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Plasma levels of efavirenz and frequency of the CYP2B6 516G>T polymorphism in people living with HIV-1 in Mexico

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Efavirenz (EFV) is a widely used antiretroviral, due to its safety, efficacy, and low cost. However, plasma concentrations have been related with an increased risk of virological failure and the appearance of serious adverse reactions. EFV is metabolized by Cytochrome P450, the main isoenzyme involved is CYP2B6 and the most relevant genetic polymorphisms found in several populations has been the CYP2B6 516G> T. The aim of this study was to identify the frequency of the *CYP2B6* 516G>T polymorphism and its effect on the plasma concentration of efavirenz (EFV) in a group of people living with HIV (PLWH) and undergoing EFV treatment in Morelos, Mexico. Ninety-six PLWH undergoing EFV treatment, at a daily dose of 600 mg orally in combination with other antiretrovirals (ARVs), were included in this study. The *CYP2B6* 516G>T polymorphism was detected using PCR-RFLP. The plasma concentrations of EFV were evaluated by high-resolution liquid chromatography coupled to a mass-mass detector, using a protein precipitation method. The median plasma EFV concentration was 4.6 µg/mL (IQR = 4.64) and 64.6% of the subjects had concentrations above the therapeutic range. The *CYP2B6* 516G>T genotype findings were as follows: 46.9% of the population presented the wild-type genotype (GG), while 45.8 % and 7.3 % showed the heterozygote (GT) and the polymorphic homozygote (TT) genotype, respectively. The homozygote G had the lowest plasma concentrations of EFV (median = 4.1 µg/mL and IQR = 1.7 µg/mL), followed by those with the GT genotype (median = 5.1 µg/mL and IQR = 3.0 µg/mL). Participants with the homozygous T genotype had the highest EFV concentrations (median = 9.7 µg/mL and IQR = 5.8 µg/mL). In conclusion, the *CYP2B6* 516G>T polymorphism was associated with plasma levels of EFV in PLWH undergoing ARV treatment. EFV plasma concentrations at 600mg doses were outside the therapeutic range in most subjects

1. Introduction

Efavirenz (EFV) is a non-nucleoside reverse transcriptase inhibitor drug used to treat HIV infection. It is usually administered as a tablet co-formulated with emtricitabine (FTC) and tenofovir (TDF) and has the advantage of being administered once a day (Apostolova et al. 2015). In recent years, the recommended guidelines for the initiation of antiretroviral (ARV) therapy have changed. The World Health Organization guidelines recommend EFV at low dose (400 mg) as one of the first-line option in combination with TDF + Lamivudine (3TC) for adults and adolescents (World Health Organization 2021).

In Mexico, EFV is included in various ARVs combinations in adults with HIV without previous treatment. (CENSIDA 2019) Thus, EFV continues to be administered frequently when access to the first-line ARV drug regimen is not possible. Also, a significant proportion of HIV patients are still on EFV at 600 mg dose regimen (CENSIDA. Secretaría de Salud, 2021).

EFV has a high therapeutic efficacy; however, it has been observed to have significant inter-individual variability in its response (Gunda et al. 2013), which usually results in a plasma concentration between 1 and 4 µg/mL. However, plasma concentrations below 1 µg/mL (subtherapeutic concentrations) have been observed and increase the risk of selective resistance, thereby resulting in ARV treatment failure. In contrast, subjects with plasma concentrations of EFV greater than 4 µg/mL (supratherapeutic concentrations) may have serious neurological adverse effects, such as depression, suicidal tendencies, or psychosis, and may require discontinuation of the drug (Marcellin et al. 2008; Marzolini et al. 2001; van Rensburg et al. 2021). EFV metabolism is mainly hepatic, through cytochrome P450 (CYP450) The main isoenzyme involved is CYP2B6, which transforms EFV into hydroxylated metabolites that subsequently undergo a glucuronidation process (McDonagh et al. 2015). A polymorphism of the *CYP2B6* gene has been described in codon 516 (*CYP2B6* 516G>T), where there may be a substitution of G to T; this refers to a change from the G / G genotype (wild type) to the G / T genotype (heterozygous) or the T / T genotype (homozygous), among which the T/ T genotype is associated with higher plasma concentrations of EFV and an increased risk of toxicity (Rodríguez-Nóvoa et al. 2006; Aurpibul et al. 2014; Dhoro et al. 2015).

