverse effects. EFV plasma levels above 4000 ng mL^{-1} are associated with an enhanced risk of central nervous system (CNS) toxicity and levels below 1000 ng mL^{-1} with higher rates of viral failure in adults.[13] In children, both under-[6–8] and overdosing[12] using standard dosing schedules have been reported, and some paediatric studies have investigated the impact of EFV plasma levels on virological outcomes.[14–21] In children, modified dosing schemes are lacking due to the more complex dosing regimens and the inherent risks for underdosing of several drugs in children at 6–24 months of age.[22–24]

EFV is primarily metabolized by cytochrome P450 2B6 (**CYP2B6**), with a minor contribution from CYP2A6, CYP3A4, CYP3A5 and UDP-glucuronosyltransferase 2B7.[25–30] Genetic polymorphisms of drug-metabolizing enzymes are associated with significant variations in EFV plasma concentrations in adults and children.[31–41] Several single nucleotide polymorphisms (SNPs) control EFV plasma exposure with marked interethnic variation.[14,25,31,34,38,42–47] Further, auto-induction may contribute to the variability of EFV plasma concentration over time, as documented in adults of different African origins[36,43,48,49] and has been suggested but not verified in a few paediatric studies.[7,35]

What is already known about this subject

- Significant variation in EFV plasma concentrations in adults and children exists due to functional single nucleotide polymorphisms in genes for drug-metabolizing enzymes.
- Pharmacokinetic modelling studies have highlighted the predictive capability of composite CYP2B6 genotypes (c.516G>T/ c.983 T>C/15582C>C) and (c.516G>T/ c.983 T>C) to adapt EFV dosing in children and pregnant women.
- Combined data on pharmacogenetics, viral resistance, EFV variability and its consequences for viral control in children is scarce.

What this study adds

- Metabolizer phenotype predicted by CYP2B6 (c.516G>T/c.983 T>C) accounted for almost 50% of the interindividual variation in EFV plasma concentration suggesting that genotype-based dosing strategies may help to control for the risk of both sub- and supratherapeutic EFV plasma concentrations in Ugandan paediatric patients.
- Previously reported occurrence of autoinduction in children was not confirmed. One-third of the plasma concentration measurements were outside the recommended therapeutic interval.
- EFV concentrations below the therapeutic interval were significantly associated with viral failure and emergence of new HIV drug-resistant mutations. Pre-treatment HIV drug resistance contributed significantly to viral failure and viral resistance development when EFV concentrations were within/above the therapeutic interval.

Genetically guided EFV dosing schedules have been proposed,[15,40,50] where CYP2B6 c.516G>T genotype alone or in combination with other genotypes, is used to estimate an individual's metabolizing capacity/phenotype.[39,40,50,51] Pharmacokinetic modelling has highlighted the predictive capability of the composite CYP2B6 genotypes c.516G>T/c.983 T>C/g.15582C>T[38,39] and c.516G>T/c.983T>C[31,52] to adapt EFV dosing in children and pregnant women. Among Ugandan and Zambian children, four distinct metabolizer phenotypes—extensive (EM), intermediate (IM), slow (SM), and ultra-slow (USM) metabolizer—were identified, based on six composite CYP2B6 genotypes of c.516G>T/c.983T>C.[31]

A number of paediatric studies[7,32,35,41,44,53–55] have explored the role of genetic polymorphisms in EFV plasma concentration variability, some using composite genotypes to estimate individual metabolizing capacity.[31,38,39,51] Others examined the association between low EFV plasma levels and the risk of viral failure,[16–21,56] but did not, with some exceptions,[14,15,40] consider pharmacogenetic data. EFV pharmacokinetic data linked to HIV drug resistance (HIVDR) remain scarce in children from sub-Saharan Africa,[14,56] where over 90% of paediatric HIV cases occur and where both pretreatment and acquired HIVDR are increasing.[57,58] To address all these aspects, we designed a prospective investigation of the impact of genetic as well as non-genetic factors on short- and long-term EFV plasma concentrations using a mixed-effects restricted maximum likelihood regression model in a Ugandan cohort of ART-naïve CLWHIV, aged 3–12 years.

We analysed genetic polymorphisms in CYP2B6, **CYP2A6**, CYP3A5, **ABCB1**, alongside predicted metabolizer phenotype. In addition, we explored the impact of pretreatment HIVDR and EFV plasma levels on viraemia and acquired HIVDR after 24 weeks of standard therapy.

2 | METHODS

2.1 | Study design, setting and clinical care

This is the second publication of the GENEFA project ("The importance of pharmacoGENetic variation on EFAvirenz levels and treatment effects in ART-naïve HIV-infected Ugandan children aged 3-12 years"). GENEFA is a prospective single-centre, observational cohort study, jointly performed by Baylor College of Medicine Children's Foundation Uganda (Baylor Uganda), Makerere College of Health Sciences, Uganda, and Karolinska Institutet, Sweden.

Enrolment and follow-up took place between February 2015 and February 2016 in Baylor Uganda, a facility offering outpatient HIV services to children and their families in Kampala metropolitan area. Participants were eligible if they were HIV-positive, previously ART-naïve, had a body weight ≥10 kg, aged 3–12 years and had no history of treatment with rifampicin, carbamazepine, phenytoin, St Johns Wort or phenobarbitone within 12 weeks before study start, as these drugs may interact with EFV. We screened 120 children and enrolled 99 (Figure 1).

Prescription of EFV tablets followed Ugandan national HIV treatment guidelines, according to the following weight bands: 10–< 14 kg, 14–< 25 kg, 25–< 35 kg and ≥35 kg with once daily doses of 200, 300, 400 and 600 mg, respectively. The two nucleotide reverse transcriptase inhibitors (NRTIs) (abacavir and lamivudine) were administered as fixed drug combination tablets. All children were prescribed co-trimoxazole prophylaxis.

Follow-up visits were scheduled at 2, 6, 12 and 24 weeks post ART initiation. Weight, height, clinical stage,nutritional status according to WHO[59,60] and adherence (measured on a scale of 0–100%, based on pill count) were assessed at each visit.[61] Families were instructed to administer the ART on an empty stomach in the evening (standard recommendation for EFV-based ART). The hour for EFV administration and other oral medication (taken within 2 weeks before EFV sampling) was documented at each visit. Missed appointments prompted a phone call and/or a home visit, to ensure adherence to treatment and follow-up.

