THE ANTI-HIV DRUG EFAVIRENZ: A CHALLENGE ON MOLECULAR MECHANISMS OF DRUG-ASSOCIATED NEUROCOGNITIVE DISORDERS

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Tese para obtenção do grau de Doutor em Ciências da Vida

na Especialidade em Ciências Funcionais e Alvos Terapêuticos (Farmacologia)

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Para os meus pais
Guida e Adelino

E para os meus avós
Clementina e Filipe

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LIST OF ACRONYMS AND ABBREVIATIONS

8,14-diOH-EFV	8-dihydroxy-efavirenz
7-OH-EFV	7-hydroxy-efavirenz
8-OH-EFV	8-hydroxy-efavirenz
7-OH-EFV-Glc	7-hydroxy-efavirenz-glucuronide
8-OH-EFV-Glc	8-hydroxy-efavirenz-glucuronide
7-OH-EFV-sulf	7-hydroxy-efavirenz-sulfate
8-OH-EFV-sulf	8-hydroxy-efavirenz-sulfate
5-HT_{2A}	Serotonin 2A receptor
ABCB1	Multidrug resistance protein 1
Aβ	Amyloid beta
AD	Alzheimer's disease
AIDS	Acquired immunodeficiency syndrome
ALT	Alanine aminotransferase
ARV	Antiretroviral drugs
ATP	Adenosine triphosphate
AUC	Area under the curve
BACE-1	β-secretase-1
BBB	Blood brain barrier
CAR	Constitutive androstane receptor
cART	Combined antiretroviral therapy
$CD4^+$	Cluster of differentiation 4 in T cells
C_{max}	Maximal concentration
CK	Creatine kinase
CNS	Central nervous system
CS	Calibration standards
CSF	Cerebrospinal fluid
CTL	Control
CV	Variation coefficient
CYP	Human cytochrome P450
Cyp	Rat cytochrome P450
CysSH	Cysteine
CysGlySH	Cysteinylglycine
CysSSCys	Cystine
CysSSP	*S*-cysteinylated protein

DAPI	4'-6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ECOD	Ethoxycoumarin-o-deethylase
EDTA	Ethylenediaminetetra-acetic acid
EFV	Efavirenz
EFV-Glc	Efavirenz-glucuronide
EPM	Elevated plus maze
FDA	U. S. Food and Drug Administration
FI	Fusion inhibitor
FS	Forced swim
GFAP	Glial fibrillary acidic protein
GSH	Glutathione
GSSG	Glutathione disulfide
GSSP	*S*-Glutathionylated protein
GST	Glutathione *S*-transferases
HIV	Human immunodeficiency virus
HIER	Heat-induced epitope-retrieval
HPLC	High-performance liquid chromatography
iNOS	Inducible nitric oxide synthase
INSTI	Integrase strand transfer inhibitor
IQR	Interquartile range
LDH	Lactate dehydrogenase
L-DOPA	L-dihydroxyphenylalanine
LLOQ	Lower limit of quantification
LMWT	Low-molecular weight thiols
LSD	Lysergic acid diethylamine
LTD	Latency-to-despair
Map2	Microtubule-associated protein 2
MDMA	3,4-methylenedioxy-methamphetamine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MWM	Morris water maze
na	not applicable
NBF	Neutral buffered formalin
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NO	Nitric oxide
NRTI	Nucleos(t)ide reverse transcriptase inhibitor

ns	not significant
NSC	Neural stem cells
NSF	*N*-ethylmaleimide-sensitive fusion protein
OF	Open field
PBMC	Peripheral blood monocular cells
PD	Parkinson's disease
PI	Protease inhibitor
PK	Pharmacokinetic
PSSCysGly	*S*-cysteinylglycinylated proteins
PXR	Pregnane X receptor
QC	Quality control
qPCR	Quantitative real time PCR
TCA	Trichloroacetic acid
TCEP	Tris (2-carboxyethyl) phosphine hydrochloride
TDM	Therapeutic drug monitoring
thioTEPA	triethylenethiophosphoramide
Tm	Melting temperature
UGT	UDP glucuronosyltransferases
UV	Ultraviolet
RNA	Ribonucleic acid
ROS	Reactive oxidative species
RSH	free reduced form
RSSP	*S*-thiolated proteins
SBD-F	7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid ammonium
SEM	Standard error of the mean
SH	Sulfhydryl group
SNARE	Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
SNAP-25	Synaptosomal-associated protein 25
SPF	Specific pathogen free
SULT	Sulfotransferase
YM	Y–Maze

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ABSTRACT

As the list of drugs becoming perceived as neurotoxic is growing, its chronic use has been raising increased concern. The anti-human immunodeficiency virus (HIV)-infection drug efavirenz (EFV) clearly illustrates these circumstances, being a drug chronically used by both adults and children, for which neurotoxic effects have been consistently recognized. In order to develop preventive/management strategies, the understanding of the mechanisms underlying EFV-induced neurotoxicity is crucial towards the mechanistic-oriented discovery of suitable biomarkers for their evaluation. Theses biomarkers will be ethically obtained and ideally will be easily accessed from peripheral biofluids, allowing the evaluation of patient´s risk, the identification of risk factors and the evaluation of the success of toxicity minimization strategies.

A significant number of individuals on the recommended dose of EFV have cen*tral nervous system* (CNS) adverse reactions, which represent the main drawback of EFV. These adverse reactions also constitute a major factor for EFV discontinuation, limiting adherence to combined antiretroviral therapy and available therapeutic options. The prevention and management of these neuro-adverse reactions are hampered by their broad-spectrum nature (from sleep disturbances to mood-changes or memory impairment) that might involve distinct underlying mechanisms. Clinical trials have consistently described a high inter-patient variability for type, time to onset, duration and severity of the CNS complaints. Most of EFV-induced CNS-adverse reactions are reversible and tend to occur during the first weeks of treatment. However, EFV discontinuation continues to occur late in the course of treatment, due to the persistence of CNS toxicity which may negatively impact the health and quality of life of patients on a long-term basis. While short-term effects are usually transient, or if not they are normally a factor for drug switch, the long-term effects are much more difficult to predict and to manage.

Patient´s individual metabolic capability is considered a factor on these neuro-adverse reactions, taking into consideration the high inter-patient variability in EFV concentrations and pharmacogenomic data. In particular, strong non-clinical evidence supports that EFV major Phase I metabolite, 8-hydroxy-efavirenz (8-OH-EFV) is a more potent neurotoxin than EFV itself. Nonetheless, the relevance of these findings to a clinically useful neuro-safety evaluation

has yet to be demonstrated. Mostly, due to difficulties on the quantification of 8-OH-EFV levels or of its reactive metabolites and/or adducts in brain, given the inaccessibility of the target tissue.

Due to these premises, the overall goals of the present translational research project were to study the mechanism underlying EFV-induced long-term CNS adverse reactions and to identify peripheral markers for the risk assessment of EFV-induced neurotoxicity and for the evaluation of strategies for prevention and management of these toxic events.

Towards these goals, firstly we have performed a clinical study aimed at investigating the CYP2B6 activity and 8-OH-EFV generation as an upstream event of mood changes in patients on long-term EFV exposure. A case-control study was performed, with two age-matched groups of HIV-infected male patients: a group without adverse CNS complaints and a group presenting mood changes. A high-performance liquid chromatography method was developed and validated to quantify the plasmatic concentrations of EFV, 8-OH-EFV and 8-hydroxy-efavirenz-glucuronide. Secondly, aiming at disclosing the role of EFV biotransformation and Cyp2b auto-induction in the mechanism underlying EFV short- and long-term toxicity, we used an animal model to study tissue- and time-dependency of biotransformation, auto-induction and oxidative stress-related *thiolomic* signature. This evaluation was performed in brain (prefrontal cortex and hippocampus) and liver from *Wistar* male rats on short (10 days) and long-term (36 days) EFV exposure. Third, in the same animal model we have evaluated the neurological, histopathological and molecular phenotype representative of long-term exposure to EFV. With this methodological approach the main findings arising from this project were:

1. The metabolism into 8-OH-EFV is associated with EFV-related mood changes upon long-term EFV exposure, which suggests that the concentration of this metabolite is a suitable parameter to perform therapeutic drug monitoring aimed at preventing/controlling these manifestations;

2. The time-course of Cyp2b mediated-EFV biotransformation justifies different mechanisms for its short- and long-term neurotoxicity. Upon long-term EFV exposure, a decrease in the neuronal function occurs in hippocampus, which is associated with learning and memory impairment. This is coincident with an accumulation of 8-OH-EFV in plasma overtime, higher expression of Cyp2b in liver and a decrease in hippocampus;

3. Cyp2b auto-induction effects and oxidative stress-related *thiolomic* signature upon EFV exposure is tissue- and time-dependent and helps to explain why this antiretroviral is mainly biotransformed at the liver but it is barely hepatotoxic.

If translated to clinical practice, these evidences will have important implications in EFV-prescription as well as short- and long-term neurotoxicity prevention and management.

Keywords: 8-hydroxy-efavirenz, neurotoxicity, CYP2B6 drug biotransformation, *thiolomic* signature, therapeutic drug monitoring, memory impairment, mood changes.

RESUMO

Como o número de fármacos com potencial neurotóxico tem vindo a aumentar, existe uma preocupação crescente com a sua utilização, principalmente de forma crónica.
O fármaco antirretroviral efavirenz (EFV) ilustra bem estas circunstâncias. Trata-se de um fármaco destinado a uso crónico, pela população adulta e pediátrica, e cujos efeitos neurotóxicos têm sido amplamente descritos. Com o objetivo de desenvolver estratégias dirigidas à prevenção e minimização destes efeitos, torna-se crucial a clarificação dos mecanismos subjacentes à sua neurotoxicidade. De facto, é imperativo encontrar biomarcadores apropriados para avaliar a neurotoxicidade associada à administração do EFV. Idealmente, a obtenção destes biomarcadores deve ser realizada de forma eticamente aceitável e através da recolha de fluidos biológicos periféricos, assegurando a avaliação de estratégias implementadas com vista a minimização da toxicidade relacionada com o fármaco.

O número de indivíduos medicados com a dose recomendada de EFV que desenvolvem reações adversas no Sistema Nervoso Central (SNC) é bastante significativo, sendo estas a principal desvantagem inerente ao uso deste fármaco. Estes eventos constituem também um fator limitante da adesão à terapêutica antirretroviral combinada por parte dos doentes e das opções terapêuticas disponíveis. O controlo e a prevenção do aparecimento destas reações de neurotoxicidade são dificultadas pela panóplia de efeitos que podem estar associados à administração deste fármaco (e.g. distúrbios do sono, alterações de humor, perda de memória) e, pela forte plausibilidade dos mecanismos que lhes são subjacentes serem distintos. Os estudos clínicos têm descrito de forma consistente a existência de uma elevada variabilidade inter-individual para o tipo, início, duração e gravidade das reações adversas. Estas são, na sua maioria, reversíveis e tendem a ocorrer durante as primeiras semanas de tratamento. Ainda assim, a descontinuação do fármaco, motivada pelo aparecimento destas manifestações, continua a observar-se em tratamentos de longa duração, condicionando um impacto negativo na saúde e qualidade de vida dos doentes. Se, por um lado, os efeitos a curto prazo são geralmente transitórios ou motivadores da interrupção da terapêutica com este fármaco, por outro, os efeitos a longo prazo são muito menos previsíveis e de difícil controlo.

Tendo em consideração a elevada variabilidade inter-individual das concentrações de EFV e aos dados farmacogenéticos que têm vindo a ser descritos, a capacidade metabólica inerente a cada doente constitui um fator preponderante para o desenvolvimento das reações adversas. Em concreto, dados obtidos de estudos não-clínicos indicam que o principal metabolito de fase I

do EFV, o 8-hidroxi-efavirenz (8-OH-EFV), é uma neurotoxina mais potente que o próprio EFV. No entanto, até ao momento, não foi elucidada a relevância destes resultados numa avaliação clínica da sua segurança neurológica. Este facto deve-se principalmente à dificuldade em quantificar os metabolitos reativos do 8-OH-EFV e/ou os níveis de adutos formados por estes no cérebro, dada a inacessibilidade a este tecido alvo.

Tendo em consideração as premissas descritas, os objetivos gerais delineados para o presente projeto translacional foram o estudo dos mecanismos subjacentes às reações adversas induzidas pelo uso prolongado do EFV e a identificação de possíveis marcadores periféricos que permitam a avaliação do risco de desenvolvimento de neurotoxicidade, bem como de estratégias destinadas à sua prevenção e controlo.

Para a concretização destes objetivos foi primeiramente realizado um estudo clínico, com o intuito de investigar a atividade do CYP2B6 e a formação do 8-OH-EFV no desenvolvimento de alterações de humor, em indivíduos medicados com EFV de modo crónico. Em concreto, foi implementado um estudo caso-controlo, que envolveu dois grupos de doentes do sexo masculino, infetados pelo VIH e com idades comparáveis: um grupo sem queixas a nível do SNC e um outro com alterações de humor. Foi também desenvolvido e validado um método de cromatografia líquida de alta eficiência para quantificar as concentrações plasmáticas de EFV, de 8-OH-EFV e do seu metabolito glucurono-conjugado. Posteriormente, com o intuito de elucidar o papel da biotransformação do EFV e da sua auto-indução no mecanismo subjacente à sua toxicidade, foi usado um modelo animal. Avaliaram-se a biotransformação, a auto-indução e o perfil "tiolómico" relacionado com o stress oxidativo em diferentes tecidos e em animais expostos ao EFV por diferentes períodos. Em concreto, esta avaliação centrou-se no cérebro (córtex pré-frontal e hipocampo) e no fígado de ratos machos *Wistar* expostos ao EFV por um período curto (10 dias) e por um período longo (36 dias). Por último, foi realizado um estudo comportamental e uma avaliação histopatológica e molecular das duas áreas do cérebro de ratos expostos a EFV por 36 dias. Através desta abordagem metodológica foi possível obter as seguintes conclusões principais:

1. A formação de 8-OH-EFV está associada a alterações de humor induzidas pela exposição ao EFV por um longo período de tempo. Este facto sugere que a concentração deste metabolito é um parâmetro adequado para a prática da monitorização da terapêutica, tendo em vista a prevenção e o controlo do aparecimento destas manifestações;

2. A biotransformação do EFV mediada pelo Cyp2b, ao longo do tempo, justifica a existência de mecanismos diferentes responsáveis pela sua neurotoxicidade a curto e longo prazo. A exposição continuada ao EFV por um longo período de tempo está associada a um decréscimo da função neuronal no hipocampo e diminuição da capacidade de aprendizagem e memória. Estes factos são coincidentes com uma acumulação de 8-OH-EFV no plasma e expressão de Cyp2b aumentada no fígado e diminuída no hipocampo;

3. A auto-indução do Cyp2b e o perfil *tiolómico* relacionado com o stress oxidativo são dependentes do tecido e do tempo de exposição ao EFV. Esta observação pode ajudar a explicar o porquê do EFV, apesar de ser maioritariamente metabolizado no fígado, não evidenciar um perfil hepatotóxico clinicamente relevante;

A translação destas observações para a prática clínica poderá ter implicações importantes na prescrição do EFV, bem como na prevenção e controlo da neurotoxicidade associada a este fármaco a curto e longo prazo.

Palavras-chave: 8-hidroxi-efavirenz, neurotoxicidade, CYP2B6, biotransformação de fármacos, perfil *tiolómico*, monitorização terapêutica de fármacos, perda de memória, alterações de humor.