The frequency of the *CYP2B6* 516G> T polymorphism varies in different populations. The frequency of the homozygous T genotype is reported to be 6% in the Caucasian population, 8.5% in the Asian population, and 16.8% in the African population (Ayuso et al. 2019).

In Mexico, there is no information related to the frequency of the *CYP2B6* 516G> T polymorphism in PLWH undergoing EFV treatment and very few studies have evaluated plasma levels of EFV. The aim of this study was to determine the frequency of this polymorphism and its effect on the plasma concentration of EFV.

2. Investigations and results

2.1. General and clinical characteristics of the PLWH

The general characteristics of the population are shown in Table 1. Of the participants, 83.3% were males. The median age of this population was 35 years (IQR= 17) A total of 85.5% of the participants were treated with FTC+TDF+EFV and 14.6% were treated with 3TC +abacavir (ABC) + EFV. The median plasma EFV concentration was 4.6 µg/mL (IQR= 4.64), with no differences between males and females (median = 4.6 µg/mL and IQR = 2.9 µg/mL vs. median = 4.7 µg/mL and IQR = 2.9 µg/mL, respectively; *p*=0.429) The intersubject coefficient of variation was 54.37%. The genotype prevalence observed for the *CYP2B6* 516G>T polymorphism in this population was as follows: the wild-type genotype (GG), the heterozygote genotype (GT), and the homozygote genotype (TT) were found in 46.9%, 45.8% and 7.3% of PLWH, respectively.

Table 1: General and clinical characteristics of the study population

Characteristic	n (%)	Median (IQR)
Age (years)		35 (17)
Sex		
Men	80 (83.3)	
Women	16 (16.7)	
BMI status		
Normal	52 (54.2)	
Overweight	32 (33.3)	
Obese	12 (12.5)	
CD4 (cells/mm^3)		
< 200	11 (11.5)	
200-350	15 (15.6)	
>350	70 (72.9)	
HIV1-RNA (copies/ml)		
<40	80 (83.3)	
≥40	16 (16.7)	
EFV plasma concentration (µg/mL)		
< 1	4 (4.2)	
1-4	30 (31.3)	
>4	62 (64.6)	
Genotypic frequency		
GG	45 (46.9)	
GT	44 (45.8)	
TT	7 (7.3)	
Allelic frequency		
Allele G	69.8	
Allele T	30.2	

IQR: Interquartile Range, BMI: body mass index, HIV1, human immunodeficiency virus type 1; EFV: Efavirenz.

2.2. Efavirenz plasma concentration and genotype

The Fig. shows that participants with the wild-type homozygote G had the lowest plasma concentrations of EFV (median = 4.1 µg/mL and IQR = 1.7 µg/mL), followed by those with the GT genotype (median = 5.1 µg/mL and IQR = 3.0 µg/mL) Participants with the homozygous T genotype had the highest EFV concentrations (median = 9.7 µg/mL and IQR = 5.8 µg/mL) (4.1 µg/mL [IQR = 1.7 µg/mL] versus 5.1 mg/ mL [IQR, 3.0 µg/mL]; p=0.004 and 4.1 µg/mL [IQR = 1.7 µg/mL] versus 9.7 mg/ mL [IQR, 5.8 µg/mL]; p< 0.001) (Fig. 1)