Mid-dose plasma samples for EFV measurements were collected in lithium-heparin tubes before ART start and at the follow-up visits 2, 6, 12 and 24 weeks thereafter. The time for blood sampling was recorded. Samples were stored at −80 °C at Baylor College except for 2 weeks at −20 °C, at Makerere University. During the 48-hour transport to Karolinska Institutet and thereafter, the storage temperature was −80 °C.

2.2 | Power calculation

The sample size was calculated using ANOVA with a .05 significance level and 80% power to detect a mean difference of at least 3 μmol/L (947 ng mL^{-1}) in efavirenz concentration among CYP2B6 c.516G>T genotypes (GG, GT, TT). Assuming a within-group standard deviation of 4.7,[62] a minimum of 100 individuals was required, with a distribution of 4.5:4.5:1 among the genotypes.

2.3 | HIV viral load and drug resistance

At baseline and Week 24, viral load (VL) and HIV drug resistance (HIVDR) were analysed in the CFAR Molecular Virology Lab/Resistance Lab of the Joint Clinical Research Centre Kampala as described.[63] The lower limit of detection was 40 copies mL^{-163} and viral failure was defined as VL ≥ 40 copies mL^{-1} after 24 weeks of treatment. Genotypic resistance was assayed by sequencing of the reverse transcriptase region. HIVDR mutations were identified by the 2015 IAS-USA mutation list[64] and further classified according to Stanford HIV Drug Resistance Database (HIVdB).[65]

2.4 | Analysis of efavirenz plasma concentrations

Plasma concentrations of unconjugated EFV were determined using liquid chromatography coupled with high-resolution tandem mass

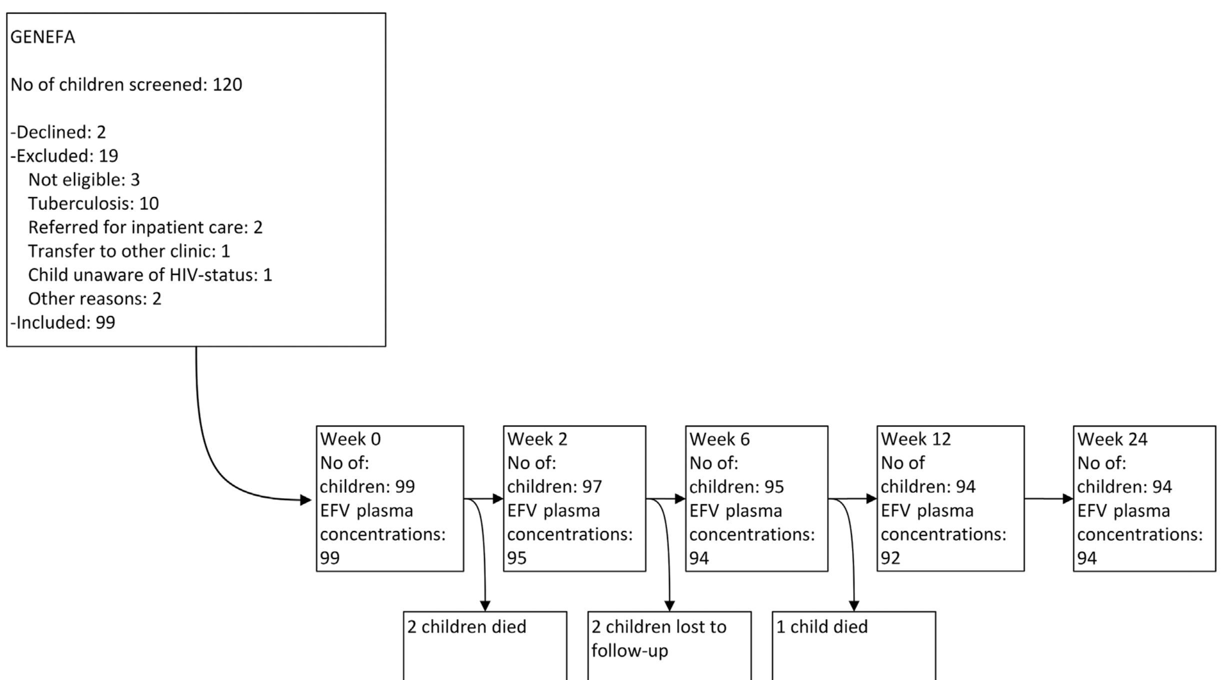

FIGURE 1 GENEFA study flow chart with number of children and successful EFV plasma analyses throughout the study. Ninety-nine ART-naive Ugandan children aged 3–12 years were enrolled and initiated EFV-based antiretroviral therapy, 2015–2016. Mid-dose EFV plasma concentration was sampled before treatment start (Week 0) and 2, 6, 12 and 24 weeks thereafter. One girl aged 8.3 years, classified as an intermediate metabolizer and two boys aged 5.2 and 3.5 years, died. The boys died after their first visit and were not genotyped nor sampled for EFV beyond baseline. The cause of death was severe acute malnutrition (SAM)/HIV-associated nephropathy, SAM, and pneumonia, respectively. One girl aged 10.4 years and one boy aged 3.5 years, both classified as extensive metabolizers, were lost to follow-up before their third visit.

spectrometry (LC-HRMS/MS). The method was developed and validated in our laboratory at the Department of Clinical Pharmacology at Karolinska University Hospital as described.[66] Briefly, samples were prepared by protein precipitation of 0.1 mL plasma and MS detection was performed using negative electrospray mode with a resolution set to 17 500. The transitions used for quantification was m/z 314.0201 > 244.0167 (efavirenz) and 318.0452 > 248.0420 (internal standard, efavirenz-d4) with a lower limit of quantification of 100 ng mL^{-1} efavirenz. Between-run precision (coefficient of variation [CV], %) was between 3.2 and 4.6% and between-run accuracy between −1.0 and 5.3%, while the within-run precision and accuracy were between 1.9–5.1% and −4.2–7.0%.