INTRODUCTION

INTRODUCTION

1. Human immunodeficiency virus (HIV)-infection in brief

At 35 years ago, the scientific community has known the human immunodeficiency virus (HIV) infection, unaware of the social and economic impact that it would have worldwide as well as it severity and transversely to sex, age and transmission route. The history of HIV-infection started in the early 80´s with a description of pneumonia and *Kaposi*'s sarcoma in five homosexual patients with compromised immune response (Gottlieb *et al.*, 1981). Once classified as gay-related immune deficiency, the occurrence of this cluster of signs and symptomatology rapidly spread independently of sexual orientation, sex, age or race and was thereafter defined as *acquired immune deficiency syndrome (*AIDS) (Herek and Glunt, 1988). Two years were gone from those observations, when HIV was identified as the cause of AIDS (Barré-Sinoussi *et al.*, 1983; Gallo *et al.*, 1983) and several studies have presented the first cases of HIV-infected individuals among those that received blood transfusions, as hemophiliacs (CDC, 1982c); inmates, mostly drug addicts (Wormser *et al.*, 1983); healthcare workers (CDC, 1982b); woman (Harris *et al.*, 1983) and children (CDC, 1982a). At the beginning of the epidemic, without available therapy, the survival rate after diagnosis was measured in weeks or months and healthcare delivery was only based in HIV diagnosis and on control and treatment of AIDS-related opportunistic infections or malignancies. During the next years this epidemic was considered an acute disease with serious implications in the patient's life expectancy, which declined considerably. Since then, HIV was responsible for about 78 million infections and more than 35 million deaths (UNAIDS, 2016).

In short, the course of non-controlled HIV-infection starts with a clinical latent period, asymptomatic, with active viral replication associated with a progressive destruction of the cluster of differentiation 4 ($CD4^+$) cells (AETC, 2014). This stage can last an average of 8-11 years, depending on several factors including the person´s health status and life style. In this latency period, there are enough immune cells to afford a response against the infection. But, a significant number of T cells are eventually destroyed and the rate of its production cannot follow the rate of its destruction, triggering the patient to the symptomatic stage. After this, an AIDS-defining condition can be defined (AETC, 2014).

2. The combined antiretroviral therapy and HIV-infection conversion into a chronic disease

The urgency of achieving a treatment to stop this pandemic, triggered a wave of research combining laboratory and clinical sciences that allowed the discovery of HIV replication cycle (Fig. 1). This was the starting point for the massive pharmacological research and ultimately opened a new era for drug development. The *Food and Drug Administration* (FDA) approved the first antiretroviral drug (ARV), zidovudine, in 1987 (Brook, 1987). Only two years were passed between the *in vitro* tests of zidovudine anti-HIV efficacy and its approval (Mitsuya *et al.*, 1985), showing the urgent need of a weapon to fight this pandemic. However, it was soon realized that its use in monotherapy would fail due to viral drug resistance (Larder and Kemp, 1989). In the 90's, to surpass drug resistance, the association of three or more drugs acting on different steps of HIV cycle, the combined antiretroviral therapy (cART), was preconized and radically changed the natural history of HIV-infection (Palella *et al*., 2006). Nowadays, there are more than 20 available ARVs and it is estimated that an individual diagnosed with HIV-infection at 20 years of age that starts cART will live 50 or more years after diagnosis (Dieffenbach and Fauci, 2011). The cART is an example of how the research and investment in drug development can change the natural course of a disease, with an unprecedented availability of several drugs in a short period of time, allowing the HIV-infection to be currently perceived as a chronic disease (Fauci, 2003; Mehellou and De Clercq, 2010).

The cART comprises different alternatives of ARVs combinations, which clinicians try to be the most adapted to each patient. ARVs do not cure HIV-infection, but reduce significantly the amount of virus (viral load) in the patients' body as well as the deleterious AIDS-related consequences. Nowadays, the European and American guidelines for HIV therapy recommend cART use at any $CD4^+$ cell count or HIV stage (AID*Sinf*o, 2016; EACS, 2016).

ARVs are distributed in six classes according to their mechanism of action in the different steps of HIV life cycle (Fig. 1). Efavirenz (EFV) (Fig. 2) belongs to the non-nucleoside reverse transcriptase inhibitors (NNRTIs) that inhibit reverse transcriptase in a non-competitive manner (De Clercq, 2004). The first-line cART is composed by three drugs, two of which are nucleos(t)ide reverse transcriptase inhibitors (NRTIs) plus one of the following options: a protease inhibitor (PI) boosted with ritonavir; a integrase strand transfer inhibitors (INSTI); or a NNRTI (AID*Sinf*o, 2016; EACS, 2016).

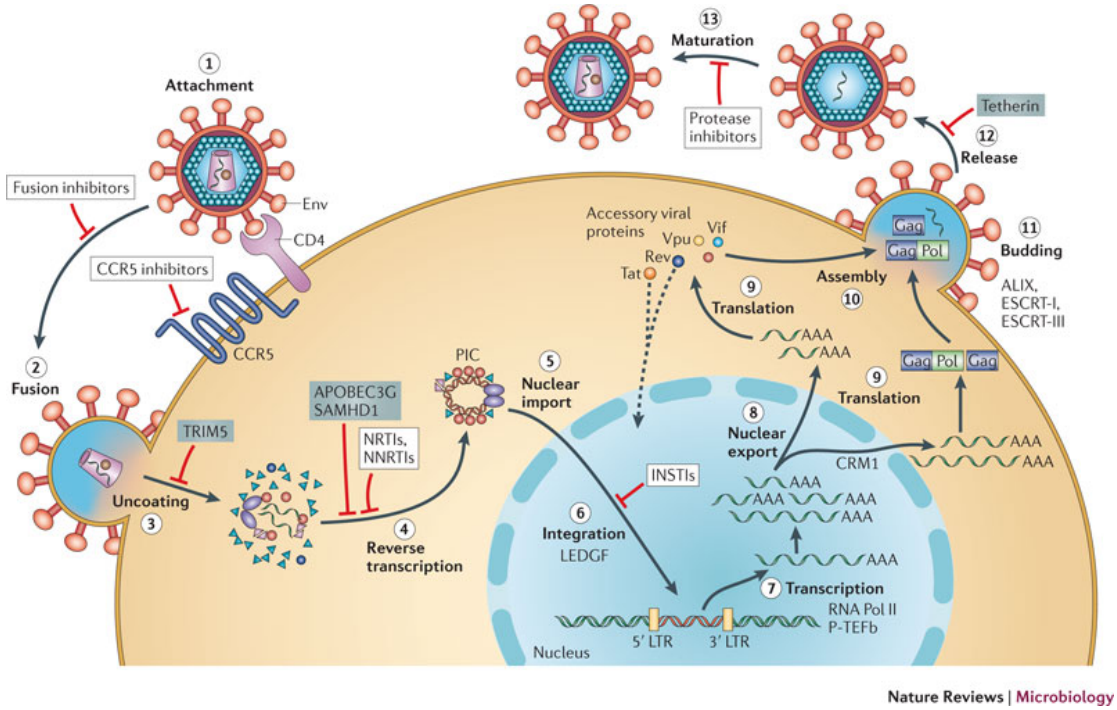

Figure 1. Schematic overview of the main steps of the human immunodeficiency virus (HIV)-life cycle and the targets for antiretroviral drugs. The **CCR5 antagonist (1)**, block the binding the viral glycoprotein 120 to the chemokine receptor 5 of the host cell; **FI (2**), fusion inhibitors bind to the viral glycoprotein 41 preventing the fusion of HIV with the host cell membrane; **NNRTIs (4),** non-nucleoside reverse transcriptase inhibitors, inhibit reverse transcriptase in a non-competitive manner; **NRTIs (4**), nucleoside/nucleotide reverse transcriptase inhibitors are analogues of the naturally occurring deoxynucleotides that first must be phosphorylated to their pharmacologically triphosphate derivate, to later competitively inhibit the virus reverse transcriptase; **INSTIs (6**), integrase strand transfer inhibitors target the integrase activity, which is the viral enzyme responsible for the integration of the viral DNA into the host cell genome; **PI (13),** protease inhibitors, block HIV protease, which is the responsible for cleavage precursor polyproteins to structural proteins (*eg*. p24) and functional proteins (*eg.* reverse transcriptase). In: Engelman and Cherepanov (2016).

Occasionally, the cART regimens need to be altered - for example in case of virological failure, adverse reactions, pregnancy, co-infections or drug interactions - and alternative cART need to be adopted. The levels of HIV RNA (viral load) are the preferred method for monitoring the response to cART. There should be at least 1 log reduction in the viral load, preferably to less than 10,000 copies/ml, within 2-4 weeks after the beginning of cART (AID*Sinf*o, 2016; EACS, 2016). If a lower reduction in viral load is observed or if it stays above 100,000 copies/mL the treatment should then be adjusted, by either adding or switching drugs. After six months, viral load should be maintained lower than 20 copies/mL (bellow quantification level). If it returns to 0.3-0.5 log of pre-treatment levels or there are two consecutive failures in attaining

undetectable value, the efficacy of cART is compromised and it should be changed. Viral load measurement should be repeated every 4-6 months in patients clinically stables (AID*Sinf*o, 2016; EACS, 2016). $CD4^+$ count is also used for monitoring cART efficacy (importantly before starting therapy). When $CD4^+$ drops to baseline or below 50% of increase from pre-treatment (approximately 30 cells.mm^{-3}) then the cART should also be changed. $CD4^+$ counts should be obtained every 3-6 months during periods of clinical stability and more frequently when symptomatic disease occurs (AID*Sinf*o, 2016; EACS, 2016).

3. HIV-infection panorama in combined antiretroviral therapy era: a matter of antiretroviral toxicity

At the end of 2015, the cART was available for 17 million people worldwide (UNAIDS, 2015). Chronic treatment with cART is presently unavoidable. Since the cART implementation, the causes of death of HIV-infected patients considerably changed from AIDS-related to non-AIDS-related diseases (Palella *et al*., 2006). This might be explained by the fact that patients live longer and several complications related with the normal aging process emerge at an earlier age, including cancer, cardiovascular, neurocognitive, liver and renal diseases (ATC, 2008; Deeks and Phillips, 2009). Furthermore, treated patients have persistent infection/inflammation, residual viremia, compromised immune system that together with life style risk factors (e.g. smoke and alcohol), co-infections (e.g. hepatitis and tuberculosis), drug-drug interactions, and importantly, the chronic exposure to antiretroviral toxicity, put HIV-infected population at higher risk of premature aging (Deeks and Phillips, 2009). Thus, nowadays there is an increasing concern about the toxic effects induced by ARVs, in particular those arising from long-term use. The adverse reactions of ARVs have a negative impact on clinical outcomes, ultimately affecting the life quality and expectancy of the patients. The management of toxicity outcomes also requires additional hospital visits and admissions, increasing the economic burden on already strained medical care systems (Núñez *et al.*, 2006).

4. The antiretroviral Efavirenz

4.1 Pros and cons

EFV (Fig. 2) was approved by FDA in 1998 (Vazquez, 1998) and is currently one of the most widely used NNRTIs, by both adults and children (Vazquez, 1998).

This antiretroviral is prescribed in combination with abacavir/lamivudine (Kivexa®) with tenofovir/emtricitabine (Truvada®) or in one-pill fixed-dose combination with tenofovir/emtricitabine (Atripla®). The availability of one pill combination significantly improved cART adherence, a major predictor of cART success, due to the simplicity and the lower quantity of pills that patients have to take daily (Maggiolo, 2009; Airoldi *et al.*, 2010). Additionally, EFV is an option during treatment with rifampicin for tuberculosis (AID*Sinf*o, 2016). These facts together with the availability of a generic EFV and its consequent lower price justify the high prescription of this drug.

Despite its benefits, EFV is associated to toxic effects that limit its use. For instance, based on animal studies and retrospective case-reports, FDA classified EFV as a pregnancy category D medication. In pregnancy this drug might be associated with neural tube and/or central nervous system (CNS) abnormalities (Ford *et al.*, 2011). Thus, special care should be given to women on EFV in reproductive age. These women may conceive while on EFV-based cART and continue EFV therapy during the high risk first trimester, before their pregnancy is diagnosed. Nevertheless, women diagnosed in the second or third trimester of pregnancy needing cART can receive EFV without a malformation concern (Bristol-Myers Squibb, 2015).

While liver and skin toxicity are less common (Phillips *et al.*, 2002; Rivero *et al.*, 2007), a significant number of individuals on the recommended EFV dose display CNS adverse reactions (Fumaz *et al.*, 2005; Kenedi and Goforth, 2011; Apostolova *et al.*, 2015b). These effects represent the main drawback of EFV and are a major factor for its discontinuation (Leutscher *et al*., 2013), limiting adherence and available cART options. EFV-CNS adverse reactions are of broad spectrum and consist mainly of sleep disturbances and other neurological symptoms (Kenedi and Goforth, 2011; Apostolova *et al.*, 2015b). Notably, up to 35% of patients on EFV-containing cART manifest mood-changes symptomatology (Fumaz *et al.*, 2005; Tashima *et al.*, 2008) and 40-50% present cognitive disorders, whereas 23-29% are related to memory (Lochet *et al.*, 2003; Ma *et al.*, 2016). In the few data available about EFV tolerability in children, the same CNS adverse reactions observed in adults were reported (Teglas *et al.*, 2001; Tukei *et al.*, 2012).

Most of EFV-neuro adverse reactions occur at a snapshot in time, during the first weeks of treatment and soon they fade away (short-term toxicity) (Fumaz *et al.*, 2005). However, they can persist for long-term in a significant proportion of patients, which may impact negatively patient's quality of life and lead to later EFV discontinuation (Leutscher *et al.*, 2013). In consequence of these adverse reactions, in the current year actualization, both European and American antiretroviral guidelines no longer recommend this NNRTI as first-line therapy as it has been since its availably in the market (AID*Sinf*o, 2016; EACS, 2016).

4.2 EFV pharmacology

The recommended dose of EFV is the oral daily intake of 600 mg for adults and adolescents (AIDS*info*, 2016). For children up to 3 years old and with more than 10 kg, EFV dose should be adjusted to their weight (AIDS*info*, 2016). Steady state maximal concentration (C_{max}) and area under the curve (AUC) are 4.07 mg/L (achieved in 3 to 5 h, Tmax) and 58.1 mg/L.h, respectively (Adkins and Noble, 1998). The half-life ($t_{1/2}$) of EFV is 19 h after long-term prescription of EFV containing cART in HIV-population (Csajka *et al.*, 2003), much shorter from what was described in non-HIV volunteers (40 – 55 h) (Adkins and Noble, 1998).

EFV is highly bounded to plasma proteins (> 99%), mainly albumin. This NNRTI is widely distributed, and it has been reported to accumulate in cells, reaching intracellular concentrations above its 90% inhibitory concentration (Almond *et al.*, 2005). EFV is able to cross the blood-brain barrier and reach the cerebrospinal fluid (CSF) with concentrations between 0.5 to 1.2% of its corresponding plasma concentrations. These concentrations represent the therapeutic concentration in brain, once these values are enough to inhibit HIV replication in CSF (Tashima *et al.*, 1999; Best *et al.*, 2011; Ene, 2011). The fact that EFV has a good penetration in brain is an advantage; since brain is an HIV reservoir, i.e., a compartment where HIV can persist for long periods of time in a quiescent state contributing to virus persistence.