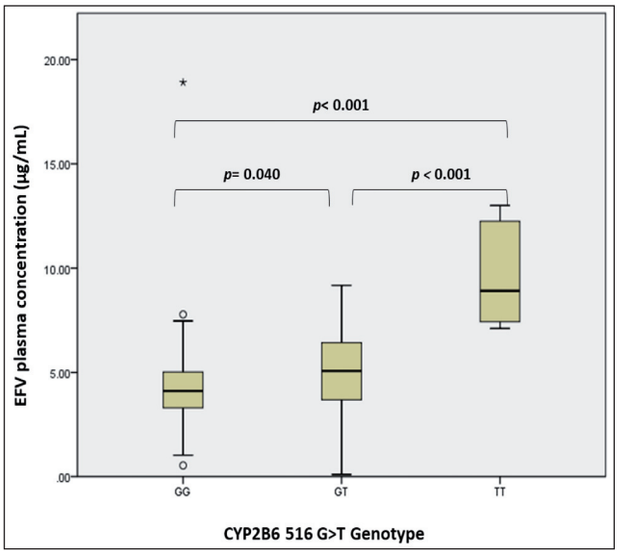

Fig. 1: Median efavirenz plasma levels according to the *CYP2B6* 516 G>T genotype. GG: homozygote genotype (n = 45; median = 4.1; IQR = 1.79); GT: heterozygote genotype (n = 44; median = 5.0; IQR = 2.86); TT: homozygote variant genotype (n = 7; median = 8.9; IQR = 5.82). Statistical analysis was performed using Kruskal–Wallis nonparametric test.

Of the 96 subjects included in this study, the supratherapeutic (>4 µg/mL), therapeutic (1–4 µg/mL), and subtherapeutic (<1 µg/mL) plasma EFV concentrations were observed in 64.5%, 31.3% and 4.2% of participants, respectively. There were no differences between plasma concentrations of EFV based on age, height, sex, and CD4 cell variables.

Table 2 shows that 16.7% and 12.9% of the subjects with therapeutic and supratherapeutic plasma concentrations presented higher values than 40 HIV-1 RNA copies/mL, while 75% of the participants were in the group with subtherapeutic concentrations (p=0.005)

Although there were no statistically significant differences between the duration of treatment with EFV and plasma EFV concentrations, it was observed that people with higher plasma levels (>4 µg/mL) had longer EFV treatment durations than people with plasma EFV less than 4 µg/mL.

To determine whether the *CYP2B6* 516 G>T polymorphism is a risk factor for supratherapeutic plasma concentrations (>4 µg/mL), the odds ratio (OR) was determined by grouping those subjects who presented at least one polymorphic allele (GT+TT) on one side and those subjects with plasma concentrations <4 µg/mL on the other side (Table 3). The GT+TT genotype was found to increase the risk of developing plasma concentrations of EFV greater than 4 µg/mL by 2.5 times ($CI_{95\%}$= 1.0-6.04, p=0.030)(data not shown). To avoid a misinterpretation of the OR that can lead to an overestimation of the risk, the RR was also calculated. Table 3 shows a RR= 1.6 ($IC_{95\%}$ =1.04-2.004)

3. Discussion

This is the first study in Mexico to evaluate the frequency of the *CYP2B6* 516 G>T polymorphism and its relationship with plasma levels of EFV in PLWH undergoing ARV treatment. It has been described that the frequency of the homozygous T genotype varies according to ethnicity, and in Caucasians and black populations, it has been found to occur at frequencies of 6% and 16%, respectively (Powers et al. 2009). In Asians, the reported frequency ranges from 6% to 14% (Aurpibul et al. 2014; Meng et al. 2015); however, few studies have investigated the frequency among hispanic populations. Notably, Haas et al. (2014) reported a frequency of

Table 2: Comparison of the characteristics of people with HIV and their plasma EFV concentrations