2.5 | Genotyping

Genomic DNA was isolated from 1.5 mL whole blood collected in EDTA tubes using QIAamp DNA MiniKit (QIAGEN GmbH, Hilden, Germany) in Kampala and stored initially at −80 °C and in −20 °C after transport to Karolinska Institutet. Genotyping was carried out with validated TaqMan assays from Life Biotechnologies (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's guidelines and a StepOnePlus Real-Time PCR system (Life Technologies, Carlsbad, CA, USA). The literature was reviewed for polymorphisms in drug-metabolizing enzyme genes and ABCB1 to identify polymorphisms of potential importance for EFV pharmacokinetics in an African setting. The following 12 polymorphisms in CYP2A6, CYP2B6, CYP3A5 and ABCB1 genes were selected and analysed: CYP2A6 g.-48T>G (rs28399433, C_30634332_10), CYP2B6 g.18492C>T (rs2279345, C_26823975_10), CYP2B6 c.516G>T (rs3745274, C_7817765_60), CYP2B6 c.136A>G (rs35303484, C_33845811_20), CYP2B6 c.983T>C (rs28399499, C_60732328_20) CYP2B6 g.-82T>C (rs34223104, C_27830964_10) CYP2B6 g.15582C>T (rs4803419, C_7817764_10), CYP3A5 g.6986A>G (rs776746, C_26201809_30), CYP3A5 g.14690G>A (rs10264272, C_30203950_10), CYP3A5 g.727131_27132insT (rs41303343, C_32287188_10), ABCB1 c.3435G>A (rs1045642, C_7586657_20) and ABCB1 c.4036T>C (rs3842, C_11711730_20).

2.6 | Classification of metabolizer phenotype

Participants were categorized according to their composite genotype of the two SNPs CYP2B6 c.516G>T and CYP2B6 c.983T>C (Table 2), and thereafter assigned a predicted metabolizer phenotype as extensive metabolizer (EM), 516GG|983TT, intermediate

metabolizer (IM), 516GG|983TC or 516GT|983TT, slow metabolizer (SM) 516GT|983TC or 516TT|983TT as described.[31,52] No ultraslow metabolizer (USM), 516GG|983CC was identified in our population.

2.7 | Data management, quality control and statistical analysis

Data from standardized clinical report forms were entered and managed with REDCap, a secure electronic research database,[67] after quality control. We used the software Stata version 14.2 and 17.0 (StataCorp LLC, TX, USA). Kruskal Wallis (KW) test was used to analyse differences in adherence, EFV plasma concentration across metabolizer phenotypes, and the Wilcoxon rank sum test when investigating gender differences. Pairwise comparisons following the KW test were performed with Holm-adjusted Conover's test. Fisher's exact test was used for testing differences between categorical variables. Wilcoxon signed rank test was performed to test for within-subject changes in EFV levels over time. *P*-values of <.05 were considered significant and were calculated two-sided. A mixed-effects restricted maximum likelihood (REML) regression model[68] was used to describe and correlate age (years), sex, dose (mg kg^{-1}), time from treatment initiation (days), assessed adherence (%), gene polymorphisms and metabolizer phenotype to EFV plasma concentrations. The outcome variable was constituted of $\log_e$ (EFV ng mL^{-1}) EFV plasma concentration. Random intercepts for individuals and random slope for treatment time was entered, while remaining explanatory variables were entered as fixed effects. All explanatory variables were investigated in individual unadjusted mixed models and thereafter in the multivariate mixed model. Bryk/Raudenbush *R*-squared level[69] was used to estimate R^2.

Due to absent adherence estimates in 23 out of 375 plasma measurements, individual mean adherence across all visits was employed in the mixed-effect modelling. Excluding two participants with a single EFV measurement and no adherence data yielded 373 concentrations for the mixed-effect modelling. In 12 samples (after treatment start), EFV was detectable but below the lower limit of quantification (LLOQ, 100 ng mL^{-1}). In order not to disregard the effect of potential polymorphisms leading to increased EFV clearance, the values below LLOQ were assigned an EFV concentration of 99 ng mL^{-1} before $\log_e$ transformation.

2.8 | Ethics

The study was approved by the Regional Ethical Review Board in Stockholm, Sweden (2016/1026–31), Baylor College of Medicine Children's Foundation IRB Texas (H35946), the Ethical Institutional Review Boards of the School of Biomedical Sciences and Higher Degrees, Makerere University College of Health Sciences (SBS-HDREC 174), and Uganda National Council for Science and Technology UNCST (HS1659). Written informed consent was obtained from participants from 8 years of age and all caretakers. Treatment and services given followed standard clinical routines of Baylor Uganda.

2.9 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24.[70,71]

3 | RESULTS

3.1 | Baseline demographics

The baseline characteristics of the 99 included study participants are summarized in Table 1. The NRTI backbone was lamivudine and abacavir, except for zidovudine and lamivudine in one child. Demographics and results from viral resistance analyses have been reported previously.[63]

3.2 | Clinical care and follow-up

A total of 94 children completed 24 weeks of treatment and follow-up. Three children died and two children were lost to follow-

TABLE 1 Pretreatment status of the 99 ART-naive Ugandan children aged 3–12 years who were enrolled and followed for 24 weeks after initiating EFV-based antiretroviral therapy.

Age (years), median (IQR)	6.2 n	(4.2–8.3) %
Sex		
Girls	59	59.6
Boys	40	40.4
WHO clinical stage[59]		
I	12	12.4
II	67	69.1
III	7	7.1
IV	11	11.3
Nutrition and growth characteristics		
Stunting (height for age, all ages)		
No (≥ − 2 SD)	59	59.6
Moderate (≤ − 2 SD and ≥ − 3SD)	19	19.2
Severe (<−3 SD)	21	21.2
Underweight (weight for age, children < 5 years)		
No underweight (≥2 SD)	22	66.7
Underweight (<−2 SD)	11	33.3
Thinness (BMI for age, children ≥ 5 years)		
No thinness (≥ − 2 SD)	58	87.9
Thinness (<−2 SD)	8	12.1
Wasting (weight for height, children < 5 years)		
No wasting (≥ − 2 SD)	31	93.9
Wasting (≤ − 2 SD)	2	6.1

TABLE 2 Genotype, variant allele and predicted metabolizer phenotype frequency distribution.

