Drug Metabolism and Pharmacokinetics 31 (2016) 349–355

Contents lists available at ScienceDirect

Drug Metabolism and Pharmacokinetics

journal homepage: http://www.journals.elsevier.com/drug-metabolism-and-pharmacokinetics

Regular article

Gene–gene interactions between DRD3, MRP4 and CYP2B6 polymorphisms and its influence on the pharmacokinetic parameters of efavirenz in HIV infected patients

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ARTICLE INFO

Article history:
Received 25 January 2016
Received in revised form
23 May 2016
Accepted 19 June 2016
Available online 27 June 2016

Keywords:
Efavirenz pharmacokinetics
Genetic factors
Gene–gene interactions
CYP2B6
MRP4
DRD3

ABSTRACT

Genetic factors have a significant impact on the PK variability of EFV, much higher than other non-genetic factors, such as demography. In this work we have performed a comprehensive PG analysis of genes encoding the major metabolizing enzymes and transporters of EFV, establishing a clear relationship between the PK parameters and genetic factors, which explain 50% of the variability in EFV PK parameters.

The most relevant associations for metabolizing enzymes were found in CYP2B6 (rs3745274), in agreement with previous studies. The influence of transporters on the kinetics of EFV was also proved with significant correlations between the PK parameters of EFV and MRP4 (rs1751034, rs2274407).

Analysis of gene–gene interactions with CYP2B6 was particularly useful to reinforce the role of MRP4 and to reveal unknown associations, such as that of DRD3. However, the role of DRD3 cannot be a direct effect but an indirect one due to physical proximity of NAT and the DRD3 locus in the genome.

1. Introduction

The widespread use of antiretroviral therapy (ART) has dramatically decreased progression to AIDS and death [1]. In developed countries, the use of HAART has made it possible to change the natural history of HIV infection into a chronic disease that nowadays requires long-term antiretroviral treatment [2]. Notwithstanding the benefits of HAART, wide intra- and intersubject variability has been observed in both efficacy and in the adverse effects associated with certain antiretroviral drugs.

In fact, this is the case of efavirenz (EFV), which shows high interindividual variability in response to the drug, despite the positive results obtained in clinical trials [3]. This could be, to a large extent, attributed to the high inter-individual variability in the disposition kinetics of EFV [4].

Currently, the role of genetic polymorphisms in genes encoding metabolizing enzymes and protein transporters is receiving particular attention because they may be implicated in many processes of the pharmacological response [5]. EFV is predominantly metabolized in the liver by CYP2B6 [6] into 8-hydroxyefavirenz,

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http://dx.doi.org/10.1016/j.dmpk.2016.06.001
1347-4367/

and also via accessory pathways involving CYP2A6, CYP3A4/5, and UGT2B7 [6]. With respect to protein transporters, the information regarding the role they play in the disposition kinetics of EFV is relatively limited [7]. Major determinants in these transport processes are ATP-dependent efflux pumps, such as MDR1 (ABC1, P-glycoprotein) and related proteins (e.g. MRPs). At present, only a few studies have evaluated genetic polymorphisms in these transporters, mainly in MDR1, with inconclusive results [7–11].

Some studies have reported an association between genetic polymorphisms of CYP2B6 and the pharmacokinetics of EFV [8,12–14]. Particularly the rs3745274 SNP in CYP2B6 has been significantly associated with high EFV plasma concentrations in different populations. Nevertheless, there is not much information on the involvement of other genetic polymorphisms and, especially, gene–gene interactions have not been explored so far.

Regarding genetic factors, it is important to note that the major adverse effects of EFV are produced in the CNS. Thus, polymorphisms in the receptors, transporters, and enzymes that belong to serotonin and dopamine transmitter systems are of special importance due to their involvement in the predisposition to mental illness and the risk of CNS toxicity when EFV is included in ART therapy [15].

In view of the importance of genetic factors in the pharmacokinetics of EFV, a study was conducted to investigate the association of gene polymorphisms and gene–gene interaction among genes encoding metabolizing enzymes, protein transporters and neurotransmitter systems and EFV PK parameters.