	EFV plasma levels			
	Subtherapeutic < 1 µg/mL n = 4	Therapeutic 1- 4 µg/mL n = 30	Supratherapeutic > 4 µg/mL n = 62	*p*
	Mean (IQR)	**Mean (IQR)**	**Mean (IQR)**	
Age (years)	33.8 (13)	36.1 (21)	37.8(16)	0.669
Sex				
Female	2 (50)	5 (16.7)	9 (14.5)	0.182
Male	2 (50)	25 (83.3)	53 (85.5)	
Height (cm)[a]	162.5 (140.3-184.6)	166 (162.7-169.3)	164.2 (161.9-166.4)	0.586
EFV duration (months)	27 (81.5)	29.7 (49.2)	38.5 (62)	0.152
	n (%)	**n (%)**	**n (%)**	
CD4 (cells/mm^3)				
< 200	1 (25)	4 (13.3)	6 (9.7)	
200-350	0	4 (13.3)	11 (17.7)	0.770
>350	3	22 (73.3)	45 (72.6)	
HIV-1 RNA (copies/mL)				
< 40	1 (25)	25 (83.3)	54 (87.1)	
≥ 40	3 (75)	5 (16.7)	8 (12.9)	0.005
CYP2B6 516G>T Genotype				
GG	1 (25)	20 (66.7)	24 (38.7)	0.047
GT	3 (75)	10 (33.3)	31 (50)	
TT	0	0	7 (11.3)	
Adherence				
Yes	3 (75)	23 (76.7)	49 (79)	0.766
No	1 (25)	7 (23.3)	13 (27)	

[a] Normal distribution (Mean, 95%CI). IQR: interquartile range EFV; efavirenz.

Table 3: Relative risk determination of supratherapeutic concentrations according to the GG vs GT+TT genotype

Genotype	< 4 µg/mL n=34 (%)	>4 µg/mL n=62 (%)	RR* (95% CI)	*p*
GG	21 (61.8)	24 (38.7)		
GT+TT	13 (38.2)	38 (61.3)	1.6 (1.04-2.004)	0.030

CI: confidence interval. *Relative Risk

6.7% in hispanic subjects, whereas a frequency of 20% has been reported in Chilean subjects (Cortes et al. 2013). In this study, for this Mexican population, the frequency of the homozygous T genotype was 7.3%. Regarding the frequency of the T allele, frequencies ranging from 18.9% for a Chinese population to 39% for an African population have been described (Ayuso et al. 2019). In this study population, the frequency of the T allele was 32.2%, which is in agreement with that reported by Cortes et al. (2013) for a Chilean population (35%)

In Mexico, as in many other countries, EFV therapy is still widely used, mainly because it is administered in a one-tablet regimen per day (in combination with FTC+TDF), is relatively inexpensive, and is well tolerated. Although the optimal plasma concentration for the safety and efficacy of EFV is 1–4 µg/mL after the administration of a standard dose of 600 mg/day, it should be noted that in this study, a high percentage (64.5%) of subject had EFV plasma concentration greater than 4 µg/mL. Although their viral load levels and CD4 cell variables were adequate, it is important to evaluate this clinical evolution, since exposure to high doses of EFV is associated with a deterioration of cognitive function and greater adverse effects related to the central nervous system (CNS) (Haas et al. 2004; Hakkers et al. 2020) Recently, a new adverse effect has been described as "late-onset efavirenz neurotoxicity syndrome" (LENS). Adults who developed this syndrome were found to have supratherapeutic efavirenz plasma concentrations, above the upper limit of the therapeutic range of 4 µg/mL (van Rensburg et al. 2021). This syndrome has been reported only in Africa. There is little information about the prevalence of LENS and it is likely to be underrecognized and underreported in most countries where efavirenz is used. Due to the high percentage of subjects with supratherapeutic EFV levels, it would be very important to follow up these subjects, in order to evaluate the presence of LENS suggestive symptoms or any other adverse effect related to the CNS.