Variant allele	rs-ID		Variant allele frequency	Genotype	Number	Frequency (%)	Expected enzyme activity
CYP2B6 c.516G>T	rs3745274	C_7817765_60	36.1%	GG	38	39.2%	Reduced
			(T)	GT	48	49.5%	
				TT	11	11.3%	
CYP2B6 c.136A>G	rs35303484	C_33845811_20	0.5%	AA	96	99.0%	Reduced
			(G)	AG	1	1.0%	
				GG	0	0%	
CYP2B6 c.983T>C	rs28399499	C_60732328_20	7.2%	CC	0	0.0%	Reduced
			(C)	CT	14	14.4%	
				TT	83	85.6%	
CYP2B6 g.18492C>T	rs2279345	C_26823975_10	21.6%	CC	60	61.9%	Reduced
			(T)	CT	32	33.0%	
				TT	5	5.1%	
CYP2B6 g.-82T>C	rs34223104	C_27830964_10	5.7%	CC	3	3.1%	Increased
			(C)	CT	5	5.2%	
				TT	89	91.8%	
CYP2B6 g.15582C>T	rs4803419	C_7817764_10	6.7%	CC	86	88.6%	Reduced
			(T)	CT	9	9.3%	
				TT	2	2.1%	
CYP3A5 g.6986A>G	rs776746	C_26201809_30	19.6%	CC	3	3.1%	Reduced
			(C)	CT	32	33.0%	
				TT	62	63.9%	
CYP3A5 g.14690_G>A	rs10264272	C__30203950_10	22.7%	CC	58	59.8%	Reduced
			(T)	CT	34	35.0%	
				TT	5	5.2%	
CYP3A5 g.727131_27132insT	rs41303343	C__32287188_10	7.7%	--	82	84.5%	Reduced
			(A)	A-	15	15.5%	
CYP2A6 g.-48 T>G	rs28399433	C_30634332_10	5.7%	AA	87	89.7%	Reduced
			(C)	AC	9	9.3%	
				CC	1	1.0%	
ABCB1 c.3435G>A	rs1045642	C_7586657_20	9.8%	AA	1	1.0%	Reduced
			(A)	AG	17	17.6%	
				GG	79	81.4%	
ABCB1 c.4036T>C	rs3842	C_11711730_20	20.1%	CC	1	1.0%	Reduced
			(C)	CT	37	38.1%	
				TT	59	60.9%	
Metabolizer phenotype Composite genotype of CYP2B6 c.516G>T and CYP2B6 c.983T>C							
EM		-			28	28.9%	
CYP2B6 (516GG|983TT)					28		
IM					54	55.7%	
CYP2B6 (516GG|983TC)					10		
CYP2B6 (516GT|983TT)					44		
SM					15	15.4%	
CYP2B6 (516GT|983TC)					4		
CYP2B6 (516TT|983TT)					11		

Note: Metabolizer phenotype: Ninety-seven out of 99 participants were categorized according to their composite genotype of the two SNPs CYP2B6 c.516G>T and CYP2B6 c.983T>C and after that assigned a predicted metabolizer phenotype as extensive metabolizer (EM), 516GG|983TT, intermediate metabolizer (IM), 516GG|983TC or 516GT|983TT, slow metabolizer (SM) 516GT|983TC or 516TT|983TT. There was no ultraslow metabolizer (USM) 516GG|983CC in this cohort.

up (Figure 1). Besides co-trimoxazole prophylaxis, reports of current/recent therapy with other oral medications were frequent at Week 2 (Figure S1) but declined with time.

3.3 | Assessment of adherence

The mean adherence per visit was 96–97% (range 52–100%). Individual mean adherence over Weeks 2–24 varied from 80 to 100% (mean of 97%; 4.4 SD). The intraindividual variability of adherence across the study expressed as CV% ranged from 0 to 31% (median 2.4; interquartile range [IQR] 0.6–6%). Adherence did not differ by sex, age or metabolizer phenotype. Individuals with a median EFV concentration below 1000 ng mL^{-1} had a significantly poorer adherence rate compared to those above 1000 ng mL^{-1}, at Week 2, median 93% (IQR; 82–100%) *vs.* median of 100% (IQR; 100–100%) ($P = .034$) and at Week 24, median 96% (IQR; 91–99%) *vs.* median 99 (IQR; 99–100%) ($P = .036$).

3.4 | Predicted metabolizer phenotype and allele frequency distribution

The variant alleles and respective genotype frequency distribution of CYP2A6, CYP2B6, CYP3A5 and ABCB1 and the metabolizer phenotype predicted from composite genotypes of CYP2B6 c.516G>T and CYP2B6 c.983TC among 97 individuals are reported in Table 2. The distribution of the metabolizer phenotype predicted from the composite genotypes of CYP2B6 c.516G>T and CYP2B6 c.983TC for the whole cohort was 28.9% EM, 55.7% IM, 15.4% SM and 0% USM. The distribution of metabolizer phenotype differed significantly between boys (21.1% EM, 50% IM, 28.9% SM) and girls (33.9% EM, 59.3% IM, 6.8% SM) ($P = .013$).

3.5 | Plasma efavirenz concentrations and metabolizer phenotype

None of the pretreatment plasma samples from the 99 participants had detectable EFV. From Week 2 onwards, we successfully quantified 375 mid-dose plasma samples from 97 individuals (including three children who died). The median (IQR) time elapsed between self-reported administration and plasma sampling was 12.9 (11.9–14.8) h. The EFV plasma levels varied 213-fold between measurements (111–23 715 ng mL^{-1}) and up to 115-fold within individuals. The median ratio between intraindividual highest/lowest EFV concentration was 2.

Across the sampling occasions, the proportion of individuals displaying plasma EFV concentration within the recommended therapeutic interval was between 60 and 66%. The corresponding percentage of children with sub- and supratherapeutic EFV levels was 12–17% and 21–24%, respectively. Children with sub−/intra−/supratherapeutic EFV levels had a median age of 4.4, 6.2 and 8.7 years, respectively ($P = .053$). Distribution over different EFV concentration intervals (defined by individual median EFV concentration), varied according to metabolizer phenotype ($P = .000$, Table 3, Figure 2). Significant differences in EFV plasma levels were also found between the metabolizer phenotypes at each visit. Pairwise group comparison showed that SM had significantly higher EFV concentrations than EM and IM across all visits and that the difference between EM and IM was statistically significant at Weeks 6, 12 and 24 (Table 3). Notably, four IM individuals repeatedly exhibited very high EFV concentrations, forming outlier observations as seen in Figure 2.