EFV is predominantly biotransformed at the liver by cytochrome P450 (CYP) 2B6, with CYP3A4, CYP3A5, CYP1A2 and CYP2A6 being minor contributors (Ward *et al.*, 2003; Bélanger *et al.*, 2009; di Iulio *et al.*, 2009; Ogburn *et al.*, 2010). CYP2B6 is mostly responsible for the hydroxylation of EFV into 8-hydroxy-efavirenz (8-OH-EFV) (Fig. 2) (Ward *et al.*, 2003). Subsequent metabolic pathways of this metabolite include phase II conjugations, primarily glucuronidation (major) by multiple UDP-glucuronosyltransferases (UGTs) (Bae *et al.*, 2011) and sulfonation (minor) (Aouri *et al.*, 2016). Further oxidation into 8,14-dihydroxy-efavirenz (8,14-diOH-EFV, Fig. 2) is also possible by CYP2B6 isoform; however, this metabolite has been barely detected in man and no correlation with EFV-induced toxic events was identified to date (Belanger *et al.*, 2009; Bae *et al.*, 2011; Cho *et al.*, 2011; Avery *et al.*, 2013a). The formation of 8,14-di-OH-EFV is somehow not consensual. *In vitro* studies have shown that this metabolite was not produced when the 8-OH-EFV was incubated in human liver microsomes (Ogburn *et al.*, 2010). Accordingly, 8,14-di-OH-EFV is barely detected plasma. While the phase II 8,14-di-OH-EFV was found in urine, suggesting that 8-OH-EFV may first undergo a phase II conjugation step prior to the second hydroxylation at position 14 (Aouri *et al.*, 2016). Phase I aromatic hydroxylation of EFV, mostly by CYP2A6 (Ogburn *et*

al., 2010), can also yield 7-hydroxy-efavirenz (7-OH-EFV, Fig. 2), which is a minor non-toxic metabolite (Tovar-y-Romo *et al.*, 2012). Phase I metabolites are present at low levels in plasma (Cho *et al.,* 2011; Avery *et al.,* 2013, Aouri *et al*, 2016) whereas the phase II metabolites (8-OH-EFV-glucuronide (8-OH-EFV-Glc), 7-OH-EFV-Glc and 7-OH-EFV-sulfate) are found at comparable or even higher levels than EFV itself (Aouri *et al*., 2016). In fact, 8-OH-EFV and 8-OH-EFV-Glc are the major phase I and phase II metabolites, respectively, and both have been quantified in plasma, urine and CSF (Aouri *et al.*, 2016). The predominant mode of EFV elimination is through glucuronide metabolites in urine, with less than 1% of EFV eliminated in the unchanged form (U.S. Food and Drug Administration, 2015).

EFV is an inducer of its own metabolism through CYP2B6 (Ngaimisi *et al.*, 2010). This auto induction is dose-, duration of treatment- and genetically-dependent, resulting in lower EFV plasma concentrations (Ngaimisi *et al.*, 2010; Habtewold *et al.*, 2011). The induction period persists for long-term and is characterized by a growing 8-OH-EFV formation (Ngaimisi *et al.*, 2010). This induction period is also highly variable among patients in extension and magnitude (Ngaimisi *et al.*, 2010).

Being a substrate (Ward *et al.*, 2003), inhibitor (Xu and Desta, 2013) or inducer (Robertson *et al.*, 2008) of CYP450 and other enzymes and drug transporters, EFV has high potential for drug interactions. Some of these interactions and their management are well defined. An example is the co-administration of EFV and the tuberculostatic rifampicin, where a dose adjustment of EFV to 800 mg per day is recommended (Bristol-Myers Squibb, 2015).

While the drug transporters influencing the pharmacokinetics (PK) of EFV and its metabolites still to be properly identified, it is known that EFV inhibits several members of multidrug resistance-associated protein from ATP-binding cassette (ABC) type C subfamily (Weiss *et al.*, 2007).

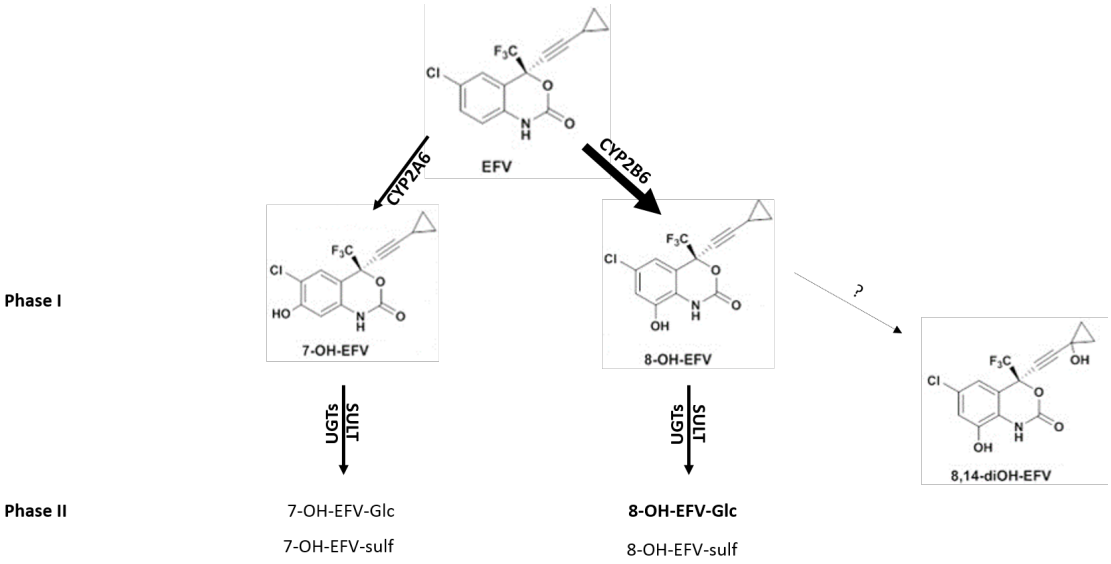

Figure 2. Efavirenz main biotransformation routes.

7-OH-EFV, 7-hydroxy-efavirenz; **7-OH-EFV-Glc**, 7-hydroxy-efavirenz-glucuronide; **7-OH-EFV-sulf**, 7-hydroxy-efavirenz-sulfate; **8-OH-EFV**, 8-hydroxy-efavirenz; **8-OH-EFV-Glc**, 8-hydroxy-efavirenz-glucuronide; **8-OH-EFV-sulf**, 8-hydroxy-efavirenz-sulfate; **8,14-diOH-EFV**, 8,14-dihydroxy-efavirenz; **EFV**, efavirenz; **SULT**, sulfotransferases; **UGT**, UDP-glucuronosyltransferases.

This NNRTI has a narrow therapeutic window (Marzolini *et al.*, 2001; Gutiérrez *et al.*, 2005; Pereira *et al.*, 2008) and exhibits considerable inter-individual variability in clinical response (Stahle *et al.*, 2004), which might be related to adherence issues, PK variability and drug interactions. In the beginning of the twenty-century, almost in parallel with the implementation of cART, the utility of therapeutic drug monitoring (TDM) was proposed as a mean to optimize response to ARV therapy (Aarnoutse *et al.*, 2003). TDM is a strategy based on repeated quantifications of drugs (drugs with a narrow therapeutic range) in patients' blood, with the aim of optimize drug response and give to the clinician the rational for an individualized treatment (Back *et al.*, 2002; Ivanovic *et al.*, 2008). The application of TDM to EFV was based on the following criteria: efficacy/toxicity relationship, high inter-patient variability, low intra-individual variability, high potential to drug interactions and a narrow therapeutic window (Ghiculescu, 2008). Marzolini and colleagues (2001) have defined the therapeutic window of EFV between 1-4 mg/L. In concentrations below 1 mg/L patients are exposed to sub-therapeutic EFV concentrations and are at higher risk of developing HIV resistance, while concentrations above 4 mg/L are related to CNS adverse reactions toxicity (Marzolini *et al.*, 2001). This study has led to a definition of a minimal toxic concentration of 4 mg/L. However, there are conflicting data regarding the relationship between higher plasma concentrations and

EFV-induced neurotoxicity. While several studies support this relationship (Marzolini *et al.*, 2001; Gutiérrez *et al.*, 2005; Rotger *et al.*, 2005) others did not establish an increased risk of neurotoxicity for that concentration (Fumaz *et al.*, 2005; Kappelhoff *et al.*, 2005; van Luin *et al.*, 2009a).

5. Mechanisms of EFV-neurotoxicity – what is known?

In the last years, several *in vitro* and *in vivo* studies have addressed plausible mechanism and key players on EFV neurotoxicity. Hereinafter, the major discoveries related to this topic are summarized. They are majorly based on *in vitro* and rodent approaches and related to EFV itself. Only recently, the role of EFV biotransformation into 8-OH-EFV has been focus of research.

To date, the existing *in vivo* studies on EFV neurotoxicity were performed in different rodent strains and only three of them performed behavior evaluation. The first, in 2005, by O´Mahony and co-authors have showed that *Wistar* rats orally exposed to EFV (10 mg/kg) during 34 days displayed spatial memory deficits (using Morris Water Maze, MWM) and higher susceptibility to stress (evaluated in the open-field test, OF) (O'Mahony *et al*., 2005). In another study, using CF-1 mice, EFV exposure (10 mg/kg during 36 days by oral intake) was associated to an anxiogenic-like effect (evaluated by elevated plus maze, EPM), without affecting the spontaneous locomotion (OF test). A substantial impairment of aversive memory, assessed by the inhibitory avoidance test, was also reported by these authors (Romao *et al.*, 2011). The most recent study described that EFV (range 10–30 mg/kg administrated intraperitoneally) induced a dose-dependent decrease in the open-field locomotor activity of male ND4 Swiss Webster mice and activated a significant head-twitch response in wild-type mice but not in 5-HT_{2A}-KO mice (Gatch *et al.*, 2013).

There are different and probably non-exclusive mechanisms that have been related to these observations, such as increase of pro-inflammatory cytokines and serotonin modulation. The first was described in *Wistar* rats orally exposed to EFV (10 mg/kg) during 34 days. The increased pro-inflammatory cytokines, interleukin 1β and tumor necrosis factor-α were partially reduced by administration of paroxetine, a selective serotonin reuptake inhibitor (O'Mahony *et al.*, 2005). Also, in *Sprague-Dawley* rats exposed to a concentration of EFV three times higher than the therapeutic (30 mg/kg, intraperitoneally), was observed that EFV has a similar response as lysergic acid diethylamine (LSD) in drug discriminative procedure, that was completely blocked by a selective antagonist of serotonin 2A (5-HT_{2A}) receptor (Gatch *et al.*,

2013). Additionally, EFV induced head-twitch response in wild-type mice but not in 5-HT_{2A}-knockout mice (Gatch *et al.*, 2013). Consubstantiated by radioligand binding assays, these authors suggested that EFV has an LSD-like effect in the manner that it can be a partial agonist of the 5-HT_{2A} receptor (Gatch *et al.*, 2013). The anomalous activity of 5-HT_{2A} receptor have been associated with several psychiatric conditions, including memory and learning impairment, mood changes, schizophrenia, and drug addiction (Zhang and Stackman, 2015).

EFV-induced neurotoxicity has also been associated to impairment in mitochondrial function and neural bioenergetics. In male CF-1 mice exposed to EFV (10 mg/kg) during 36 days, creatine kinase (CK) was diminished in cerebellum, hippocampus, striatum and cortex (Streck *et al.*, 2008). CK is important for energy homeostasis (Wallimann *et al*., 1992) and a decrease in CK activity is associated with neuronal loss following brain ischemia (Tomimoto *et al.*, 1993), neurodegenerative diseases (Aksenov *et al.*, 2000). Regarding mitochondrial impairment EFV affects cortex, striatum and hippocampus, at the level of complex IV activity (Streck *et al.*, 2008). In neural stem cells (NSC), EFV reduces cell proliferation, intracellular adenosine triphosphate (ATP), mitochondrial membrane potential as well as it increases lactate dehydrogenase (LDH) release, p38 MAPK (protein kinases that participate in the response to cell stress) activation and Bax expression (pro-apoptotic gene) (Jin *et al.*, 2016). The authors replicated the reduction in proliferating NSCs in the subventricular zone of C57BL/6 mice (Jin *et al.*, 2016). Other *in vitro* studies using SH-SY5Y (neurons) and U-251MG (glial cells) cell cultures, described a decrease in mitochondrial respiratory function, in a concentration-dependent manner upon acute EFV exposure (10 μM and 25 μM) (Funes *et al.*, 2015). EFV induced a drop in ATP production, which coincided with an increase in autophagy, mitochondrial fragmentation and depolarization (Funes *et al.*, 2015). Cell viability/proliferation was only reduced in SH-SY5Y. The authors also reproduce these results in primary cultures of rat neurons and astrocytes, confirming mitochondrial dysfunction and a higher impairment in neurons than astrocytes, which were only slightly affected at a higher concentration of EFV (Funes *et al.*, 2014). On opposite, no alterations in mitochondrial respiration were observed when rat primary astrocytes culture were exposed to 10 μM of EFV (Brandmann *et al.*, 2013). EFV only compromised cell viability at 10-times higher concentration (100 μM) (Brandmann *et al.*, 2013). Increasing evidence have been showing the role of mitochondrial dysfunction in CNS diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), dementia, multiple sclerosis, ataxia, encephalomyopathies, and depression (Maes *et al.*, 1990; Witte *et al.*, 2010; Moran *et al.*, 2012; Chaturvedi and Flint Beal, 2013).

EFV also promotes inducible nitric oxide synthase (iNOS) expression in cultured glial cells and generates nitric oxide (NO) (Apostolova *et al.*, 2015a), which might affect mitochondrial function and enhance reactive oxygen species (ROS) generation in a concentration-dependent manner and in both neurons and glia (Funes *et al.*, 2014). Neuroinflammation and oxidative stress are also common features in neurodegenerative diseases. In both AD and PD, oxidative stress activates inflammatory signaling pathways through several mechanisms, including exacerbating the production of ROS, mitochondrial dysfunction, activating microglia and astrocytes to release pro-inflammatory mediators (Whitton, 2007; Schapira, 2010; von Bernhardi and Eugenin, 2012).

EFV increases soluble amyloid beta (Aβ) and β-secretase-1 (BACE-1) expression, that is a key enzyme with an important role in Aß formation (Cole and Vassar, 2007). The role of Aß plaques formation in AD development is well known (Golde *et al.*, 2000; Sambamurti *et al.*, 2002) as these peptides have recognized neurotoxic, oxidative and inflammatory potential (Bradt *et al.*, 1998; Murakami *et al.*, 2005). By using a murine N2a cells transfected with the human "Swedish" mutant form of amyloid precursor protein (SweAPP N2a cells) and Tg2576 mice exposed to EFV (15 mg/kg in chow for a period of 10 days) it was shown that this drug is capable of promote BACE-1 activity and impair microglial clearance mechanisms, ultimately increasing Aß plaques production and leading to the disease profile of Alzheimer (Brown *et al.*, 2014).

More recently, *in vitro* studies have link neurotoxicity with EFV main metabolite, 8-OH-EFV. Using incubated rat hippocampal neuronal cultures with EFV or 8-OH-EFV or 7-OH-EFV it was shown that 8-OH-EFV was one order of magnitude more potent in inducing neurotoxicity than the others. Also, the minimal neurotoxic 8-OH-EFV concentration was three times lower than what is found in the CSF of HIV-infected patients (Tovar-y-Romo *et al.*, 2012). The exposure to 8-OH-EFV, in dose-dependent manner (0.01 μM – 10 μM), caused significant dendritic spines damage and induced rapid calcium influx in neurons, mediated by L-type voltage-operated calcium channels. Neurons appeared to undergo a dramatic loss of membrane integrity and release of the calcium probe. Other study showed that 8-OH-EFV stimulates the glycolytic flux in rat primary astrocytes culture in a time- and concentration-dependent manner, with maximal effects at 10 μM, and enhanced both lactate release and glucose consumption (Brandmann *et al.*, 2013). However, on contrary to what was expected no effects were observed regarding mitochondrial respiration at10 μM (Brandmann *et al.*, 2013). Another mechanism to 8-OH-EFV neurotoxicity was proposed by Harjivan and coauthors (2014), that reported the generation of toxic quinoid derivatives upon *in vitro* oxidation of 8-OH-EFV (Harjivan *et al.*, 2014).