Due to the high plasma concentration of EFV found in this population, the reduction of the EFV dose from 600 to 400 mg/day, as indicated by the WHO guidelines (World Health Organization, 2021) is highly recommended, both for PLWHs starting EFV therapy as for people already using EFV who present supratherapeutic levels of the drug.

In contrast, it was found that 3 out of 4 subjects with EFV levels below 1 µg/mL had ≥ 40 HIV-1 RNA copies/mL. Although the number of subjects was very low, these data could suggest that exposure to subtherapeutic doses of EFV leads to virological failure, as described by Marzolini et al. (2001), who observed that 50% of subjects with plasma EFV values less than 1 µg/mL had a virological failure (Marzolini et al. 2001). This can lead to higher risk of selecting and transmitting EFV-resistant viruses. In addition to viral factors or ARV adherence related to the development of EFV-resistant virus, pharmacogenetics may be playing an important role. In the case of the G allele of CYP2B6, it has been described that is directly associated with the risk of EFV-resistance development and people with this allele were more likely to carry HIV EFV-resistant infections (Maseng et al. 2021).

The efficacy of EFV may be compromised in most subjects included in this study because they did not have therapeutic plasma concentrations of 1–4 µg/mL. This emphasizes the importance of

identifying other potential factors. Although the *CYP2B6* 516 G>T polymorphism represents the most relevant *CYP2B6* variant in the metabolism of EFV (Ayuso et al. 2019), other variants, such as *CYP2B6* 785A>G, *CYP2B6* 983 T>C, and to a lesser extent CYP2A6 1836G>T and others, have been described (Desta et al. 2019).

In addition to identifying the genetic polymorphisms that may cause this variability, early treatment, therapy optimization, and improvements in pharmacological efficacy will help minimize resistance development and serious adverse effects.

Our study has several limitations. First, the sample size is relatively small, participants were enrolled at a single center which may have led to bias in the interpretation of the results. Secondly, EFV plasma samples were obtained 8 to 15 h after not observed dosing. Third, data on neurological adverse effects of ARV treatment were not collected. Fourth, other variants, such as *CYP2B6* 785A>G, and *CYP2B6* 983 T>C could not be evaluated in this study.

In conclusion, the *CYP2B6* 516G>T polymorphism significantly influences plasma EFV levels. However, it is important to identify other genetic variants and potential factors that influence EFV pharmacokinetics to implement customized treatment strategies that increase the safety and efficacy of this drug.

4. Experimental

4.1. Study population

PLWH who regularly visited the *Centro Ambulatorio para la Prevención y Atención en SIDA e Infecciones de Transmisión Sexual* (CAPASITS) of the Morelos State Health Services during the period from February to July 2015 were invited to participate in this study. All individuals aged above 18 years who agreed to participate and sign an informed consent letter and were on EFV treatment at a daily dose of 600 mg orally in combination with other ARV agents were included. A previously published secondary analysis described the characteristics of the site, questionnaires applied, and calculation for determining sample size (Martínez-Salazar et al. 2017).

Treatment adherence was evaluated through the implementation of the ESPA questionnaire which consists of six questions with dichotomous responses; patients were considered adherent (5-6 points) or non-adherent (0-4 points) (Knobel et al. 2002).

The project was approved by the Ethics Committee of Henry Dunant Hospital in Mexico.

Blood samples with anticoagulant were obtained for DNA extraction and plasma collection between 8 and 15 hours after EFV administration.