2. Materials and methods

2.1. Subject recruitment

The present study involved HIV-positive adult European patients treated with EFV at the outpatient unit of the Pharmacy Service of the University Hospital of Salamanca (Spain) during a study-period of five years. All patients received EFV in combination with two NRTIs as part of their antiretroviral regimen, and had to meet the following criteria in order to participate in the study: confirmed HIV infection, treatment with a dose of 600 mg oral EFV once a day for at least 3 months, age $\geq$18 years, >90% adherence to the treatment regimen and no co-medication with known inducer or inhibitor drugs of EFV metabolism. The patients were included in a therapeutic drug monitoring (TDM) program and plasma samples for the EFV assay were drawn periodically at 3–6 month intervals on follow-up visits to the hospital, along with viral and biochemical tests. Individual information was carefully recorded at the time of collecting the blood samples and included dose history, sampling time, time of last dose, gender, age, weight, height, concomitant pathologies and treatment, habits and adherence.

2.2. Ethics statement

The study was approved by the Ethics Committee of the University Hospital of Salamanca. Each patient provided written informed consent and blood samples were obtained for genetic testing.

2.3. Pharmacokinetic analysis

The number of samples taken per patient to determine plasma concentrations was 2–3 samples per year, at the time of the analytical clinical follow-up (viral load and CD4 lymphocytes). Most blood samples were collected at midpoint of the dosage interval, between 8 and 20 h after EFV administration, under steady-state conditions ($\geq$3 month after the initiation of EFV treatment).

Blood samples (5 ml) were collected, and plasma was isolated by centrifugation at 3000 $\times$ *g*. Samples were stored at −20 °C (following virus inactivation in a water bath at 60 °C for 60 min) until analysis. The stability of EFV under these conditions is adequate, since the slope of the calibration curves of EFV, established in samples subjected to the thermostatting procedure, was only slightly lower (3.93% ± 1.57%) than that obtained with non-heated samples. EFV concentrations were measured by high-performance liquid chromatography (HPLC) (Waters, Milford, MA) with UV detection at 215 nm, following solid-phase extraction using a GX-271 ASPEC (Gilson, Villiers le Bel, France). This method was validated over a concentration range of 0.5–10 μg/ml using 600 μl of plasma. Recovery of EFV from human plasma was 107.4%. Intra- and inter-day CV precisions were consistently <5.7% for all internal quality controls (0.5, 2.0, and 10.0 μg/ml). The quantification limit was 0.25 μg/ml, and the absence of interference from the 21 drugs frequently used in HIV patients was confirmed. Our analysis laboratory successfully participates in the International Inter-laboratory Quality Control Program for Therapeutic Drug Monitoring in HIV Infection (Dutch Association for Quality Assessment in Therapeutic Drug Monitoring and Clinical Toxicology [KKGT]).

The PKs of EFV were characterized assuming an open one-compartment model with a fixed absorption constant and first-order elimination, and using the nonlinear mixed effect modeling program [16]. Estimates of the PK parameters (the apparent oral clearance [CL/F], the maximum steady-state plasma concentration [C_{maxss}], the minimum steady-state plasma concentration [C_{minss}]) from the plasma concentration data for each individual were generated using Bayesian algorithms.

2.4. SNP selection and genotyping

The selection of genetic polymorphisms was performed taking into account the fact that they were occurring in genes encoding enzymes and transporters of antiretrovirals (EFV mainly) and in genes encoding neurotransmitter systems.

SNPs were selected based on three main criteria: (1) already identified SNPs or the reports of putative enzymes and transporters of EFV, (2) functional SNPs (based on potential protein changes, or (3) SNPs reported by other groups from public databases: CYPalleles [17], dbSNP [18] and PharmGKB [19].

After an initial selection, SNPs showing minor allele frequencies <1% and those not being in Hardy–Weinberg equilibrium were discarded. In total, a set of 68 SNPs was finally available for statistical analysis. The final panel included SNPs of genes involved in the metabolism (10 genes) and transport (11 genes) of EFV. In addition, we also included genes coding for proteins involved in neurotransmitter systems (5 genes) (Table 1 and supplementary Table 1).

The genotypes of metabolizing CYP enzymes and drug transporter genes were mainly determined using Sequenom's high-throughput matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) in six multiplexes. Furthermore, some SNPs on CYP2A6 (rs28399454 and rs34816076) and CYP3A5 (rs28365095) could not be analyzed by MALDI-TOF and therefore, they were analyzed by real-time PCR using TaqMan (Applied Biosystems) technology according to the specifications of the manufacturer.