3.6 | EFV plasma concentrations over time

The median concentration increased between the sampling occasions (Table 3) for the total cohort, as well as for those classified as IM and SM (Figure 2). To investigate possible within-subject variations of EFV over time, the participants' EFV plasma concentration from Week 24 was compared with samples from Weeks 2–12. The EFV concentrations for the entire cohort were higher at Week 24, but statistically significant only when compared to Week 6 ($z = 3.52$, $P = .0003$). Also, in IM and SM individuals, Week 24 concentrations were significantly higher when compared to Weeks 2 ($z = 2.03$, $P = .042$) and 6 ($z = 2.74$, $P = .0056$) for IM and to Week 12 ($z = 2.01$, $P = .035$) for SM. In EM, EFV concentrations were higher at Week 24 compared to Week 6, but lower compared to samples from Week 2 and Week 12 (all n.s.) (Figure 2).

3.7 | EFV plasma concentration and association to viraemia and HIV drug resistance mutations

By Week 24, 72/93 (77%) were virally suppressed.[63] Viraemia was more frequent in children with a median EFV concentration ($P = .013$) or a Week 24 EFV concentration ($P = .025$) below 1000 ng mL^{-1}. Children with a median EFV plasma level below 1000 ng mL^{-1} were more likely to develop new drug-resistant mutations ($P = .035$) (Table 4). Among children with a median EFV plasma level within or above the therapeutic interval, both viral failure and acquisition of new HIVDR mutations were significantly related to pretreatment HIVDR (Table 4). There was no significant difference in the distribution of pretreatment HIV drug resistance in relation to whether participants' median EFV plasma concentration was below or within/above the therapeutic interval (Table S1).

3.8 | Viral outcomes and metabolizer phenotype

HIV drug resistance at baseline, viraemia and acquisition of new HIV drug-resistant mutations at Week 24 were all more frequent among EM and IM compared to SM (n.s.) (Table S2).

TABLE 3 Distribution of metabolizer phenotype over EFV plasma concentration intervals and median EFV concentrations per visit.

Metabolizer classification based on Composite genotypes	Number (%) of children at different concentration intervals (defined by individual median EFV plasma concentrations)				Median EFV concentration (ng mL^{-1}) (IQR) per visit			
	<1000 ng mL^{-1} (%)	1000–4000 ng mL^{-1} (%)	>4000 ng mL^{-1} (%)	N (100%)	Week 2[a]	Week 6[a]	Week 12[a]	Week 24[a]
All	9 (9.3)	66 (68.0)	22 (22.7)	97	2204 (1358–3954)	2003 (1285–3225)	2473 (1536–3761)	2618 (1524–3875)
EM	5 (17.9)	23 (82.1)	0	28	1577 (1181–2185)	1525 (1073–1853)	1909 (1486–2340)	1537 (1058–2196)
516GG|983TT	5 (17.9)	23 (82.1)	0	28				
IM	4 (7.4)	43 (79.6)	7 (13)	54	2120 (1358–3142)	2237 (1197–2977)	2456 (1425–3410	2786 (1767–3294)
516GG|983TC	1 (10)	7 (70)	2 (20)	10				
516GT|983TT	3 (6.8)	37 (84.1)	4 (9.1)	44				
SM	0	0	15 (100)	15	10 052 (7078–12 821)	11 621 (7155–14 961)	9864 (5121–16 260)	12 725 (8192–18 706)
516GT|983TC			4	4				
516TT|983TT			11	11				

Note: Distribution over different EFV concentration intervals varied significantly between metabolizer phenotypes (based on composite genotype of the two single nucleotide polymorphisms CYP2B6 c.516G>T and CYP2B6 c.983T>C (P = .000). [a]Kruskal Wallis equality of population rank test showed statistically significant differences in EFV plasma levels among categories of slow (SM), intermediate (IM) and extensive metabolizers (EM) across the study period: visit 2 (chi^2 = 36.541, P = .0001); visit 6 (chi^2 = 32.525, P = .0001); visit 12 (chi^2 = 34.759, P = .0001) and visit 24 (chi^2 = 44.127, P = .0001).

3.9 | Factors influencing $\log_e$ efavirenz plasma concentration

The association between age, sex, dose, time from treatment initiation, adherence, gene polymorphisms, metabolizer phenotype and $\log_e$ EFV mid-dose concentration were analysed in a restricted maximum likelihood regression model (Table 5). Both IM and SM phenotype were significantly associated with higher $\log_e$ EFV plasma concentration compared to EM (P = .03 and .00, respectively).

Two more variants in CY2B6 displayed a significant correlation to $\log_e$ EFV plasma concentrations. Homozygosity for CYP2B6 15582C>T predicted lower $\log_e$ EFV, while heterozygosity in CYP2B6 c.136A>G predicted higher $\log_e$ EFV plasma concentration. The latter SNP occurred in only one participant (id. 79), who despite being predicted as an IM (composite genotype 516GG|983TC), maintained very high EFV plasma levels (across study median of 12 539 ng mL^{-1}, range 11 163–23 715 ng mL^{-1}). No significant association of the polymorphisms in CYP2A6, CYP3A5 and ABCB1 with $\log_e$ EFV plasma concentration was found in the multivariate analysis.

Time from treatment initiation and mean adherence were significantly linked to increased $\log_e$ EFV plasma concentration in both univariate and multivariate analysis. Age had a significant positive association in univariate analysis only. Sex did not predict $\log_e$ EFV plasma concentration. Visual inspection of plotted residuals indicated an acceptable fit of the multivariate model (Figure S2). Sensitivity analyses with multivariate REML were performed first by excluding participants with repeated outlier observations (id. 37, 53 and 79) and secondly by replacing EFV concentrations measured as 0 with 50 ng mL^{-1} (instead of 99). Both sensitivity analyses identified adherence and metabolizer phenotype as significantly positive predictors of $\log_e$ EFV plasma concentration, while time on treatment was indicated as a positive predictor, but only significantly so in the first analysis.