4.2. Identification of the CYP2B6 516G>T polymorphism

Genomic DNA was isolated from the peripheral blood using heparin as an anticoagulant and a Thermo Scientific GeneJET Gel Extraction Kit (#K0699DNA) Polymerase chain reaction (PCR) was performed as described by Lang et al (2001) The sequence of primers for *CYP2B6* were as follows: forward primer 5′-GGTCTGCCCATCTATAAAC-3′ and reverse primer 5′-CTGATTCTTCACATGTCTGCG-3′. The PCR reaction was performed in a volume of 25 µL, which contained 15 pmol of each primer, 100 ng of DNA, 1x Taq Buffer ([NH4] 2SO4), dNTPs at 200 mM, $MgCl_2$ at 1.25 mM, 1 U Taq DNA polymerase, and sterile ddH_2O. The amplification program included an initial denaturation stage at 92°C for 5 min, followed by 40 denaturation cycles at 92°C for 30 s, hybridization at 58°C for 30 s, extension at 72°C for 40 s, and a final extension at 72°C for 7 min. The thermocycling method was applied using a Perkin Elmer Gene Amp PCR System 2400 thermal cycler.

After amplification of a 526 bp product by PCR, *CYP2B6*G>T polymorphism was detected by restriction fragment length polymorphism (RFLP) analysis. For RFLP analysis, 10 µL of the amplified product was incubated with *BsrI* enzyme (Thermo Scientific catalog No. ER0881) at 60°C for 1 hour. Digested fragments were separated by electrophoresis using a 2.5% agarose gel. Allele G was represented by fragments of 268, 241, and 17 bp, and the mutated allele T was represented by fragments of 509 and 17 bp. The digestion readings were obtained by two investigators in a double-blind manner. Concordance with quality control samples was 100%.

4.3. Determination of plasma EFV levels

The fasting plasma efavirenz level at 8-15 h post-dose of EFV was measured using a validated high-performance liquid chromatography assay (lower limit of quantification: 50 ng/mL) in compliance with the Official Mexican Standard NOM-177-SSA1-2013(*NOM-177-SSA1-2013*, 2013). Briefly, patient plasma samples (285 µL) were pretreatment with 600 µL of acetonitrile, vortex agitation for 1 min, and centrifugation for 10 minutes at 13000 rpm. Lastly, the supernatant was transferred to a chromatography vial. LC20AD (Shimadzu, Japan) high-resolution liquid chromatograph with a Zorbax® C18 XCB (50 x 4.6 mm Di) chromatographic column, stored in a column oven at 40°C, was used.

The mobile phase consisted of a buffer solution of sodium acetate 0.02 M and acetonitrile, at a ratio of 20:80, in an isocratic mode, at a flow rate of 0.4 mL/min. The temperature of the self-sampling device was maintained at 10°C, the injection volume was 1 µL, and the injection time was 2.5 min. An ABSciex Qtrap 4500 triple quadrupole mass spectrometer detector was used. Ionization was performed with electrospray in a negative mode, at a temperature of 450°C and an electrospray voltage of -4500 V, using nitrogen as the collision gas. Efavirenz was detected in the multi-reaction monitoring (MRM) mode using precursor→production of m/z 314→244 m/z. Data were acquired using Analyst® software, with 1/x used to weigh the calculated line by least squares.

According to the therapeutic range established for EFV from 1 to 4 µg/mL (Marzolini et al. 2001), three categories of plasma concentrations were defined: subtherapeutic (<1 µg/mL), therapeutic (1–4 µg/mL), and supratherapeutic (>4 µg/mL).

4.4. Statistical analysis

The results of molecular assays, plasma levels of EFV, age, sex, height, and weight were entered into a database using Statistical Package for the Social Sciences (SPSS), for processing and analysis (SPSS software v.20 for Windows™, SPSS Inc. Chicago, IL) Quantitative variables were analyzed using Student's *t*-test or Mann–Whitney *U* test and analysis of variance or Kruskal–Wallis test, depending on the normality or non-normality of the variables (Kolmorogov-Smirnov test) Chi-square test was used to analyze qualitative variables. Odds Ratio (OR) was transformed into relative risk (RR) with the formula: $RR = OR/([1 - P_0] + [P_0 \times OR])$, where P_0 is the baseline risk (Zhang 1998) The existence of Hardy-Weinberg equilibrium was confirmed (p=0.8032).

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Conflicts of interest: The authors declare that they have no conflict of interest.

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