2.5. Statistical analysis

Statistical calculations were made using PASW Statistics 18 (IBM SPSS Statistics) and R for Windows (SNPassoc library [20]). Haploview [21] and Beagle [22] were used to obtain and visualize haplotypes for some genes.

Table 1
List of analyzed genes and selected SNPs.

Genes	SNPs
Metabolism	
CYP2A6	rs1801272, rs28399435, rs8192726
CYP2B6	rs2279343, rs3211371, rs35303484, rs3745274, rs8192709
CYP2C8	rs10509681, rs11572080
CYP2C9	rs1057911
CYP2C19	rs12248560, rs4244285
CYP2D6	CYP2D6_1661, CYP2D6_2097, CYP2D6_31, rs1065852, rs3892097
CYP3A4	rs2740574
CYP3A5	rs10264272, rs28371764, rs4646453, rs776746
TPMT	rs1142345, rs1800460
UGT2B7	rs7439366
Transporter	
ABCA1	rs4149313
APOA5	rs3135506, rs662799
APOC3	rs5128
APOE	rs429358, rs7412
BCRP	rs2231137, rs2231142
CETP	rs1800775
MDR1	rs1045642, rs1128503, rs2229109, rs2235046, rs9282564
MRP1	rs246221, rs35587, rs45560437
MRP2	rs17222723, rs2273697, rs3740066, rs717620, rs8187707, rs8187710
MRP4	rs11568658, rs12875235, rs1557070, rs1751034, rs2274405, rs2274406, rs2274407, rs3742106, rs899494
OAT1	rs4149170
Neurotransmitter	
5HT2A	rs6313, rs6314
5HT2C	rs6318
ADRB3	rs1042714
DRD3	rs1486012, rs2134655, rs7631540, rs963468
OPRM2	rs1799971

Initially, before the statistical analysis, the EFV PKs data (all dependent variables) were log-transformed to enhance the normality of their distribution and the homogeneity of their variances. Besides this, the genotyping data were filtered using the genotype call rate (>90% completeness), the Hardy–Weinberg equilibrium test (p-value > 0.001) and a minor allele frequency (MAF) criterion (>1%) in order to ensure adequate quality of the PG data.

A linear regression was carried out to evaluate the association between individual SNP markers and EFV PK parameters. All analyses were performed assuming a log-additive model; however, in cases where the SNPs analyzed had less than five observations for the patients with a homozygous genotype, the dominant genetic model (in which both the heterozygous variant and the rare homozygous variant were combined) was used instead. Age, gender and BMI were included as covariates in this regression model. Because of the known influence of rs3745274 SNP in CYP2B6 on EFV PK parameters and to enhance the detection of weaker effects, this SNP was also included as a covariate in the model.

Additionally, the statistical interaction between each SNP and rs3745274 was tested adding the product between both SNPs to the multivariate regression model.

3. Results

One hundred and twenty-one HIV-infected European patients treated with EFV who met all the inclusion criteria were enrolled into the study for genotype–phenotype analysis. The baseline demographic characteristics and EFV PK parameters are summarized in Table 2. All patients were Caucasian and most had a good clinical evolution because the mean CD4+ lymphocyte cell count was 388 cells/μL; 94 patients (77.6%) had an undetectable (<40 copies/mL) plasma HIV RNA load. At baseline, according to the therapeutic range of EFV (C_{minss} = 1–4 mg/L) [23], a considerable percentage of patients (21.5%) did not reach therapeutic concentrations.

Table 2
Demographic characteristics and efavirenz pharmacokinetic parameters of study population (n = 121).

Characteristics	Values Mean ± SD (range) or n (%)
Demographic factors	
Age (years)	44.8 ± 9.38 (18–77)
Male	82 (67.8)
BMI (kg/m^2)	23.0 ± 3.39 (14.51–36.24)
Pharmacokinetic parameters	
C_{minss} (mg/L)	2.19 ± 1.67 (0.62–12.84)
C_{maxss} (mg/L)	4.17 ± 1.77 (2.48–14.15)
CL/F (L/h)	9.33 ± 3.19 (0.61–16.10)

SD: standard deviation; BMI: body mass index; C_{minss}: minimum steady-state plasma concentrations; C_{maxss}: maximum steady-state plasma concentrations; CL/F: apparent oral clearance.