6. An overlook on cytochrome P450 2B6 and efavirenz biotransformation

CYPs isoenzymes are heme-containing mixed function oxidases, which are involved in the metabolism of several endogenous and exogenous substrates. This superfamily of isoenzymes is localized in the endoplasmic reticulum of cells. Currently, there are 17 mammalian CYP gene families described that translates into approximately 60 distinct CYP forms (Nelson, 1999), displaying species-, sex-, tissue- and age-specific expression patterns, which will influence differently drug metabolism, activation, and detoxification. CYP450 enzymes are well conserved throughout species and are derived from ~1.36 billion years old ancestral gene (Lin, 1995). Members of the CYP2B subfamily have been identified among many mammalian species including man, monkeys, dogs, rabbits, and rodents (Martignoni *et al.*, 2006). The family member CYP2B6 was firstly described in 1989 and it is located on the long-arm of chromosome 19 (Yamano *et al.*, 1989). While primarily expressed at the liver, representing 2% to 10% of the total hepatic CYP450 content (Wang and Tompkins, 2008), CYP2B6 has also been detected in several extrahepatic tissues including brain, kidney, intestine, endometrium, peripheral blood lymphocytes and skin (Gervot *et al.*, 1999; Janmohamed *et al.*, 2001; Ding and Kaminsky, 2003).

Studies using human liver microsomes have revealed that the formation rate of 8-OH-EFV displays considerable variability between samples (Ward *et al.*, 2003), anticipating that EFV systemic exposure is likely to rely on inter-individual variability in CYP2B6 activity and with drug interactions involving this isoform. This has subsequently been corroborated by several pharmacogenetics studies focusing CY2B6 polymorphisms in HIV-infected patients on EFV (Nolan *et al.*, 2006). Importantly, EFV hydroxylation into 8-OH-EFV was elected as phenotyping tool for the evaluation of CYP2B6-mediated metabolism for *in vitro* a clinical studies by Food and Drug Administration (U. S. Food and Drug Administration, 2016).

Several factors contribute to CYP2B6 activity and might impact EFV biotransformation, such as genetics, sex, age, smoking and drug interactions (Zanger and Klein, 2013). Lamba and coauthors (2003), by using liver donors (from 80 ethnically mixed samples), showed higher amounts of CYP2B6 mRNA (3.9-fold), protein (1.7-fold) and enzyme activity (1.6-fold) among females compared to male subjects (Lamba *et al.*, 2003). In another study including 235 Caucasian individuals, female liver samples had 1.6-fold higher CYP2B6 expression levels than man, but this difference did not translate into higher protein or activity levels (Hofmann *et al.*, 2008). Contrariwise, higher plasma concentrations were found in female on EFV-containing cART compared to male individuals, highlighting the contribution of other

factors as race (these results were observed in non-Caucasians), age or body fat into EFV concentrations (Burger *et al.*, 2006; Nyakutira *et al.*, 2008; Mukonzo *et al.*, 2009). In fact, CYP2B6 phamacogenetics may have a higher contribution to EFV biotransformation than sex differences (Burger *et al*., 2006).

6.1. Efavirenz pharmacogenetics

The impact of CYP2B6 genetic variability on EFV response has been one of the most studied examples on pharmacogenetic utility in therapeutic response optimization. Most of the available data relate genetic variants with EFV concentrations. However, its relation with each type of CNS adverse reaction, with the treatment time-point that they occur (shortly after beginning or at long-term) and with PK of 8-OH-EFV still very poorly documented.

The variant CYP2B6 516G>T (*6) has been associated to a reduction in enzyme activity and elevated EFV plasma concentrations (Arab-Alameddine *et al.*, 2009; Mukonzo *et al.*, 2009; Yimer *et al.*, 2012). The studied populations are from different ethnic groups, among which CYP2B6*6 occurs at frequencies of 15% to over 60% (Zanger and Klein, 2013). As CYP2B6*6 is most frequent in the African American and African populations, this has been raising concern about EFV dose adjustments in these populations (Zanger and Klein, 2013). CYP2B6*6 (516G>T) was the genetic variant with the strongest association with EFV neurotoxicity (without discriminating the CNS adverse reaction). Also, it was associated to higher plasma concentrations at short-term treatment (Gounden *et al.*, 2010) and after one year on EFV (Sanchez Martin *et al.*, 2013). In other cohort, CYP2B6*6 was only associated with CNS adverse reactions at the first week of treatment but not at week 24 (Haas *et al.*, 2004), when the auto-induction of biotransformation period is already near equilibrium (Ngaimisi *et al.*, 2010). CYP2B6*6 was associated with a lower magnitude of the auto-induction of EFV biotransformation and to a lower 8-OH-EFV formation (Ngaimisi *et al*., 2010).

Other functionally alleles of CYP2B6 have been studied, for example *18, which is also responsible for a reduced expression and activity of the enzyme and its associated with higher EFV concentrations (Zanger and Klein, 2013). On the contrary, CYP2B6*22 is responsible for a gain of function (higher expression and activity) (Rotger *et al.*, 2007), but until now no relation with EFV, 8-OH-EFV or neurotoxicity was investigated.

In a limited number of studies, CYP3A4 and CYP2A6 allelic diversity were also shown to influence EFV PK variability, especially in CYP2B6 poor metabolizers (Arab-Alameddine *et al.*, 2009). The CYP2A6 polymorphisms are rare among Caucasians and more frequent in African populations. The variants CYP2A6*10, *17 and *9 are responsible for a decrease in protein activity and increased plasma concentrations of EFV (Nakajima *et al.*, 2006; Kwara *et al.*, 2009b).

Most of the pharmacogenetic studies have so far been focused on genetic variations of CYP450 enzymes, but genetic variations in nuclear receptors, drug transporters or conjugation enzymes can also contribute to the observed variability in EFV disposition. Genetic variants in constitutive androstane receptor (CAR) (540C>T) contribute to early treatment discontinuation of EFV-based regimens (Wyen *et al.*, 2011) and to lower EFV plasma concentrations (Cortes *et al.*, 2013). In fact, the induction of CYP2B6 by EFV is promoted by the upregulation of this receptor (Meyer Zu Schwabedissen *et al.*, 2012). The UGT2B7 enzyme genetic variants might also contribute to variations in EFV plasma concentrations, particularly UGT2B7*1 (low enzyme activity), which is responsible for an increase in EFV plasma concentration (Kwara *et al.*, 2009a). The 4036A>G and 4036G>G genotypes of ABCB1 were significantly associated with lower EFV plasma concentrations, while the ABCB1 1236C>T and 1236T>T genotypes were associated with higher EFV concentrations (Swart *et al.*, 2012a).

6.2. CYP2B6 substrates, inhibitors and inducers

The number of drugs recognized to be metabolized by CYP2B6 has been continuously increasing. This isoenzyme is responsible for the biotransformation of several therapeutic drugs that include antiepileptic *(e.g.* phenytoin (H. Wang *et al.*, 2004)); anticancer (*e.g.* prodrug of cyclophosphamide (Roy *et al.*, 1999)). Bupropion has been considered the probe substrate for CYP2B6 activity (Faucette *et al.*, 2000; Hesse *et al.*, 2001). CYP2B6 also metabolizes drugs of abuse as nicotine (Yamazaki *et al.*, 1999) and 3,4-methylenedioxy-methamphetamine (MDMA/ecstasy) (Kreth *et al.*, 2000)); pesticides (*e.g.* chlorpyrifos), pollutants (*e.g.* benzene) and neurotoxic contaminants (*e.g.* 1-methyl--4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)) (D. Lewis, 1996). Anandamide, testosterone and serotonin (Rosenbrock *et al.*, 1999; Fradette *et al.*, 2004; Sridar *et al.*, 2011) are endogenous substrates of this enzyme.

Among CYP2B6 inhibitors, triethylenethiophosphoramide (thioTEPA) is a strong inhibitor (Rae *et al.*, 2002) and clopidogrel, ticlopidine and prasugrel are weak inhibitors (Nishiya *et al.*,

2009). The antiretroviral tenofovir, which might be combined with EFV in cART (AIDS*info*, 2016) and the antifungal voriconazole are also weak inhibitors (weak) of CYP2B6 (U.S Food and Drug Administration, 2016).

Besides EFV, several drugs can induce CYP2B6, being phenobarbital the most recognized (Gervot *et al.*, 1999). Others examples include rifampicin (Faucette *et al.*, 2004), phenytoin (Wang *et al.*, 2004) and carbamazepine (Oscarson *et al.*, 2006). The ARVs nevirapine, which is never co-administrated with EFV (AIDS*info*, 2016) and ritonavir (only administrated in baby doses to HIV-infected patients) are also CYP2B6 inducers (Faucette *et al.*, 2007).
CYP2B6 expression is regulated by both CAR (Sueyoshi *et al.*, 1999) and pregnane X (PXR) (Goodwin *et al.*, 2001) receptor. When activated by inducers, these nuclear receptors are transported from cytoplasm to the nucleus, bind to DNA and increase CYP2B6 transcription (Meyer zu Schwabedissen *et al*., 2012). The target genes of CAR and PXR broadly overlap and both receptors interact with the same response elements in target gene promoters (Smirlis *et al.*, 2001). It is also known that together with EFV, nevirapine, carbamazepine, phenytoin preferentially and directly interacts with CAR. CAR-mediated EFV auto-induction is compartment-specific, occurring at liver and peripheral blood monocular cells (PBMC) but not at intestine (Meyer zu Schwabedissen *et al*., 2012).

6.3. Brain CYP2B6

CYP2B6 in the human brain are expressed in a cell- and region-specific manner and possibly have different putative roles within the brain (Miksys *et al.*, 2003; Dutheil *et al.*, 2009). CYP2B6-expressing brain cells include neurons and astrocytes (Miksys *et al.*, 2003). Together with the blood brain barrier (BBB), they create microenvironments in which CYP2B6 play a significant role in the local metabolism of substrates (Miksys and Tyndale, 2002) and may induce different responses to drugs and their metabolites. For example, most of CYP2B6 inducers have been studied at the hepatic microsomes (Faucette *et al.*, 2004), while scarce information exists about induction in the brain. In human brain CYP2B6 is inducible by nicotine and alcohol (Miksys *et al.*, 2003).

6.4. Rat CYP2B

Whereas tissue availability makes difficult to study CYP2B6 regulation in man, several similarities make the rat a suitable animal model in drug metabolism studies. CYP2B family is similar between humans (CYP2B6) and rodents (Cyp2b1 and Cyp2b2), with high homology and sharing substrates (Miksys *et al.*, 2000). Similarly, to human brain, Cyp2b1/2 is also present in rat brain in a tissue- and cell- dependent manner (Miksys *et al.*, 2000). Its expression is mainly regulated by CAR in man (Sueyoshi *et al.*, 1999) and rat Cyp2b has also been described to be regulated by the orthologous CAR (Muangmoonchai *et al.*, 2001). The prototypal CYP2B6 inducer phenobarbital has been proved as a Cyp2b inducer in rat (Ganem *et al.*, 1999) as well as there is Cyp2b induction in brain by nicotine (Miksys *et al.*, 2000), phenobarbital (Schilter *et al.*, 2000) and phenytoin (Rosenbrock *et al.*, 1999).

7. Bioactivation of 8-hydroxy-efavirenz – a plausible mechanism for EFV neurotoxicity

In vitro studies have recently supported the plausibility of 8-OH-EFV to be bioactivated. When exposed to oxidative conditions (by the oxidative agent Frémy's salt, which is frequently used to obtain quinones from phenolic compounds), the 8-OH-EFV was considerably more prone to oxidative degradation than 7-OH-EFV, yielding a quinone-imine derivative (Fig. 3) (Wanke *et al.*, 2012; Harjivan *et al.*, 2014). Despite the possibility of a catechol intermediate to be formed upon 7-OH-EFV oxidation, no quinoid derivative was obtained, under similar oxidative conditions. In contrast, a stable metabolite was obtained. These contrasting behaviors under oxidative conditions may explain the distinct toxicities of the two phenolic EFV metabolites observed in primary rat neuron cultures (Tovar-y-Romo *et al.,* 2012). In fact, quinoid species (quinone and quinone-imines) are reactive electrophiles (as Michael acceptors) and can easily react with nucleophiles from macromolecules (*e.g.,* proteins and DNA) and generate covalent adducts (Bolton *et al.*, 2000; Monks and Jones, 2002). Additionally, due to their pro-oxidant activity and redox cycling, these quinoid intermediates are involved in the formation of reactive oxygen species. Therefore, the role of quinoid derivatives to electrophilic/oxidative stress, is frequently involved in the onset of toxic events. Many electrophilic quinoid species can disrupt brain function (Lopachin and Decaprio, 2005). One example is the oxidation of dopamine or L-dihydroxyphenylalanine-(L-DOPA) to a quinone metabolite, that is neurotoxic via formation of covalent adducts with proteins (Asanuma *et al.*, 2003).Notably, EFV has been associated with selective genotoxicity in brain, but not in heart, liver or PBMCs, supporting the hypothesis

of DNA-adducts formation in CNS compartments (de Oliveira *et al.*, 2014). This observation was true for mice exposed to EFV (orally treated with 10 mg/kg EFV) during 36 days (chronic administration), but not to those exposed only to a single-dose (acute administration).