The multivariate REML regression model explained, according to Bryk/Raudenbush R-squared Level 1, 1.04% of the intraindividual variation and, according to Bryk/Raudenbush R-squared Level 2, 70.1% of the interindividual variation. In univariate mixed models, the corresponding Bryk/Raudenbush R-squared Level 1 and Level 2 were 0.03% and 49.9% for metabolizer phenotype, 0% and 3.22% for CYP2B6 c.136A>G and −0.06% and 12.8% for CYP2B6 g.15582C>T), respectively.

4 | DISCUSSION

This prospective cohort study of previously ART-naïve children living with HIV (CLWHIV) in Uganda, aged 3–12 years, showed that both genetic and non-genetic factors affected EFV plasma concentration. Metabolizer phenotype based on CYP2B6 c.516G>T|CYP2B6 c.983T>C accounted for almost 50% of the interindividual variation. Standard dosing schemes based on age and weight were suboptimal, as more than a third of the children did not achieve adequate plasma EFV exposures. EFV concentrations below 1000 ng mL^{-1} were

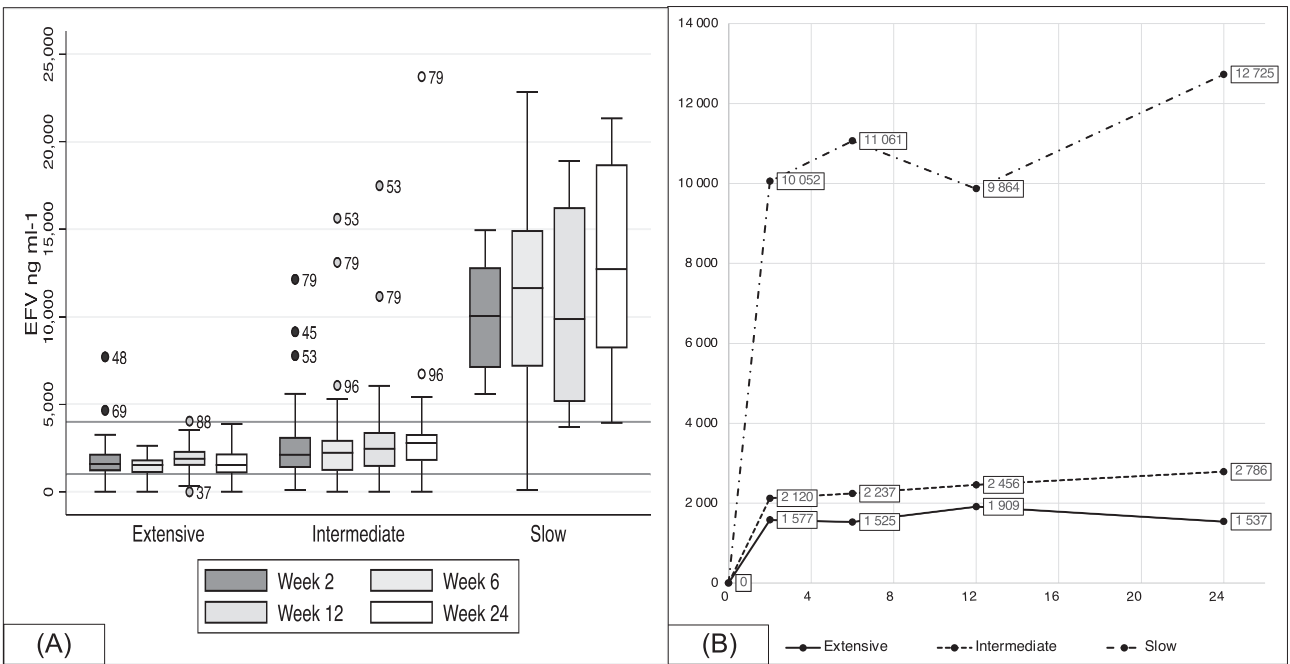

FIGURE 2 Efavirenz plasma concentration by metabolizer type in Ugandan children aged 3–12 years. Extensive (EM), intermediate (IM) and slow metabolizer (SM) type based on composite genotype of CYP2B6 516G>T/983T>C, with 28, 54 and 15 children in each group. In total, 97 individuals contributed to 375 EFV plasma samples with 95, 94, 92 and 94 samples at Weeks 2, 6, 12 and 24, respectively. A: EFV concentrations (ng mL^{-1}) Weeks 2–24. Lines inside box denote median, while lower and upper box boundaries represent 25th and 75th percentiles, respectively. Data points more than 1.5 box-lengths away from 25th or 75th percentiles are represented by dots, marked with study ID. Horizontal lines display therapeutic EFV plasma concentration interval of 1000–4000 ng mL^{-1}. B: Median EFV concentration per visit and metabolizer type, by weeks from therapy start. EFV concentrations (ng mL^{-1}) on *y*-axis and weeks on *x*-axis.

significantly associated with viral failure and emergence of new HIVDR mutations after 6 months of EFV-based therapy.

The adherence was generally good, but it had a significant independent effect on EFV plasma concentrations. As the model used individual mean adherence, this effect reflected the interindividual rather than the intraindividual differences.

Change in plasma EFV exposure over time due to autoinduction has been indicated in adults within 4 months of therapy and considered clinically relevant for EM but of marginal importance for individuals with slower metabolic capacity.[33,48,49] A similar pattern of autoinduction was recently suggested but not verified in an Ethiopian paediatric longitudinal population pharmacokinetic study,[35] where children with the genotypes CYP2B6 516 GG (EM) and CYP2B6 516 GT (IM) displayed a non-significant increase of EFV clearance between the first and eighth weeks. Increased clearance over the first 2 weeks was observed in a small Dutch paediatric cohort, among CYP2B6 516 GG classified subjects.[7] In contrast, the median EFV plasma concentration in our larger study remained stable for the EM group but increased for both IM and SM. The within-subject comparisons between EFV concentrations after 24 weeks of treatment implied decreasing, but not statistically significant, EFV levels with time in EM. The mixed effects REML regression model showed that time on ART had an independent minor positive effect on EFV plasma concentrations, suggesting that autoinduction had little or no effect on EFV variability in this cohort of Ugandan CLWHIV.