As expected, the already reported strong association between rs3745274 SNP in CYP2B6 and PK parameters was clearly confirmed in a preliminary linear regression analysis (b = 0.62, p-value = 7.10 e^{-19}, b = 0.33, p = 7.76 e^{-18}, b = −3.38, p = 3.20 e^{-16} for C_{minss}, C_{maxss} and CL/F respectively).

The linear regression analysis of the effect of genetic polymorphisms on these PK parameters, when adjusting for this SNP and the demographic variables, revealed that only four SNPs (one in CYP2B6, one in CYP2A6 and two in MRP4) showed suggestive probabilities (nominal p-value <0.05). These results are presented in Table 3.

Because SNPs were coded according to log-additive model (genotype coded as 0, 1, 2 in function of the count of minor alleles) or dominant model (frequent homozygous coded as 0 and heterozygous or minor allele homozygous coded as 1), the slope shown in Table 3 could be easily interpreted as the effect of the minor allele: a positive (negative) slope indicates an increase (decrease) effect of the minor allele on the PK value.

Among the SNPs in the MRP4 gene, two SNPs (rs2274407 and rs1751034) had a good correlation with C_{maxss}. Both SNPs were shown not to be linked (data not reported) and therefore they are contributing independently to the results. This fact explains the influence of MRP4 variability on EFV PK parameters.

When a more complete regression model was applied, adding an interaction term between rs3745274 and each analyzed SNP, several interesting interactions were found. Table 4 shows a summary of the results for those SNPs showing a nominal significance (p-value < 0.05) for the interaction term with rs3745274 in some of the three PK parameters analyzed.

In addition to some interesting results, such as the association found (p < 0.01) between C_{maxss} and interaction term with rs6318 (5HT2C) and rs662799 (APOA5), two main results were obtained: the interactions found for several independent SNPs of MRP4 (other than those shown in Table 2) and, especially, those found for four SNPs of DRD3. For two of these SNPs (rs1486012 and rs963468), the probability observed for the interaction term remained significant when corrected for multiple comparisons (p < 0.00037, Bonferroni threshold for 136 tests: 68 SNPs and two regression models, with and without the interaction term).

In both cases the slope of the interaction term was positive, indicating that the highest C_{maxss} values were obtained for the combinations of both minor allele homozygous genotypes (coded as 2). In other words, the increase in C_{maxss} related to the genotype of rs3745274 in CYP2B6 (from GG to GT and TT) was stronger within homozygous individuals for the SNP of DRD3 (rs1486012 or rs963468) and vice-versa. Conversely, negative interactions (as

Table 3
Results of linear regression of each SNP adjusting for rs3745274 and phenotypic covariables.

SNP	Gene	Major/minor allele	C_{minss}		C_{maxss}		CL/F	
			Slope	p-value	Slope	p-value	Slope	p-value
rs1751034	MRP4	A/G	−0.10	0.09610	−0.07	0.04797	0.71	0.06180
rs2274407	MRP4	G/T	0.20	0.03010	0.11	0.02486	−0.98	0.07733
rs8192709	CYP2B6	C/T	−0.31	0.11550	−0.17	0.13902	2.42	0.04402
rs8192726	CYP2A6	G/T	0.25	0.03123	0.15	0.02528	−1.35	0.05966

C_{minss}: minimum steady-state plasma concentrations; C_{maxss}: maximum steady-state plasma concentrations; CL/F: apparent oral clearance.

Table 4
Significance of the interaction term between each SNP and rs3745274. Significant probabilities ($p < 0.00037$, multiple correction threshold) are marked in bold.