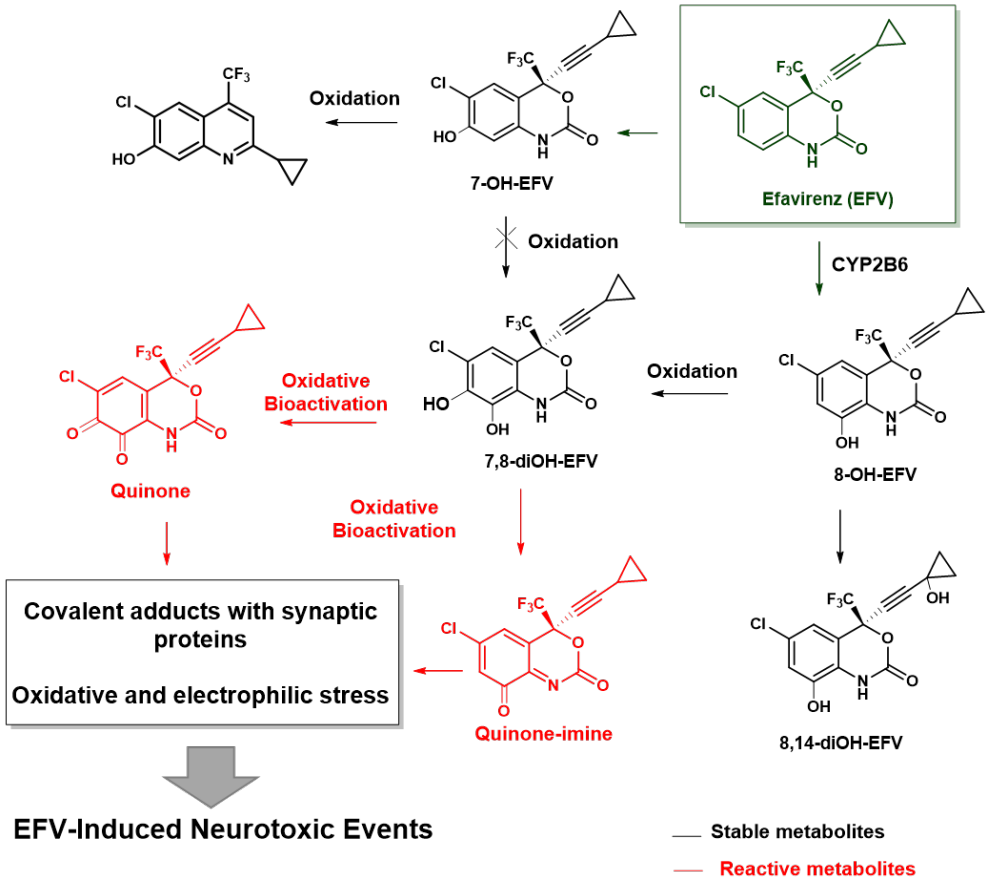

Figure 3. Distinct behaviors of EFV Phase I metabolites, 7-OH-EFV and 8-OH-EFV, under oxidative conditions *in vitro*. Contrasting with 7-OH-EFV, the main EFV metabolite, 8-OH-EFV, affords reactive quinoide metabolite under oxidative conditions. The capacity of these reactive metabolites to form covalent adducts with proteins and of promoting oxidative/electrophilic stress may explain the role of 8-OH-EFV bioactivation in the onset of EFV-induced neurotoxic events. **7-OH-EFV**, 7-hydroxy-efavirenz; **8-OH-EFV**, 8-hydroxy-efavirenz; **8,14-diOH-EFV**, 8,14-dihydroxy-efavirenz; **EFV**, efavirenz;

It is well known that covalent modifications negatively impact structure and/or function of proteins and thereby interfere with energy metabolism, axonal transport or presynaptic neurotransmitter release. Therefore, protein adducts are involved in primary pathophysiological brain processes (Asanuma *et al.*, 2003; Lopachin and Decaprio, 2005). Moreover, the main targets of these reactive metabolites are cysteine residues within proteins, which have a

sulfhydryl/thiol group (-SH) (Dickinson and Forman, 2002), and the redox status of brain thiols is determinant for neurotransmission (LoPachin and Barber, 2006).

Thiols with high nucleophilic reactivity are found in diverse synaptic proteins (Fig. 4). Briefly, during a synapse, the action potential in the presynaptic nerve depolarizes the presynaptic membrane, opening the voltage-gated Ca^{2+} channels. The rise of Ca^{2+} causes the fusion of the plasma membrane with the transmitter-filled synaptic vesicle, followed by the release of neurotransmitter into the synaptic cleft by exocytosis. The transmitter molecules diffuse across the synaptic cleft, bind to the receptor in the postsynaptic membrane and thus modulate the excitability of the cell. On the presynaptic membrane occurs the retrieval of the synaptic vesicle by endocytosis and the refill of the vesicle with neurotransmitters (Siegel *et al.*, 2006). In this process many critical steps exist (e.g., ligand-gated ion flux, receptor binding, membrane fusion, presynaptic release of neurotransmitter) that are influenced by the redox state of the sulfhydryl groups (Barber and LoPachin, 2004). In the case of presynaptic release of neurotransmitters, the neural soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex (involved in the vesicle-membrane fusion (Sudhof and Rizo, 2011)) and the *N*-ethylmaleimide-sensitive fusion (NSF, involved in the dissociation of SNARE after vesicle fusion (Whiteheart *et al.*, 2001)) are cysteine rich. In fact, through mass spectrometry analysis, the formation of acrylamide adducts with NSF was observed, which might be responsible for reduced NSF activity and synaptosomal neurotransmitter release, and increased SNARE complex in acrylamide exposed synaptosomes (Barber and LoPachin, 2004). The vulnerability of synapses to toxicants action is also related to a higher rate of NO modulation and relatively slow turnover of NO in neuronal tissue (Esplugues, 2002). Additionally, the nerve terminal lacks the ability to initiate transcription based cytoprotective responses (LoPachin and Barber, 2006).

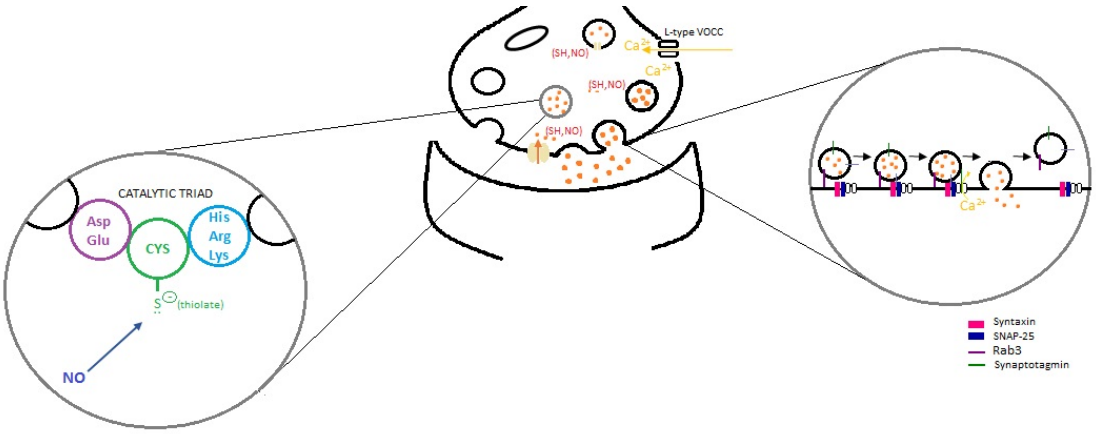

Figure 4. Cysteine sulfhydryl groups of synaptic proteins and synaptic activity regulation.
NO, nitric oxide; **CYS,** cysteine; **-SH,** sulfhydryl group.

Thiols have important roles in metabolism and homeostasis of the biological systems, as the maintenance of the antioxidant system and in the detoxification of several molecules and xenobiotics (Bald *et al.*, 2004; Prakash *et al.*, 2009). Also, most protein thiols have non-catalytic functions, such as regulation of protein activity or protein folding (Jacob *et al.*, 2003). Both redox signaling and sulfhydryl homeostasis are important features in the context of brain diseases (Sabens Liedhegner *et al.*, 2012).

The –SH group exist in low molecular weight thiols (LMWT), *e.g*., as glutathione, and in cysteine residues of proteins (Dickinson and Forman, 2002). LMWT can be found in their free reduced form (RSH) (*e.g.*, glutathione (GSH), its precursor cysteine (CysSH), and its product of degradation cysteinylglicine (CysGlySH)) and as disulfides (RSSR). This latter form include homodisulfides, formed between two identical thiols (e.g., cystine (CysSSCys), glutathione disulfide (GSSG)) and heterodisulfides/mixed disulfides, formed between two different thiols (Rossi *et al.*, 2009). GSH is the major non-protein thiol involved in antioxidant defenses and its redox status is critical for a variety of biological process including cell regulation, inflammation, and apoptosis (Bojes *et al.*, 1999; Rahman and MacNee, 2000; Guoyao Wu *et al.*, 2004).

Protein thiols include the sulfhydryl groups of cysteine and protein mixed disulfides with thiols as cysteinylglycine (protein cysteineglicinylation, PSSCysGly), cysteine (protein cysteinylation, CysSSP) and glutathione (protein glutathionylation GSSP), referred as protein *S*-thiolation (RSSP) (Eaton, 2006). The largest pool of LMWT in human plasma is the CysSH)/CysSSCys redox couple while in cells is GSH, mainly in its reduced form (Rossi *et al.*, 2009). Therefore, *S*-glutathionylation occurs mainly in intracellular proteins whereas *S*-cysteinylation is predominantly in the extracellular compartments (Rossi *et al.*, 2009). In human plasma, the concentration of protein sulfhydryl groups is much higher (mM range) than LMWT (μM range) (Mansoor *et al.*, 1992; Giustarini *et al.*, 2006). Protein *S*-thiolation can be reversible or irreversible; both cases are associated with important biological functions and brain pathological states (Cooper *et al.*, 2011). Several factors can influence this linkage; from the enzymes involved *e.g*. glutaredoxin enzymes (Dalle-Donne *et al.*, 2009) or glutathione *S*-transferases (GST) (which can also be glutathionylated) (Townsend *et al.*, 2009) to the accessibility of cysteine residues (Newman *et al.*, 2007). It is not completely understood if this modification serves for the activation of oxidative sensitive signaling pathways (Dalle-Donne *et al.*, 2003; Baty *et al.*, 2005) and/or as an adaptive protection response from irreversible oxidation of thiol groups of cysteine residues (Seres *et al.*, 1996; Grant *et al.*, 1999; Dalle-Donne *et al.*, 2005a; Dalle-Donne *et al.*, 2005b; Dalle-Donne *et al.*, 2006).

Thesis outline

The results of this thesis and their discussion are divided in three chapters. **Chapter 1** comprises the results from the clinical study performed in HIV-infected individuals. This chapter is an original article published in *Toxicology Letters*. In **Chapter 2** are presented the results regarding the study of time- and tissue-dependence of EFV biotransformation, performed in an animal model. This chapter is submitted for publication. **Chapter 3** contains the results regarding neurological, histological and molecular evaluation performed in an animal model long-term exposed to EFV. This last chapter is being prepared for publication. Lastly, it is presented the **final considerations** regarding all the work here discussed.

General and Specific Aims

GENERAL AND SPECIFIC AIMS

Which were the main five facts that represented the starting points for our study design?

1. Despite the correlation between EFV and CNS adverse reactions was consolidated for a long time, the mechanisms underlying these events were yet to be elucidated;
2. These neuro-adverse reactions are variable among patients in type, severity, time of onset and persistence.
3. EFV clearance is highly variable among patients and higher EFV concentrations were related with EFV neuro-adverse reactions. Nonetheless, the benefit of therapeutic drug monitoring and dose adjustment for the minimization of neuro-adverse reactions was not consensual.
4. CYP2B6 polymorphisms are associated to high inter-patient variability in EFV clearance. The 8-OH-EFV is the main Phase I metabolite generated through CYP2B6 activity.
5. There was a demand for a suitable animal model that could allow to understand the mechanisms underlying EFV-CNS adverse reactions.

Facing these facts, the **general aim** of this thesis was to study EFV biotransformation as an upstream event of the mechanisms underlying EFV-induced CNS adverse reactions. Additionally, it was aimed to identify mechanistically oriented markers of EFV-CNS adverse reactions that allow neurotoxicity risk assessment as well as the evaluation of strategies for the optimization of EFV use.

To achieve these aims a translational approach was carried out by performing a clinical study with HIV-infected patients on EFV and an *in vivo* animal model of *Wistar* rats exposed to EFV.

The **specific aims** of the present work are the following:

1. Develop and validate a method for the quantification of EFV and its main metabolite;
2. Measure 8-OH-EFV plasma concentrations and phenotype CYP2B6 activity in a case-control study of HIV-infected patients on long-term combined antiretroviral therapy-containing EFV with or without mood changes;
3. Investigate the time-dependency of EFV biotransformation via CYP2B6 activity in *Wistar* rats with short (10 days) and long (36 days)-term EFV exposure;
4. Explore the tissue-dependency of EFV biotransformation via CYP2B6 activity, comparing two brain areas (prefrontal cortex and hippocampus) with the liver;

5. Determine EFV-induced short- and long-term oxidative stress-related *thiolomic* signature and its tissue-dependence;
6. Investigate the behavioral phenotype of *Wistar* rats exposed to long-term EFV;
7. Compare prefrontal cortex and hippocampus histological changes, synaptic and neuronal function induced by long-term EFV exposure.

Chapter 1

Efavirenz biotransformation as an up-stream event of mood changes in HIV-infected patients

CHAPTER 1
Efavirenz biotransformation as an up-stream event of mood changes in HIV-infected patients

1.1 SUMMARY

EFV is a drug of choice for adults and children infected with the human immunodeficiency virus. Notably, up to 35% of patients on EFV suffer from mood changes.
This work aimed to investigate EFV biotransformation into 8-OH-EFV as an up-stream event of mood changes and to evaluate the suitability of 8-OH-EFV biomonitoring for the minimization of these manifestations. A case-control study with two age-matched groups of HIV-infected male patients was performed in a group without adverse central nervous system complaints (28 patients) and a group presenting mood changes (14 patients). The plasma concentration of non-conjugated 8-OH-EFV was higher in patients with mood changes (p=0.020). An association between EFV and 8-OH-EFV-Glc was found (*Spearman* r=0.414, p<0.010), only within therapeutic EFV concentrations. This correlation was not observed in patients with toxic (> 4 mg/L) plasma concentrations of the parent drug. We conclude that metabolism to 8-OH-EFV is associated with EFV-related mood changes, which suggests that the concentration of this metabolite is a suitable parameter for therapeutic drug monitoring aimed at controlling these manifestations. Moreover, our data suggest that 8-OH-EFV is able to cross the blood-brain barrier and that the peripheral detoxification of 8-OH-EFV by glucuronidation may be inhibited by toxic EFV concentrations.

1.2 MATERIALS AND METHODS

1.2.1 Patients

The study protocol received prior approval from the Ethics Committee of *Centro Hospitalar de Lisboa Central, EPE (*115/2013). Patients gave their written informed consent in accordance with the Declaration of Helsinki (attachment #1). Compliance was controlled by the clinician. All patients were male adults, with documented HIV-infection, on EFV-containing cART (600 mg once daily) for at least one month prior to the study and regardless of past therapeutic history. Patients that fulfilled the inclusion criteria were sequentially included during 12 months. Whenever present, the psychiatric effects including mood changes (anxiety, agitation,

euphoria, mental confusion, paranoia, hallucinations, and/or depression) were recorded according to the evaluation by the clinician in charge and the self-reported complaints made by the patients. For each EFV-treated patient with mood changes (study group, 14 patients) two age-matched EFV-treated patients without any CNS complaints were selected (control group, 28 patients). Exclusion criteria were having AIDS-defining conditions and compliance issues. The following data were also gathered for each patient: age, time on EFV-containing cART, antiretroviral co-medication, time between blood sampling and last EFV dose intake, viral load, $CD4^+$ T-cell count (attachment #2).

Blood samples (2 mL) were collected into ethylenediaminetetraacetic acid (EDTA)-containing tubes. Plasma was obtained by centrifugation at 3000 *g* for 10 min, at 4 ºC. Samples were stored at -80 ºC until further analysis.

1.2.2 High performance liquid chromatography method: development and validation

1.2.2.1 Materials

EFV was kindly provided by Dr. Frederick A. Beland (National Center for Toxicological Research, Arkansas, USA). The 8-OH-EFV and 7-OH-EFV metabolites were synthesized as described previously (Harjivan *et al.*, 2014). The 8,14-diOH-EFV metabolite was obtained from *Toronto Research Chemicals Inc.* (Canada). The pure compounds used for method interference screening were obtained from the *NIH AIDS Reagent Program* (Protocol PT090513.01). Rifampicin was kindly supplied by *Aventis Pharma* (Mem Martins, Portugal). β-Glucuronidase (Type VII-A from *E.coli*, E.C. 3.2.1.31, 1000 U/mL) was purchased from *Sigma-Aldrich* (St. Louis, MO, USA). *n*-Hexane, acetonitrile, methanol, *tert*-butyl methyl ether and sodium acetate were supplied by *VWR* (Belgium). Formic acid was purchased from *Carlo Erba* (Milano, Italy). Calibrants and quality control samples were prepared using a pool of plasma from healthy volunteers provided by *Instituto Português do Sangue*, Lisbon, Portugal, under protocol FCM/IPS-P-0207/CD/3HT/sr.