Few studies have explored the relationship between EFV plasma concentrations and HIVDR in children. One study in ART-naïve children observed no correlation between EFV levels and the emergence of new drug-resistant mutations, but authors suggested the study to be underpowered.[14] In our larger study, as many as four out of ten efavirenz plasma concentrations were outside the recommended therapeutic plasma concentration interval (1000–4000 ng mL^{-1}) (Table 3) and this significantly influenced viral suppression (Table 4). We show that the risk of poor viral control is high with subtherapeutic EFV concentrations since nearly two-thirds of these participants experienced viral failure and two-fifths acquired at least one new drug-resistant mutation. In contrast, less than one-fifth of children with EFV measurements above 1000 ng mL^{-1} displayed viraemia and 9% developed new drug-resistant mutations, and both these outcomes were significantly associated to pretreatment HIVDR.

We confirmed that CYP2B6 c.516G>T is a frequent SNP in Ugandans, with an allele frequency of 35%.[31,34] Based on the composite CYP2B6 (c.516G>T|c.983T>C) genotype, 28.9% of our cohort was classified as EM, 55.7% as IM and 15.5% as SM. Bienczak et al. reported 33.1%, 40% and 25.5% of children with Ugandan and Zambian origin to match EM, IM and SM status, respectively.[31] Boys only accounted for 40% of the cohort at large but were overrepresented among SM. Nearly 50% of the interindividual variation of EFV plasma concentration exposure was explained by the CYP2B6 (c.516G>T|c.983T>C)-predicted metabolizer phenotype.

TABLE 4 Association between EFV concentrations, pretreatment HIV drug resistance and viral outcomes (viraemia and new HIV drug resistance mutations at Week 24).

Panel A: Viral outcomes and individual median/Week 24 EFV concentration below and within/above therapeutic interval

		Viraemia (VL ≥ 40 Copies ml^{-1})	No viraemia (VL < 40 Copies ml^{-1})	*P*-value		New DRM	No new DRM	*P*-value
Individual EFV median concentration								
n = 8	Subtherapeutic (<1000 ng mL^{-1})	5 (62.5%)	3 (37.5%)		*n* = 7	3 (42.9%)	4 (57.1%)	
n = 85	Therapeutic-Supratherapeutic (≥1000 ng mL^{-1})	16 (18.8%)	69 (81.2%)		*n* = 85	8 (9.4%)	77 (90.6)	
				.013				.035
EFV concentration at Week 24								
n = 12	Subtherapeutic (<1000 ng mL^{-1})	6 (50%)	6 (50%)		*n* = 12	3 (25%)	9 (75%)	
n = 81	Therapeutic-Supratherapeutic (≥1000 ng mL^{-1})	15 (18.5%)	66 (81.5%)		*n* = 80	8 (10%)	72 (90%)	
N = 93				.025	*N* = 92			.153

Panel B: Viral outcomes and pretreatment HIV drug resistance according to individual median EFV concentration below or within/above therapeutic intervals

		Viraemia (VL ≥ 40 copies mL^{-1})	No viraemia (VL < 40 copies mL^{-1})	*P*-value		New DRM	No new drug mutations	*P*-value
Individual EFV median concentration ≥1000 ng mL^{-1}								
n = 16	Pretreatment DRM	7 (43.8%)	9 (56.2%)		*n* = 16	11 (68.8%)	5 (31.2%)	
n = 61	No pretreatment DRM	8 (13.1%)	53 (86.9%)		*n* = 61	3 (4.9%)	58 (95.1%)	
N = 77				.006	*N* = 77			.008
Individual EFV median concentration <1000 ng mL^{-1}								
n = 3	Pretreatment DRM	1 (33.3%)	2 (66.7%)		*n* = 2	1 (50%)	1 (50%)	
n = 8	No pretreatment DRM	5 (62.5%)	3 (37.5%)		*n* = 5	2 (40%)	3 (60%)	
N = 11				1.00	*N* = 7			1.00

Note: Drug resistance mutations (DRM) and viral load (VL) were investigated at baseline and Week 24. HIV drug resistance (HIVDR) was classified according to the Stanford HIV Drug Resistance Database (HIVdB). HIVDR was defined as the presence of DRMs known to confer *any* level of impaired susceptibility to any NRTI/NNRTI.

Results for the SNP CYP2B6 g.15582 C>T were ambiguous, contradicting previous findings that linked this polymorphism to increased EFV plasma exposure.[37,38] Homozygosity for this SNP was observed in two participants and predicted decreased EFV concentration in the multivariate analysis. However, other factors may explain their low EFV exposure: both were EM and in one, EFV was consistently undetectable, suggesting adherence issues (although pill count indicated otherwise). In contrast, participant 79 (Figure 2) with the 516GG|983TC composite genotype (IM), exhibited unexpectedly high concentrations of EFV and was also the only carrier (heterozygous) for CYP2B6 c.136A>G which was indicated as a predictor for increased EFV levels in the REML model. Similarly, among ethnically mixed adults, an IM with heterozygosity for 136A>G showed notably higher plasma exposure to EFV.[72] This supports earlier findings that this rare variant allele could contribute to elevated EFV plasma levels.[33,41] However, we did not perform an exhaustive genomic analysis and may therefore have neglected the influence of other functional SNPs.

Previous findings of linkage between the polymorphism CYP2B6 g.18492T>C and moderately increased EFV clearance,[46,73,74] CYP2B6 g.-82T>C (CYP2B6*22) and enhanced liver metabolism of EFV[75,76] or CYP2A6 g.-48T>G and increased EFV levels in children[41] were not confirmed in our cohort. Further, the REML model did not identify any additional predictors of $\log_e$ EFV plasma concentration among the CYP3A5 and ABCB1 polymorphisms.

Identifying factors affecting EFV plasma exposure is essential to minimize the risk of treatment failure and adverse reactions.

TABLE 5 Association between log(e) transformed EFV plasma concentrations, genetic polymorphisms, metabolizer phenotypes, and other covariates.