SNP	Gene	C_{minss}		C_{maxss}		CL/F	
		Slope	p-value	Slope	p-value	Slope	p-value
rs6318	5HT2C	0.14	0.03303	0.10	0.00626	−0.72	0.07598
rs6314	5HT2A	−0.32	0.03571	−0.22	0.01122	1.97	0.03312
rs662799	APOA5	−0.42	0.01852	−0.26	0.00945	2.70	0.01350
rs8192726	CYP2A6	0.28	0.12756	0.21	0.04099	−0.79	0.48586
rs3211371	CYP2B6	0.22	0.20273	0.21	0.03108	−0.61	0.57101
CYP2D6_1661	CYP2D6	−0.14	0.05754	−0.09	0.04582	0.85	0.06273
rs1486012	DRD3	0.23	0.00394	0.17	**0.00016**	−0.63	0.20547
rs2134655	DRD3	−0.27	0.00737	−0.19	0.00065	1.16	0.05922
rs7631540	DRD3	−0.20	0.01220	−0.14	0.00160	0.49	0.31827
rs963468	DRD3	0.21	0.00805	0.16	**0.00033**	−0.47	0.34357
rs9282564	MDR1	0.23	0.20497	0.24	0.01811	−0.50	0.65318
rs717620	MRP2	0.13	0.14067	0.11	0.02141	−0.17	0.75162
rs12875235	MRP4	−0.23	0.14942	−0.19	0.03168	1.20	0.22278
rs2274405	MRP4	0.16	0.04576	0.11	0.01008	−0.78	0.10576
rs2274406	MRP4	0.17	0.03576	0.12	0.00947	−0.87	0.07629
rs3742106	MRP4	−0.18	0.04322	−0.14	0.00633	0.85	0.12761
rs1799971	OPRM2	−0.18	0.15617	−0.14	0.04209	0.73	0.33458

those suggested by other SNPs of DRD3) highlight different genotype combinations with increasing C_{maxss} values.

The linkage pattern between the four SNPs of DRD3 is shown in Fig. 1. The linkage between several of these SNPs is relatively strong, and, as a consequence, these four SNPs have often been analyzed as haplotypes in association studies [24]. For this reason, the relative frequency of the estimated haplotypes (EM algorithm) was also analyzed.

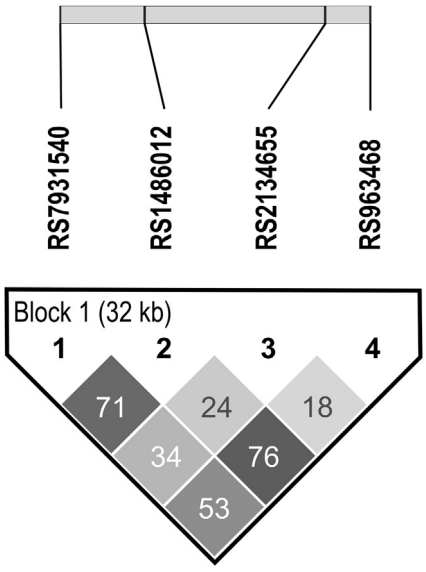

Fig. 1. Linkage disequilibrium values (R-squared) at DRD3. The grayscale represents pair-wise R-squared.

The most frequent haplotype (37%) corresponded with the combination C-A-G-T for rs7631540, rs1486012, rs2134655 and rs963468 (in order of their position within the chromosome), and contained the three alleles of rs1486012 (A), rs2134655 (G) and rs963468 (T), which were associated with higher C_{maxss} levels. Other frequent haplotypes (T-T-G-C and T-T-T-C) were checked for individual association with PK parameters, but no association was found.

To analyze the possible role of the haplotypes in an interaction term with rs3745274, the individual phase was inferred, coded under an additive model (number of copies of the haplotype) and dominant model (individuals coded as 1 or 0 for carriers and non-carriers of this haplotype, respectively), and the cross-product with rs3745274 was checked in a similar model to that of the SNP analysis.

As expected, the interaction between rs3745274 and the common haplotype C-A-G-T was found to be highly significant ($p = 8.2^{*}10^{-7}$) whereas, no other haplotypes were found to be significant.

To understand the meaning of this interaction, Table 5 shows the results of the association analysis between C_{maxss} and rs3745274, stratified within each haplotype class (under additive and dominant models). All of them were clearly significant, but the lowest slope of the regression model was found in the subset of observations without the haplotype CAGT ($b = 0.23$). The slope increased in the subset of individuals heterozygous ($b = 0.52$) or homozygous ($b = 0.64$) for this haplotype. An increase in this slope was also observed when CAGT carriers (heterozygous and homozygous for haplotype) were compared to non-carriers (interaction term, $b = 0.54$, $p = 7.48^{*}10^{-11}$). Table 5 also shows the C_{maxss} average value for each genotype combination. The number of observations in some of these combinations was low because of the use of the additive model in CYP2B6 (there were only seven TT individuals for rs3745274, see supplementary Table 1). The existence of this interaction was confirmed when this SNP was coded under a dominant model, increasing the number of observations within each group ($b = 0.436$, $p = 3^{*}10^{-5}$ for the interaction term). Fig. 2 shows this interaction graphically: each combination of