1.2.2.2 Stock solutions, standard solutions and sample pre-treatment

Calibration standards (CSs) were prepared by successive dilutions to obtain eight different concentrations in plasma, 0.1 to 10 mg/L for EFV and 0.25 to 10 mg/L for 8-OH-EFV.

Quality control (QC) samples were prepared from a distinct stock solution by successive dilutions to obtain final concentrations in plasma of 5, 1.5, and 0.25 mg/L (QC1, QC2, QC3, respectively) for EFV and 5, 2.5, and 1.5 mg/L (QC1, QC2, QC3) for 8-OH-EFV.
Aliquots of plasma samples from patients, CSs and QCs (600 µL) were heated at 60 ^{o}C for 60 min, for virus inactivation, before handling at room temperature and then 2 mL sodium acetate (0.2 M, pH 6.8) was added. One aliquot was treated with β-glucuronidase (60 µL, 1000 U/mL) and the other without the enzyme (60 µL of deionized ultrapure water) and both were incubated at 37 ^{o}C, for 60 min. Analytes were extracted with 7.5 mL of *tert*-butyl methyl ether and the organic phase was isolated and dried under vacuum (-80 kPa, at 60 ^{0}C). The dried residue was reconstituted in 200 µL of acetonitrile: 0.1% formic acid buffer (1:1) plus 2 mL *n*-hexane and the mixture was homogenized and centrifuged. The aqueous phase was analyzed by high performance liquid chromatography (HPLC).

1.2.2.3 High-performance liquid chromatography

The separation of the analytes by HPLC was performed on an Agilent 1100 Series equipment (*Agilent Technologies*, Santa Clara, CA, USA), using a reversed-phase Luna C18 column (250 mm ×4.6 mm; 5 µm; 100 Å; *Phenomenex*, Torrance, CA, USA). The mobile phase consisted of 0.1% formic acid buffer (pH 2.65) (A, 65%) and acetonitrile (B; 35%), delivered at a flow rate of 1.2 mL/min for the first 5 minutes. Then the percentage of solution A was gradually decreased to 55% for 10 min and maintained at this value for a period of 16 minutes. Finally, this percentage was gradually diminished to 49% during 5 minutes and maintained for the subsequent 7 minutes. The column temperature was set to 30 °C, the injection volume was 100 µL, and ultraviolet (UV) absorbance was monitored at 246 nm.

1.2.2.4 Method validation

The linearity, lower limit of quantification (LLOQ), carry-over effect, accuracy, intra- and inter-assay precision, and recovery of the method, as well as the stability of samples after two sequential freezing cycles at -80 ºC, were evaluated. The validation criteria followed standard procedures for bioanalytical methods (European Medicines Agency, 2011; Services, 2013).
The interference of plasma compounds, 7-OH-EFV and 8,14-diOH-EFV, and other antiretroviral drugs (abacavir, atazanavir, darunavir, emtricitabine, lamivudine, lopinavir, raltegravir, ritonavir, saquinavir, tenofovir and zidovudine) with EFV and 8-OH-EFV was also

evaluated. Due to the common co-administration with antiretrovirals, the interference of the anti-tuberculosis drug rifampicin was also assessed.

1.2.3 Data analysis

Systemic exposure to EFV and 8-OH-EFV was assessed in terms of absolute concentrations and metabolite ratios. The concentrations of 8-OH-EFV-Glc (expressed in mg/mL) were calculated as the difference between the analyte concentrations obtained with and without β-glucuronidase treatment, corrected for the molecular weight change upon conjugation.
Statistical analysis was performed using Graph Prism® 5.0 (GraphPad Software Inc., San Diego, CA, USA). Data were expressed as the mean ± standard error of the mean (SEM), median (interquartile range; IQR), percentage or coefficient of variation (CV), whenever applicable. To test normality among groups, the *Shapiro-Wilk* test was used. The comparisons between the groups were performed using the *Mann Whitney* test. The *F*-test was used to explore differences between the slopes of the calibration curves in the method validation.

1.3 RESULTS

1.3.1 Method Validation

Under our HPLC conditions, 8-OH-EFV (eluting at 30 minutes) and EFV (eluting at 41 minutes) were well separated (not shown). With the exception of lopinavir, which co-eluted with EFV, no interference from the other tested drugs was observed at the retention times of EFV or any of its metabolites. This did not affect our measurements, since none of the patients included in the study were on lopinavir-containing cART.

The evaluation of linearity was performed using CSs ranging from 0.25 to 10 mg/L for 8-OH-EFV and from 0.1 to 10 to mg/L for EFV. The linear regression model showed to be the most suitable for fitting a function to the experimental data (*Run Test* $p > 0.05$). The concentration of the standard samples significantly influenced the chromatographic signal area (*F tests* $p < 0.001$) for the two analytes. The correlation coefficient was > 0.99 for both 8-OH-EFV and EFV. For the calibration curves of both analytes, the 95% confidence interval for the intercept contained zero. The average back-calculated concentrations were close to the

expected theoretical values at each tested concentration and presented differences lower than 12% for both analytes.

The intra- and interassays of multiple measures of each standard showed a CV lower than 11% for both EFV and 8-OH-EFV.

The LLOQ of the method was 0.1 mg/L for EFV and 0.25 mg/L for 8-OH-EFV and no carry-over effect or significant deviation from the nominal concentration after two freezing cycles were observed.
For both analytes, the accuracy ranged between 89-101%, the intra- and interassay precision between 2-11%, and recovery was higher than 90%.

1.3.2 Patients

A total of 42 male patients (14 with mood changes) were included in the study. The most prevalent mood change was anxiety, in 71% of the patients.
Anthropometric and clinical data of the patients are presented in Table 1. There were no differences between groups regarding race, age, time on EFV and time between sampling and last EFV intake. The patients had no detectable viral load and their immunologic ($CD4^+$ T cell counts) and hepatic condition (alanine aminotransferase) did not differ between groups. Hepatitis C co-infection occurred in three patients from the control group. Smoking and alcohol intake habits were self-reported, but the distribution was similar in the control and study groups.

Table 1. Anthropometric and clinical data.

Parameter		Control Group	Study Group	*p* value
Number of patients		28	14	
Non-Caucasians (%)		14	7	ns [a]
Age (years)		44 (36 - 52)	42 (36 - 51)	ns [b]
Alcohol intake (%)	No	46	69	ns [a]
	Social	32	23	ns [a]
	Chronic	21	8	ns [a]
Smokers (%)		50	31	ns [a]
Time on EFV (years)		4 (2 - 7)	5 (2 - 10)	ns [b]
$CD4^+$ T-cell count (cells/mm^3)		626 (494 - 798)	610 (464 - 778)	ns [b]
ALT (U/L)		29 (20 - 39)	27 (19 - 38)	ns [b]
Time between sampling and last EFV intake (h)		17 (16 - 19)	17 (15 - 19)	ns [b]

Control group: HIV-treated patients without any CNS complaint; **Study group:** HIV-treated patients presenting mood changes. Data are presented as median (IQR) or number (%). [a] χ^2 test. [b] *Mann–Whitney*-U-test. **ALT**, alanine aminotransferase; **EFV**, efavirenz; **ns**, not significant.

The plasma concentrations of EFV, 8-OH-EFV, and 8-OH-EFV-Glc are presented in Table 2. No differences between groups were found in EFV levels. Two patients from the control group and one from the study group had EFV concentrations higher than 4 mg/L, the proposed minimal toxic concentration (Marzolini *et al.*, 2001).

Patients with mood changes had higher plasma concentrations of 8-OH-EFV than those in the control group and this represented the only significant difference in analyte levels found between groups. The 8-OH-EFV/EFV ratio tended to be higher in the study group, although the difference did not reach statistical significance.

The major metabolite found in circulation was 8-OH-EFV-Glc in both groups. Its concentration was 11-fold higher than that of 8-OH-EFV in the control group and 8-fold higher in the study group.

Table 2. Plasma concentrations of efavirenz, 8-hydroxy-efavirenz and 8-hydroxy-efavirenz-glucuronide.

Analyte	Control Group 28 patients	Study Group 14 patients	*p* value
EFV (mg/L)	1.55 (1.38 - 2.11)	1.88 (1.50 - 3.11)	ns
8-OH-EFV (mg/L)	1.04 (0.80 - 1.69)	1.69 (1.03 - 3.19)	0.020
8-OH-EFV-Glc (mg/L)	11.74 (6.15 - 14.05)	13.85 (9.17 - 27)	ns
8-OH-EFV/EFV	0.68 (0.40 - 0.92)	0.81 (0.31 - 1.84)	ns

Control group: HIV-treated patients without any CNS complain; **Study group**: HIV-treated patients presenting mood changes. Data are presented as median (IQR). *Mann–Whitney* U-test. **EFV**, efavirenz; **8-OH-EFV**, 8-hydroxy-efavirenz; **8-OH-EFV-Glc**, 8-hydroxy-efavirenz-glucuronide; **ns**, not significant.

We found that the 8-OH-EFV and EFV concentrations were not related. Conversely, within the therapeutic range of EFV concentrations (1 - 4 mg/L) (Marzolini *et al.*, 2001) the present data indicate a positive association between the concentrations of 8-OH-EFV-Glc and EFV (*Spearman* r = 0.414, $p < 0.010$) (Fig. 5). This correlation was not observed at toxic (> 4 mg/L) concentrations of EFV (Fig. 5).

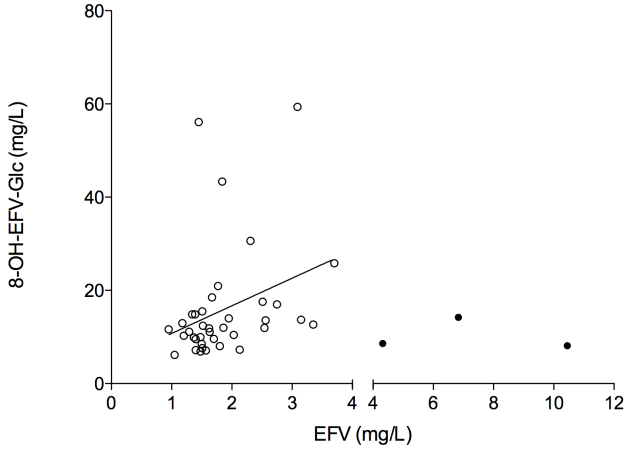

Figure 5. Correlation between EFV and 8-OH-EFV-Glc. EFV plasma concentration is associated with 8-OH-EFV-Glc plasma concentration (Spearman r = 0.414, p = 0.010, n=39) at non-toxic EFV levels. This association was not observed for patients with EFV concentrations higher than minimal toxic EFV concentration (4 mg/L). EFV, efavirenz; 8-OH-EFV-Glc, 8-hydroxy-efavirenz-glucuronide. Open circles: patients with therapeutic EFV concentrations; Full circles: patients with toxic (> 4 mg/L) EFV concentrations.

1.4 DISCUSSION

This work was aimed at evaluating the plasma concentrations of EFV and its metabolites, 8-OH-EFV and 8-OH-EFV-Glc, in a case-control study comparing HIV-infected male patients with or without mood changes complaints. Herein we have demonstrated the feasibility of using this straightforward methodology, rather than more expensive and elaborate liquid-chromatography-mass spectrometry approaches, to monitor EFV and 8-OH-EFV at clinically relevant levels.

Literature data reporting the dependence of CNS adverse reactions on EFV concentration are contradictory (Ngaimisi *et al.*, 2010; Cho *et al.*, 2011; Habtewold *et al.*, 2011; Tovar-y-Romo *et al.*, 2012; Aouri *et al.*, 2016). This is probably due to the high inter-individual PK variability of EFV (Pereira *et al.*, 2008), which is largely attributed to its biotransformation (Marzolini *et al.*, 2001; Fumaz *et al.*, 2005; Pereira *et al.*, 2008;Tashima *et al.*, 2008; van Luin *et al.*, 2009b; Kenedi and Goforth, 2011; Pereira *et al.*, 2012; Apostolova *et al.*, 2015b; Winston *et al.*, 2015; Aouri *et al.*, 2016). The discrepant data reported might also reflect the broad type of symptoms considered. Moreover, literature data on relationships between EFV metabolites and a particular group of CNS manifestations have yet to be provided (Winston *et al.*, 2015; Aouri *et al.*, 2016). These uncertainties prompted us to investigate EFV-induced mood changes.

The most frequent mood change recorded in our study group was anxiety, which is in agreement with previously reported clinical studies (Fumaz *et al.*, 2002; Fumaz *et al.*, 2005; Rihs *et al.*, 2006). This is also in line with data reporting an anxiogenic effect in male CF-1 mice upon chronic exposure to EFV (Romao *et al.*, 2011). Additionally, our results suggest that mood changes are related with plasmatic concentrations of 8-OH-EFV. This contrasts with the lack of consistent correlations reported in the literature, which might be a reflection of our effort to eliminate potential variability factors from the current study (*e.g.*, type of CNS effect, the time on EFV, PK parameter and sex).

EFV is a moderate inducer of CYP2B6 (U.S. Food and Drug Administration, 2012), the major isoform responsible for the formation of 8-OH-EFV. The drug has a long-term auto-induction effect that is dose-, duration of treatment- and genetically-dependent, resulting in lower EFV plasma concentrations after multiple dosing (Ngaimisi *et al.*, 2010; Habtewold *et al.*, 2011). Previous studies have shown that the proportion of patients with EFV concentrations higher than 4 mg/mL, which is considered a neurotoxic concentration (Marzolini *et al.*, 2001), decreased by 44% from week 4 to week 16 of therapy, due to auto-induction of EFV metabolism (Ngaimisi *et al.*, 2010). Accordingly, the concentration of 8-OH-EFV was much higher in week 16 than week 4, showing an accumulation of 8-OH-EFV in the first months of therapy (Ngaimisi *et al.*, 2010). Interestingly, this might indicate that the contribution of 8-OH-

EFV differs between EFV-induced short- and long-term effects. While short-term effects would probably be more dependent on EFV concentration, long-term adverse reactions will probably be more related to 8-OH-EFV accumulation. This is in accordance with the fact that the majority of EFV-induced adverse CNS reactions is reversible and tends to occur during the first weeks of treatment (Fumaz *et al.*, 2005), when the auto-induction period of EFV biotransformation is starting (Ngaimisi *et al.*, 2010). After this period, the plasmatic concentrations of EFV are considerably lower (Ngaimisi *et al.*, 2010) and have low intra-individual variability (Pereira *et al.*, 2008). One major difference between our study and others is the time on EFV. In fact, in the few studies mentioning the time on EFV, metabolites were quantified during the first 16 weeks (Ngaimisi *et al.*, 2010; Cho *et al.*, 2011; Habtewold *et al.*, 2011). As this corresponds to the auto-induction period, the maximum levels of metabolites were not attained and adverse reactions are likely to have been mainly EFV-dependent. By contrast, all patients in our study were users of EFV-containing cART beyond the auto-induction stage and time on EFV did not differ between control and study groups. Accordingly, the EFV concentration was similar in the two groups but differences were observed in the levels of 8-OH-EFV. Taken together, these data demonstrate the need to include time of exposure to EFV in neuro-safety evaluation studies. In fact, Leutscher *et al.* (2013) suggested that EFV discontinuation occurs late in the course of treatment due to the persistence of CNS toxicity, which may impact quality of life negatively on a long-term basis. Herein, we present convincing data that patients on EFV for a long period show anxiety. Actually, our data emphasize the relevance of the 8-OH-EFV metabolite, rather than EFV, when a persistent CNS effect is considered. While the EFV concentration remains stable after the first year, the documented variability during the auto-induction period supports the need for therapeutic drug monitoring based on quarterly sampling in the first year of therapy (Pereira *et al.*, 2008). It remains to be established if this is also applicable to 8-OH-EFV.