Variables					Univariate mixed model				Multivariate mixed model			
Enzyme	Variant allele	rs-ID	Genotype	Metabolizer phenotype	Coefficient	*P* > |z|	[95% Conf. interval]		Coefficient	*P* > |z|	[95% Conf. interval]	
CYP2B6	c.516G>T/c.983 T>C	3 745 274/28399 499	Composite genotypes	**IM**	0.45	0.008	0.12	0.79	0.42	**0.033**	0.35	0.80
				SM	2.03	0.000	1.57	2.49	1.94	**0.000**	1.39	2.50
	g.18492T>C	2 279 345	CT	-	0.19	0.68	−0.73	1.13	−0.15	0.64	−0.79	0.48
			TT	-	0.49	0.29	−0.41	1.39	−0.30	0.38	0.97	0.37
	g.82T>C	34 223 104	CT	-	−0.42	0.36	−1.33	0.48	−0.07	0.83	−0.69	0.55
			CC	-	−0.65	0.28	−1.83	0.52	0.05	0.93	−0.90	0.99
	g.15582C>T	4 803 419	CT	-	0.26	0.43	−0.38	0.89	0.29	0.29	−0.24	0.81
			TT	-	−2.37	0.00	−3.67	−1.09	−1.93	**0.00**	−2.87	−1.00
	c.136A>G	35 303 484	**AG**	-	1.87	0.06	−0.05	3.79	1.68	**0.01**	0.40	2.96
			-	-								
CYP3A5	g.6986A>G	776 746	CT	-	0.28	0.19	−0.14	0.71	0.21	0.16	−0.081	0.50
			TT	-	0.37	0.53	−0.78	1.52	0.21	0.58	−0.54	0.97
	g.14690G>A	10 264 272	CT	-	0.42	0.05	0.00	0.83	0.17	0.27	−0.13	0.47
			TT	.-	0.13	0.77	−0.76	1.03	0.26	0.41	−0.36	0.88
	g.727131_27132_insT	41 303 343	A-	-	−0.57	0.04	−1.12	−0.04	−0.33	0.13	−0.75	0.10
CYP2A6	g.-48T>G	28 399 433	AC	-	−0.04	0.91	−0.72	0.65	0.01	0.95	−0.43	0.46
			CC	-	−0.07	0.95	−2.05	1.94	0.27	0.68	−1.02	1.56
ABCB1	c.3435C>T	1 045 642	AG	-	0.05	0.85	−0.47	0.57	−0.08	0.64	−0.43	0.27
			AA	-	−0.09	0.93	−2.03	1.86	0.80	0.29	−0.67	2.27
	c.4036A	3842	CT	-	−0.25	0.23	−0.65	0.16	0.021	0.88	−0.25	0.30
			CC	-	−0.81	0.41	−2.74	1.12	−0.60	0.34	−1.84	0.63
Age at treatment start (years)					0.11	0.004	0.03	0.18	0.05	0.08	−0.01	0.11
Treatment duration (days)					0.001	0.04	0.00	0.002	0.001	**0.037**	0.0001	0.002
Dose/weight (mg kg^{-1})					−0.03	0.18	−0.08	0.02	0.001	0.95	−0.05	0.05
Mean Adherence (%)					0.07	0.003	0.02	0.11	0.04	**0.014**	0.01	0.07
Sex (ref. female)					−0.45	0.03	−0.84	−0.05	0.11	0.49	−0.19	0.41
Constant					n/a	n/a	n/a	n/a	2.88	0.07	−0.26	6.01

Note: The association between EFV plasma concentration, genetic polymorphisms, predicted metabolizer phenotype and other covariates were investigated in a mixed-effects restricted maximum likelihood (REML) regression model. EFV plasma concentration was log(e) transformed to normalize the distribution. The analysis included 373 EFV plasma concentrations from 95 children sampled at 2, 6, 12 and 24 weeks from antiretroviral therapy start.

Genotyped-based dosing strategies may help to control for both sub- and supratherapeutic EFV plasma concentrations. Yet, in the presence of HIV drug resistance, therapeutic EFV levels may not prevent viral failure, as demonstrated in our cohort, where the prevalence of pretreatment NNRTI drug resistance was 20%.[63]

Our study had several strengths. We examined both pre-existing and emerging viral resistance in relation to pharmacogenetics, EFV plasma levels and viraemia. The study design was prospective and included a high number of repeated EFV concentration measurements. Additionally, our study achieved a thorough follow-up with a low lost-to-follow-up rate. The relatively short follow-up period is a limitation. Another limitation is the uncertainty of EFV intake, due to unobserved drug administration and adherence that to a varying degree is captured by pill count. Features of symptomatic HIV disease such as malnutrition, infections and co-therapy with other medications might also influence the administration, uptake and metabolism of EFV. This could lead to inaccurate assessments of the impact of genetic and non-genetic covariates on mid-dose efavirenz levels.

In conclusion, duration of therapy, adherence and metabolizer phenotype based on composite genotypes of CYP2B6 c.516G>T|CYP2B6 c.983T>C predicts EFV plasma exposure among Ugandan CLWHIV. Changes in EFV plasma exposure due to autoinduction were not observed. More than 33% of the measurements were outside the recommended EFV plasma concentration interval, and concentrations below the interval resulted in worse virological control and increased risk for viral drug resistance. Poor virological outcome was also observed with EFV exposures within therapeutic range in the presence of pretreatment drug resistance. Multivariate modelling explained 70% of the interindividual variation of EFV plasma concentrations, and in univariate analysis, metabolizer phenotype explained 50% of this variation, which supports that adapted EFV dosing schedules should take into account metabolizer phenotype based on composite genotypes of CYP2B6 c.516G>T|CYP2B6 c.983T>C.

AUTHOR CONTRIBUTIONS

Sandra Soeria-Atmadja (SSA), Jaran Eriksen (JE), Johanna Rubin (JR), Lars L. Gustafsson (LLG), Adeodata Kekitiinwa (AK), Celestino Obua (CO) and Lars Navér (LN) designed the study. Pauline Amuge (PA), Dickson Bbuye (DB), Sarah Nanzigu (SN), SSA, JE and LN participated in the acquisition of data. Marja-Liisa Dahl (MLD) participated in the genotyping and interpretation of the analytical data. Madeleine Pettersson Bergstrand (MPB) and Anton Pohanka (AP) performed the efavirenz analyses and participated in the interpretation of the analytical data. SSA, LLG and LN drafted the manuscript. SSA, JE, JR, LLG and LN participated in data analysis. SSA, PA, SB, SN, JE, JR, AK, CO, LLG and LN participated in the interpretation of data and critically revised the manuscript. All authors have read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed during the current study are not publicly available due to data protection regulations but are available from the corresponding author upon reasonable request.

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