Table 5
Results of linear regression of rs3745274 on C_{maxss} stratified within DRD3 haplotype classes.

rs3745274	DRD3 haplotype			
	−/−	−/CAGT	CAGT/CAGT	CAGT carriers
	C_{maxss} (N)	C_{maxss} (N)	C_{maxss} (N)	C_{maxss} (N)
GG	3.57 (54)	3.47 (7)	3.45 (13)	3.46 (20)
GT	4.29 (33)	5.43 (5)	5.26 (2)	5.38 (7)
TT	7.13 (3)	7.21 (2)	13.55 (2)	10.38 (4)
Slope	0.23	0.52	0.64	0.54
p-value	$1.05^{*}10^{-8}$	$7.5^{*}10^{-4}$	$8.18^{*}10^{-8}$	$7.48^{*}10^{-11}$

−/−: individuals without CAGT haplotype; −/CAGT: heterozygous individuals for the CAGT haplotype; CAGT/CAGT: homozygous for CAGT haplotype; CAGT carriers: individuals carrying one or two copies of the CAGT haplotype (−/CAGT + CAGT/CAGT).

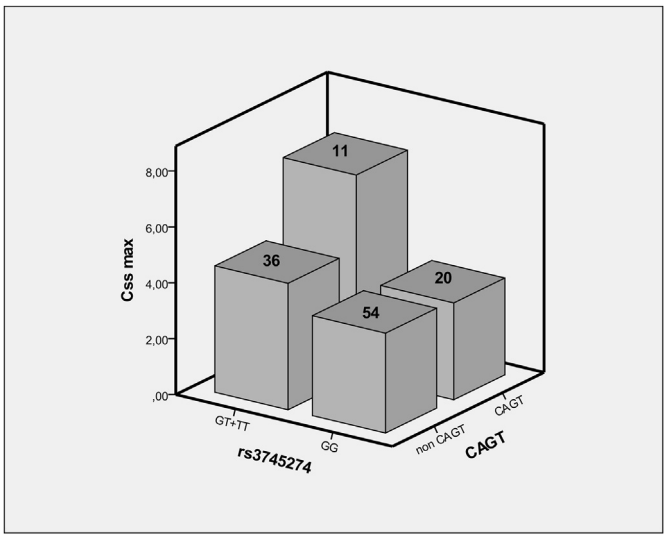

Fig. 2. Mean values of C_{maxss} for genotype combinations of CYP2B6 (rs3745274) and the haplotype CAGT for DRD3, coded both under dominant model. The height of each bar shows the average value for C_{maxss} calculated from the individuals showing that genotype combination. The number of individuals belonging to each class is shown on the bar.

genotypes for CYP2B6 (coded as 0 for GG individuals and 1 for GT + TT in rs3745274) and the haplotype of DRD3 is illustrated by a bar. The height of each bar is the C_{maxss} average value calculated for the individuals showing that genotype combination, and the number of observations is indicated on the bar. The difference in C_{maxss} values between GG individuals and T-carriers (GT + TT) for rs3745274 was lower within the individuals without the DRD3 haplotype (from 3.57 to 4.52, $b = 0.22$, $p < 0.001$), and clearly higher within the individuals carrying at least one CAGT haplotype (from 3.46 to 7.20, $b = 0.72$, $p < 0.0001$).

4. Discussion

The main objective of this study was to investigate the impact of genetic factors on the PK parameters of EFV due to the high inter-individual variability that has not been fully explained by other factors. This variability was demonstrated in the present study. Thus, the percentage of patients with EFV concentrations outside the therapeutic range was approximately 20%, which is consistent with other studies [25–29]. This implies that the response to EFV treatment also differs from one patient to another, further emphasizing the need to find the factors involved in the response.

To understand this variation some previous genetic studies were focus in individual genes [8,13,25,30–32]. However, the pharmacological response is much more complex and it is essential to consider all potential genes involved, and to explore the existence of interactions between them.