Induction-driven changes in the levels of EFV and its metabolites differ between women and men (Habtewold *et al.*, 2011) and this might explain conflicting literature results, depending on the number of women included in the studies. To reduce sex-related confounding factors, all patients in our study were men, which differs greatly from other studies in which the percentage of males varied from 20 to 80% (Ngaimisi *et al.*, 2010; Cho *et al.*, 2011; Habtewold *et al.*, 2011; Tovar-y-Romo *et al.*, 2012).

Our data support a role for 8-OH-EFV in the genesis of mood changes in patients on long-term EFV exposure. This suggests that the plasma concentration of 8-OH-EFV may be more appropriate than EFV levels for therapeutic drug monitoring towards the minimization of these CNS effects. Nonetheless, this raises an interesting question: if mood changes are dependent on EFV biotransformation and EFV predominantly undergoes hepatic clearance, why is the

CNS compartment a vulnerable milieu for EFV toxicity? Several factors can be invoked to explain this apparently conflicting scenario, namely the fact that EFV tends to accumulate in the CNS compartment (Decloedt *et al.*, 2015). Indeed, although EFV is highly protein-bound, the CSF penetration of unbound EFV is high (Yilmaz *et al.*, 2012; Avery *et al.*, 2013a; Thompson *et al.*, 2015) and the drug is not actively cleared from the CNS (Decloedt *et al.*, 2015). One additional potential key player is the fact that EFV is a substrate for CYP2B6 (U.S. Food and Drug Administration, 2012), the major isoform responsible for its metabolic conversion into 8-OH-EFV. Whereas CYP2B6 is abundant in the liver, it is also expressed in the brain, albeit at much lower levels (Miksys and Tyndale, 2013), and its expression can vary by more than two-fold across brain areas (Miksys *et al.*, 2003). The concentration of this isoform in specific regions and cell types may originate particular biotransformation environments (Miksys *et al.*, 2000; Miksys *et al.*, 2003), plausibly predisposed to local accumulation of 8-OH-EFV. The non-CYP2B6-mediated metabolic pathways of EFV are diminished in the brain, namely the minor phase I formation of 7-OH-EFV via CYP2A6 (Dutheil *et al.*, 2009) as well as phase II metabolism (Ouzzine *et al.*, 2014) (Fig. 4), which can also contribute to 8-OH-EFV accumulation. Once in the brain, the formation of electrophilic quinone and quinone-imine species upon oxidation of 8-OH-EFV is a conceivable event (Harjivan *et al*., 2014), similarly to what happens with endogenous neurotoxins (Baumgarten and Lachenmayer, 2004; Kato *et al.*, 2012; Kato *et al.*, 2014). Quinone and quinone-imine metabolites have a recognized toxicological relevance, not only due to their ability to generate reactive oxygen species but also to their capacity to yield covalent adducts with proteins (Bolton *et al.*, 2000; Monks and Jones, 2002). In fact, the formation of these reactive metabolites may be at the genesis of CNS effects by distinct but not mutually exclusive mechanisms. For instance, adduct formation could negatively impact the function of proteins and thereby interfere with energy metabolism, axonal transport or pre-synaptic neurotransmitter release (Asanuma *et al.*, 2003; Lopachin and Decaprio, 2005). Moreover, electrophilic species have the ability to reduce glutathione (GSH) content (Rabinovic and Hastings, 1998), which has been described in several psychiatric conditions (Eskiocak *et al.*, 2005; Gawryluk *et al.*, 2011). The brain is highly susceptible to oxidation, due to its high oxygen consumption rate, and has low levels of GSH, when compared to other tissues (Ballatori *et al.*, 2009). Thus, depletion of brain GSH levels may be critical since this molecule is not only an antioxidant but also plays key roles as neuromodulator, neurotransmitter, and enabler of brain cells' survival (Ballatori *et al.*, 2009; G. Morris *et al.*, 2014).

Our observation of higher plasma concentrations of 8-OH-EFV in the study group suggests that peripheral 8-OH-EFV contributes to the brain pool of this toxic metabolite. Although the ability of 8-OH-EFV to cross the blood-brain barrier has yet to be demonstrated, our results appear consistent with this capacity.

One additional important observation was the trend for a higher 8-OH-EFV/EFV ratio in the study group compared to the control group, albeit not reaching statistical significance. This observation, together with the higher concentrations of 8-OH-EFV in the study group, suggests that this group of patients had a higher CYP2B6 activity than the control group. In fact, the 8-OH-EFV/EFV ratio has been considered a metabolic phenotype for CYP2B6 activity (U. S. Food and Drug Administration, 2014). This is an important finding since the metabolic phenotype is central to clinical implementation and monitoring in pharmacovigilance throughout the study of PK drug-drug interactions and individualization of drug dosages (Llerena and Penas-Lledo, 2015). In this regard, it is noteworthy that brain CYP2B6 will not only metabolize EFV to 8-OH-EFV but also influence the levels of endogenous metabolites such as mood-related steroids and neurotransmitters (*e.g.*, testosterone (Imaoka *et al.*, 1996; Fink *et al.*, 1999), and serotonin (Fradette *et al.*, 2004).

It is also noteworthy that the plasma concentrations of 8-OH-EFV-Glc were similar in both groups and much higher than the levels of 8-OH-EFV. Taking into consideration that, as opposed to 8-OH-EFV, the glucuronide cannot be metabolized to toxic electrophiles, this observation suggests that phase II glucuronidation is the main route of 8-OH-EFV detoxification. Thus, controversies regarding PK-neurosafety relationships between studies on EFV may result from the fact that these studies typically considered the total 8-OH-EFV concentrations (*i.e.,* 8-OH-EFV plus 8-OH-EFV-Glc) and no correlations were established with the non-conjugated metabolite *per se*. Toxic EFV concentrations (> 4 mg/L) (Marzolini *et al.*, 2001) were herein associated with lower 8-OH-EFV-Glc concentrations. This suggests that higher EFV concentrations are able to inhibit UGT enzymes, which is in line with *in vitro* data (Belanger *et al.*, 2009; Ji *et al.*, 2012). In fact, EFV is reported as a non-competitive inhibitor of UGT1A1 and a competitive inhibitor of UGT1A9 (Ji *et al.*, 2012) and both isoforms participate in 8-OH-EFV-Glc formation (Bae *et al.*, 2011). This inhibition may represent a new toxicity mechanism at high EFV concentrations. Indeed, the diminished peripheral detoxification of 8-OH-EFV under these conditions suggests a synergistic effect of EFV on the toxic events induced by its major metabolite. Figure 6 illustrates the different mechanistic hypotheses described for EFV-neurotoxicity (O'Mahony *et al.*, 2005; Streck *et al.*, 2008; Apostolova *et al.*, 2010; Tovar-y-Romo *et al.*, 2012; Brandmann *et al.*, 2013; Blas-Garcia *et al.*, 2014; Brown *et al.*, 2014; de Oliveira *et al.*, 2014; Funes *et al.*, 2014; Apostolova *et al.*, 2015a), which are plausibly not exclusive and will dictate the patients' susceptibility to mood changes.

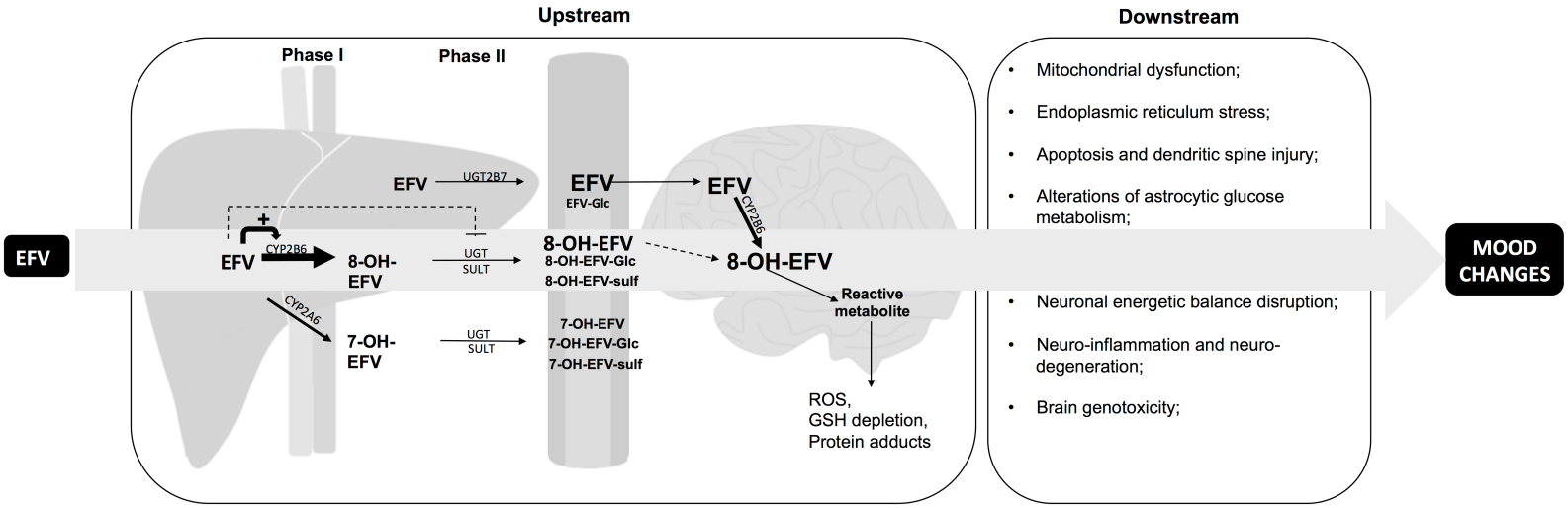

Figure 6. Schematic representation of the events preceding mood changes upon EFV exposure. Upstream events: in the liver, EFV is mainly metabolized via CYP2B6 to 8-OH-EFV, to a slighter degree to 7-OH-EFV via CYP2A6 and barely conjugated by UGT2B7 to EFV-Glc. The hydroxylated metabolites undergo phase II conjugation through glucuronidation (major) and sulfonation (minor). Formation of 8-OH-EFV may occur to a lesser extent in the brain and further oxidation can generate a reactive quinoid metabolite, ultimately leading to detrimental ROS generation, GSH depletion and protein modification that may result in downstream neurotoxicity. EFV, efavirenz; 8-OH-EFV, 8-hydroxy-efavirenz; 7-OH-EFV, 7-hydroxy-efavirenz; EFV-Glc , efavirenz- glucuronide; 8-OH-EFV-Glc, 8-hydroxy-efavirenz-glucuronide; 7-OH-EFV- Glc, 7-hydroxy-efavirenz-glucuronide; 8-OH-EFV-sulf, 8-hydroxy-efavirenz-sulfate; 7-OH-EFV-sulf, 7-hydroxy-efavirenz-sulfate. UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase.

Chapter 2

Time-Course of efavirenz biotransformation: a cause for its short- and long-term neurotoxicity

CHAPTER 2
Time-course of efavirenz biotransformation: a cause for its short- and long-term neurotoxicity

2.1 SUMMARY

EFV is an anti-HIV drug that presents relevant short- and long-term central nervous system adverse reactions. Its main metabolite (8-OH-EFV) was demonstrated to be a more potent neurotoxin than EFV itself. This work was aimed to understand how EFV biotransformation to 8-OH-EFV is related to its short- and long-term neuro-adverse reactions. To access those mechanisms, the expression and activity of Cyp2b enzymes as well as the *thiolomic* signature (low molecular weight thiols plus *S*-thiolated proteins) were longitudinally evaluated in the hepatic and brain tissues of rats exposed to EFV during 10 and 36 days. EFV and 8-OH-EFV plasma concentrations were monitored at the same time points. Cyp2b induction had a delayed onset in liver ($p<0.001$), translating into increases in Cyp2b activity in liver and 8-hydroxy-efavirenz plasma concentration ($p<0.001$). Moreover, an increase in *S*-cysteinylglycinylated proteins ($p<0.001$) and in free low molecular weight thiols was also observed in liver. A distinct scenario was observed in hippocampus, which showed an underexpression of Cyp2b as well as a decrease in *S*-cysteinylated and *S*-glutathionylated proteins. Additionally, the observed changes in tissues were associated with a marked increase of *S*-glutathionylation in plasma. Our data suggest that the time course of EFV biotransformation results from different mechanisms for its short- and long-term neurotoxicity. The difference in the redox profile between liver and hippocampus might explain why, despite being mostly metabolized by the liver, this drug is neurotoxic. If translated to clinical practice, this evidence will have important implications in EFV short- and long-term neurotoxicity prevention and management.

2.2 MATERIALS AND METHODS

2.2.1 Drugs and Chemicals

EFV was kindly provided by Dr. Frederick A. Beland (*National Center for Toxicological Research*, Arkansas, USA). HPLC-grade solvents were purchased from *VWR* (Belgium). *β*-Glucuronidase (Type VII-A from *E.coli*, E.C. 3.2.1.31, 1000 U/mL), Arylsulfatase (Type H-1, from *E. coli*, E.C. 3.1.6.1., 1000 U/mL) and reagents used for *thiolomic* profile determination were purchased from *Sigma-Aldrich* (USA), with the exception of trichloroacetic acid (TCA),

which was purchased from *Roth* (Germany). A kit from *NZYTech* (Portugal) was used for the cDNA synthesis. Primers and PowerUp™ SYBR® Green Master Mix used for quantitative real time PCR (qPCR) were purchased from *Applied Biosystems, Thermo Fisher Scientific* (USA).

2.2.2 Animals

Experiments were performed with male *Wistar* rats (*Rattus norvegicus* L.), aged 13 weeks, with a mean body weight of 293 ± 60 g, obtained from the *NOVA Medical School* animal facility. Animals were housed two *per* cage in polycarbonate cages with wire lids (*Tecniplast*, Italy), under 12 h light/dark cycles (8 am - 8 pm), at room temperature (22 ± 2.0 °C) and a relative humidity of 60 ± 10%. Rats were maintained on a standard laboratory diet (SDS RM1, *Special Diets Services*, UK) and *ad libitum* reverse osmosis water.

Rats were randomly assigned into four groups: 10-day control (CTL), 10-day EFV-exposed, 36-day CTL, and 36-day EFV-exposed. In order to reduce the number of animals used in the experiments (*3Rs approach)*, the same rat was used for at least two experiments (*i.e.*, one hippocampus was used to determine ethoxycoumarin O-deethylase (ECOD) activity and the other for Cyp2b expression). A number of 8 animals *per* group was used for *thiolomic* profile (RSSP+LMWT) analysis. For Cyp2b expression and activity analysis a number of 6 animals *per* group were used.

NIH Principles of Laboratory Animal Care (NIH Publication 85-23, revised 1985), the European guidelines for the protection of animals used for scientific purposes (European Union Directive 2010/63/EU) and the Portuguese Law nº 113/2013 concerning ethical use of animals were followed. The experimental procedures (protocol nº 14/2016/CEFCM) received prior approval by the *Institutional Ethics Committee* of the *NOVA Medical School* for animal care and use in research.