In this regard, the selection of candidate genes is key since it has not yet been clearly defined which metabolizing enzymes and transporters are primarily involved in the ADME process of EFV. Thus, although the main route of EFV metabolism is through the CYP2B6 isoenzyme [6], we must also take into account the genes encoding enzymes involved in secondary metabolism. Therefore, a total of 26 SNPs from 10 genes were selected. Transporter genes were analyzed in detail to explore the role that transport proteins play in the distribution of this drug, which can affect both efficiency and toxicity (33 SNPs from 11 genes were selected). Finally 9 SNPs of neurotransmitter genes were selected to explore the role of these genes given the risk of CNS toxicity when EFV is included in ART therapy.

Our results showed that the SNPs most significantly associated with the response (in addition to the already known effect of CYP2B6) were of those genes encoding the MRP4 transport protein, the CYP2A6 enzyme and, more surprisingly, DRD3 through an interaction effect with CYP2B6.

The SNP rs3745274 in CYP2B6 showed a significant relationship with EFV PK variability in the preliminary analysis, which has been widely studied and linked to a decrease in the activity of this isoenzyme. In our study, this polymorphism affected all PK parameters. Thus, in patients with a homozygous genotype (TT), when compared with the wild type homozygote (GG), an increase from 1.56 to 7.11 μg/mL in mean C_{minss}, 3.54 to 8.99 μg/mL in mean C_{maxss} and a decrease from 10.79 to 3.10 L/h in mean CL/F were observed. These data are consistent with the results of other studies [33,34]. This SNP explained about 45% of the total variance when included in a univariate regression model. Our data confirm the important impact this polymorphism might have on treatment with EFV, especially regarding the toxicity of this drug, since several studies showed that high EFV plasma concentrations are related to an increased risk of adverse effects [15,35]. In this sense, the possibility of knowing the genotypes before prescribing this drug would be very useful in clinical practice for optimizing EFV treatment [36].

Despite the undoubted importance of the CYP2B6 isoenzyme, other metabolizing enzymes were also identified as being potentially responsible for the PK variability. Thus, rs8192726 in CYP2A6 remained possibly associated when adjusting for rs3745274. Some studies have recently examined the influence of this enzyme [25,31], but the results have been contradictory. Our data support the notion that the impact of CYP2A6 on the PK parameters of EFV could be independent of the activity of CYP2B6.

Regarding the rest of the CYPs, no further individual SNP associations were found, in accordance with the results of previous studies [7,8,37,38]. There may be several reasons for this, including the low frequency found for the selected SNPs in the European population and, moreover, the possible but small influence of these SNPs on the metabolism of EFV. However, more studies with a larger sample size are needed to confirm these hypotheses.

One of the most remarkable results of this work concerned the influence of transporters on the kinetics of EFV. Several SNPs in MRP4, mainly rs1751034 and rs2274407, are correlated with the PK parameters of EFV. Other SNPs in this gene are involved in possible interactions with rs3745274 in CYP2B6, especially in the analysis of the effects on C_{maxss} (see Table 4). In addition, two other SNPs from MRP4 (rs1557070 and rs2274407) also showed suggestive probabilities ($p < 0.10$) for the interaction term. However, in most of these cases, the trends observed for each SNP are similar within each rs3745274 genotype from CYP2B6. So, interactions probably indicate the existence of a greater slope for some genotypes, but these results could be biased because of the low number of observations in some combination of genotypes.

The impact that these polymorphisms might have on EFV therapy is not known since no previous studies have examined their effects. This transporter is located in many epithelial barriers including the blood–brain, acting as an efflux pump of xenobiotics, so that genetic polymorphisms in this protein may decrease their expression and increase EFV concentrations in cerebrospinal fluid, and therefore the presence or intensity of central nervous system (CNS) adverse effects. These effects have been observed in a previous study [15], in which certain SNPs in other transporters (MRP1 and BCR) located in the same tissue as MRP4 were associated with CNS adverse effects in patients treated with EFV.

It is also necessary to highlight that previous studies involving other antiretrovirals, describing that MRPs can efflux nucleoside reverse transcriptase inhibitors (NRTIs) from intracellular

compartments [39] have also confirmed the results obtained in our study. In addition, a study addressing pharmacogenetics of zidovudine therapy in HIV-infected adults reported a trend for elevated zidovudine-triphosphate concentration in MRP4 variant carriers [40].

Therefore, the influence of MRP4 in the pharmacokinetic of EFV could play an important role, given that it could also be involved in the toxicity and efficacy of this drug. In this sense, further studies on different populations and larger numbers of patients are essential.

Concerning gene–gene interactions, the most interesting result was the influence of four SNPs of DRD3, in combination with CYP2B6 (rs3745274), on C_{maxss} values. In two of these SNPs (rs1486012 and rs963468), and in the CAGT haplotype analysis, the probability of the interaction term was clearly significant (after multi-test correction). These findings could be partially influenced by the low number of observations in some genotype combinations, as rs3745274 was coded under the additive model. The use of this genetic model was justified by previous association studies [8,13] and by the PK values observed in this study (for instance, the average C_{maxss} value was 3.54, 4.48 and 8.99 for GG, GT and TT individuals, respectively). To evaluate the relative importance of the seven minor homozygous (rs3745274-TT) individuals in the interactions found, the analyses were repeated under the dominant model (results not shown), and once again a SNP of DRD3 (rs7831540) showed a significant interaction ($b = -0.43$, $P = 0.00002$). Results of the analysis of interactions for the CAGT haplotype (shown in Fig. 2 using the dominant model) also support the existence of a true interaction between CYP2B6 and DRD3.

DRD3 encodes the D3 subtype of the five (D1–D5) dopamine receptors. The activity of the D3 subtype receptor is mediated by G proteins which inhibit adenylyl cyclase. This receptor is localized to the limbic areas of the brain, which are associated with cognitive, emotional, and endocrine functions, but how it can influence pharmacokinetic parameters still remains unknown.

In a study recently carried out by Cabaleiro et al. [41], they found a clear correlation between clearance of quetiapine and polymorphism in DRD3, pointing out an additional effect of this drug on pharmacokinetic parameters.

Whereas a direct action of DRD3 on this type of parameters is difficult to explain, a possible explanation is the fact that N(alpha)-acetyltransferase 50, NatE catalytic subunit locus is in the vicinity of the DRD3 locus and in linkage disequilibrium. Changes in efavirenz apparent clearance in patients taking both efavirenz and antituberculosis treatment was highly dependent on NAT2 polymorphism, as a possible surrogate of isoniazid exposure.

Patients carrying the rs3745274 GG genotype in CYP2B6 and fast-acetylation NAT2 phenotype had the highest efavirenz apparent clearance and this could account for the high dose that they required [42,43]. Conversely, individuals carrying the rs3745274 TT genotype and low-acetylation NAT2 phenotype would show lower clearance and therefore, higher values of C_{maxss}, probably demanding a dosage correction. In fact, in the present work, the only two individuals who required a strong dosage reduction (200 mg/day) were TT for rs3745274 and homozygous for the DRD3 haplotype that we found significant in the interaction term. This finding and possible explanation requires further research and opens up the possibility of new ideas to understand variation in response to antiretroviral drugs.

5. Conclusions

This work performed a comprehensive PG analysis of genes encoding the major metabolizing enzymes and transporters of EFV, establishing a clear relationship between genetic factors and the PK parameters of this drug.

As expected, the most relevant association were found in metabolizing enzymes, mainly CYP2B6 (rs3745274), but the influence of transporters on the kinetics of EFV was also shown by the correlations found between the PK parameters of EFV and MRP4 alleles (rs1751034 and rs2274407). Other SNPs in this gene are involved in interesting interactions with rs3745274 SNP in CYP2B6, particularly in the analysis of the effects on C_{maxss} (especially for rs2274406 and rs3742106, showing a probability for the interaction term lower than 0.01).

In addition, analysis of gene–gene interactions was particularly useful to discover other associated genes, the most relevant finding being the role of DRD3, which could not be a direct effect but an indirect one due to physical proximity of NAT and the DRD3 locus in the genome.

This genetic information, in the near future, could be utilized to improve clinical efficacy and/or reduce or avoid the toxicity associated with treatment.

Funding

This research was supported by funding granted by the Europharma Foundation through a collaboration agreement between the University of Salamanca and the University Austral of Chile.

The study was also supported by grants from the Instituto de Salud Carlos III FIS (PI13/01136 to AC) and by King Abdulaziz University, grant no. (1-117-1434-HiCi).

Conflict of interest

The authors confirm that there are no conflicts of interest.

Acknowledgments

Efavirenz, as a pure compound, was kindly provided by the Bristol Myers Squibb Laboratories. This substance was used as the standard for validating the analytical technique and as the standard in all quantitative determinations.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dmpk.2016.06.001.

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