2.2.3 Experimental protocol

Animals from the EFV groups were administered 9 mg/kg/day of EFV by oral gavage, suspended in 2 mL of reverse osmosis water, and CTL groups were administered the same volume of reverse osmosis water. The administrations were performed using a sterile polypropylene feeding tube (15 gauge; tip diameter: 3 mm; length: 78 mm; *Instech Laboratories*, Inc., USA) to reduce the risk of trauma, perforation and cross contamination (Morton *et al.*, 2001). All animals underwent a 7-day period of acclimatization, handled daily

by the same individual for a period of 2 minutes each, and accustomed to the gavage position, in a different animal facility room.

The animals were weighed at baseline and twice a week during the entire study. The amounts of EFV were adjusted accordingly, in order to ensure a constant daily dose. EFV or vehicle were administered daily in the morning, at approximately the same schedule.

At day 10 or 36, approximately 1-2 hours after EFV administration, rats were anesthetized by intraperitoneal injection with medetomidine (0.5 mg/kg body weight; Domitor®, *Pfizer Animal Health*) and ketamine (75 mg/kg body weight; Imalgene 1000®, *Mérial*, Lyon, France). Cardiac puncture was performed for blood sampling and the plasma samples were stored at -80 °C until use. The animals were then decapitated under deep anesthesia, brains were removed from skull, and hippocampus and prefrontal cortex were dissected. The liver was also rapidly removed.

2.2.4 Cyp2b1 and Cyp2b2 gene expression

Liver, hippocampus and prefrontal cortex were collected and homogenized in Trizol® (*Life Technologies*) using a tissue homogenizer (*Heidolph* DIAX 900). Total RNA extraction was performed according to the Trizol® manufacturer's instructions. The RNA concentration was determined prior to cDNA synthesis by measuring the absorbance at 260 nm on a SPECTROstar Omega spectrometer (*BMG Labtech*, Ortengerg, Germany) operating in the LVis Plate mode. cDNA was synthesized from 1 µg RNA according to the manufacturer's instructions. qPCR was carried out in a final volume of 15 µL with 2× PowerUp™ SYBR® Green Master Mix and 0.3 µM of each primer, plus 2 µL of cDNA was added as template.

Rat specific primers were used for the housekeeping gene *ß-actin* (Forward 5'-AAGTCCCTCACCCTCCCAAAAG-3'; Reverse 5'-AAGCAATGCTGTCACCTTCCC-3') (Peinnequin *et al.*, 2004) and for the target genes *Cyp2b1* (Forward 5'-GCTCAAGTACCCCCATGTCG-3'; Reverse 5'-ATCAGTGTATGGCATTTTACTGCGG-3') and *Cyp2b2* (Forward 5'-CTTTGCTGGCACTGAGACCG-3'; Reverse 5'-ATCAGTGTATGGCATTTTGGTACGA-3') (Schilter *et al.*, 2000). The efficiency of each reaction was estimated with a calibration curve built using serial cDNA dilutions (1, 10^{-1}, and 10^{-2}) in order to construct a standard curve for each gene and tissue. The reaction was performed on an *Applied Biosystems* 7300 Real Time PCR System, consisting of a denaturation step at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 60 °C for 1 min and extension at 72 °C for 30 seconds. A dissociation stage was added to determine the melting temperature (Tm) of a single nucleic acid target sequence as a quality and specificity

measure. The comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to quantify the target genes, which were normalized to the reference *ß-actin* gene, and fold change was calculated in EFV-treated rats relative to the respective CTL group.

2.2.5 Determination of ethoxycoumarin-O-deethylase (ECOD) activity

ECOD activity measures rat Cypla1/2, Cyp2a1, Cyp2b1/2, Cyp2c6/7, Cyp2c11, Cyp2c13, and Cyp2el, among which the 1a and 2b subfamilies are the most effective forms (Kern *et al.*, 1997). The procedure was performed as previously described (Cipriano *et al*., 2016; Pinheiro *et al*., 2016). Briefly, liver, hippocampus and prefrontal cortex slices were incubated for 90 min with 7-ethoxycoumarin (0.8 mM) in Dulbecco's Modified Eagle Medium (DMEM). The 7-hydroxycoumarin concentrations were determined after 2 h of enzymatic digestion with a *β*-glucuronidase/arylsulfatase solution (pH 4.5 in 0.1 M acetate buffer) followed by a liquid-liquid extraction with chloroform. The 7-hydroxycoumarin fluorescence was measured using the SPECTROstar Omega spectrometer (λ_{exc} 340 nm; λ_{em} 460 nm). The results are presented as the EFV/CTL fluorescence ratio for each tissue and exposure time. Total protein concentration was determined by absorbance at 280 nm using the same spectrometer in the LVis Plate mode.

2.2.6 Determination of efavirenz and 8-hydroxy-efavirenz plasma concentrations

EFV and 8-OH-EFV were quantified as previously described (Grilo *et al*, 2016). Briefly, the plasma samples were incubated with *β*-glucuronidase, at 37 ºC, for 1 h. Analytes were extracted with *tert*-butyl methyl ether and the organic phase was isolated and dried under vacuum (-80 kPa, at 60 ºC). The dried residue was reconstituted in acetonitrile: 0.1% formic acid plus *n*-hexane (50/50 v/v) and the mixture was homogenized and centrifuged. The samples were analyzed by HPLC on an Agilent 1100 Series equipment (*Agilent Technologies*, Santa Clara, CA, USA), using a reversed-phase Luna C18 column (250 mm ×4.6 mm; 5 µm; 100 Å; *Phenomenex*, USA). The mobile phase consisted of 0.1 % formic acid (pH 2.65) (solvent A, 65%) and acetonitrile (solvent B, 35%) for the first 5 min, at a flow rate of 1.2 mL/min. The percentage of solution A was linearly decreased to 55% for 10 min and maintained at this value for 16 minutes. For the next 5 min the percentage of A was linearly diminished to 49% and

maintained for the following 7 min. The column temperature was set at 30 °C, the injection volume was 100 µL, and UV absorbance was monitored at 246 nm.

2.2.7 Determination of the *thiolomic* profile

GSH, its precursor CysSH and its catabolism product CysGlySH were quantified. These three moieties were analyzed in their RSSP forms (GSSP, CysSSP and PSSCysGly) and in their LMWT forms. The latter were composed by the RSH fractions, including GSH, CysSH and CysGlySH, and the respective disulfides.

Liver (approximately 50 mg), hippocampus (one) and prefrontal cortex (one) were collected, kept on ice, and immediately homogenized in 400 µL of iced phosphate-buffered saline (1x), using a tissue homogenizer (*Heidolph* DIAX 900). An initial volume of 50 µL from the tissue homogenate and plasma were used to assess thiol fractions.

The total 'thiol' fraction (RSSP+RSH+RSSR) was obtained by reducing the sulfhydryl groups with tris (2-carboxyethyl)phosphine hydrochloride (TCEP; 100g/L, 5 µL). After a 30 min incubation at room temperature, the samples were treated with TCA (100 g/L) containing 1 mM EDTA (45 µL) for protein precipitation. Each mixture was then centrifuged (13000 *g*, 10 min, 4°C) and the supernatant collected to a new tube containing 1.55 M NaOH (5 µL), 125 mM sodium tetraborate buffer ($Na_2B_4O_7$, pH 9.5) with 4 mM EDTA (62.5 µL) and 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid ammonium (SBD-F) 1 g/L) in $Na_2B_4O_7$ buffer (125 mM with 4 mM EDTA) (25 µL). The final mixture was vortexed and incubated in the dark, at 60 ºC for 1 h, to complete the derivatization of the free sulfhydryl groups. Finally, a volume of 10 µL was analyzed by HLPC.

The LMWT (RSSR+ RSH) and the reduced (RSH) fractions were also analyzed. Two aliquots from the same sample were submitted to protein precipitation with TCA, with subsequent centrifugation (13000 *g*, 10 min, at 4ºC), as described (Przemyslaw *et al.*, 2011). Then, while one aliquot was reduced with the TCEP reagent for total non-bound fraction quantification, the other was incubated with reverse osmosis water in order to obtain the naturally reduced RSH fraction. After incubation at room temperature, for 30 min, the protocol described above was followed. The RSSP contribution for each particular thiol was obtained by subtracting the LMWT (RSH+RSSR) from the total thiol concentration.

The quantifications were performed by HPLC-FD analysis on a Shimadzu LC-10AD VP (Shimadzu Scientific Instruments Inc) system using a reversed-phase C18 LiChroCART 250-

4 column (LiChrospher 100 RP-18, 5µm, VWR, USA), at 29 ºC with an adapted from the methodology reported by Nolin and co-authors (Nolin *et al.*, 2007). The detector was set at excitation and emission wavelengths of 385 and 515 nm, respectively. The mobile phase consisted of 100 mM acetate buffer (pH 4.5) and methanol [99:1 (v/v)]. The analytes were separated in an isocratic elution mode for 20 min, at a flow rate of 0.8 mL/min.

2.2.8 Data analysis

Statistical analysis was performed using Graph Prism® 5.0 (GraphPad Software Inc., San Diego, CA, USA). The comparisons between groups were performed using the *Unpaired t*-test or *ANOVA,* whenever applicable.

2.3 RESULTS

2.3.1 Animals

At the beginning of the experiments, the animals were age-matched and no differences were found regarding animal body weights. However, at the end of the 36-day period, the EFV-exposed rats weighed significantly less than the age-matched controls (102 ± 5 g versus 87 ± 3 g, $p<0.05$).

2.3.2 Cyp2b expression

Regardless of treatment time, liver was the tissue that presented higher expression of Cyp2b1 upon EFV exposure (Fig. 7A). Long-term (36 days) exposure to EFV resulted in a 4-fold increase in liver Cyp2b1 expression compared to short-term exposure (10 days) ($p<0.001$). A similar pattern was observed for the prefrontal cortex (13-fold increase; $p<0.001$) while the hippocampus presented a decrease in Cyp2b1 expression with time ($p<0.001$). Expression of Cyp2b2 was also lower in the hippocampus, while liver and prefrontal cortex had comparable levels upon short-term EFV exposure (Fig. 7B). However, while time increased Cyp2b2 expression in the prefrontal cortex ($p<0.001$), the level in the liver remained unaffected.

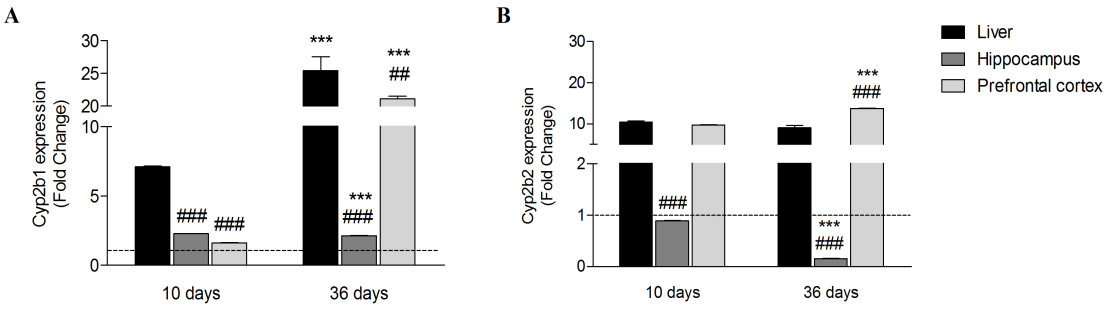

Figure 7. Cyp2b1 (A) and Cyp2b2 (B) expression in liver, hippocampus and prefrontal cortex of rats exposed to efavirenz during 10 and 36 days. n = 6 animals per group. The data are expressed in fold change compared to non-treated controls. * represents time-dependent differences (*Unpaired t-test*, ***p<0.001) and # represents differences among tissues using liver as control (*Two-way ANOVA* with *Bonferroni* post-test, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001).

2.3.3 Quantification of ECOD activity

The tissue and time dependence of Cyp2b expression were not reflected in ECOD activity (Fig. 6), for which an increase with the duration of EFV-exposure was only observed in the liver (p<0.001).

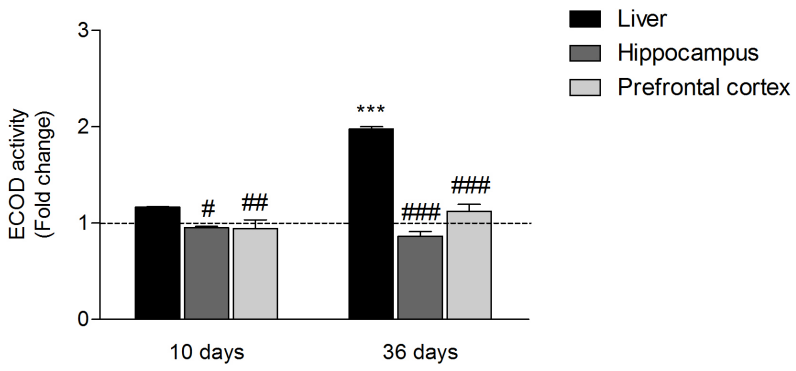

Figure 8. ECOD activity in liver, hippocampus and prefrontal cortex of rats exposed to efavirenz during 10 and 36 days. n = 6 animals per group. Data are expressed in fold change compared to non-treated controls. * represents time-dependent differences (*Unpaired t-test*, ***p<0.001) and # represents differences among tissues using liver expression as control (*Two-way ANOVA* with *Bonferroni* post-test, $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001).

2.3.4 Determination of efavirenz and 8-hydroxy-efavirenz plasma concentrations

While no differences were found for EFV plasma concentration between 10 and 36 days (Fig. 9), the 8-OH-EFV concentrations raised over time (p=0.02) (Figure 3).

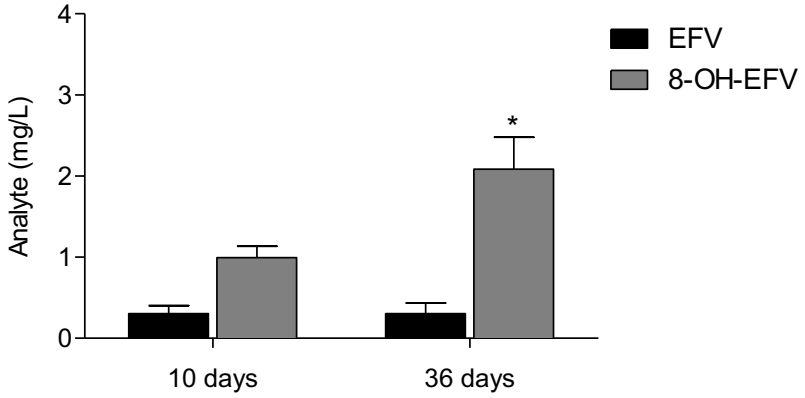

Figure 9. Efavirenz and 8-hydroxy-efavirenz quantification in rat plasma at 10 and 36 days of efavirenz exposure. n = 8 animals per group. Data are expressed as mean ± standard error of the mean in mg/L.* represents time-dependent differences (*unpaired t-test*, *p=0.02).

2.3.5 *Thiolomic* signature

No differences over time were found for any of the analytes in control animals (Table 3). With the exception of $_{LMW}$Cys, the liver presented higher levels of the analytes, when compared to prefrontal cortex and hippocampus at both time points. Additionally, while in the liver the predominant LMWT fraction was the reduced form, the oxidized form accounted for approximately 40% in the brain.

Regarding differences in EFV-treated animals, the liver had a general increase in all analytes over time (Table 4). Exceptions were the GSSP and CysSSP levels. In hippocampus, a marked decrease in GSSP (p<0.01) and CysSSP (p<0.001) was observed, in parallel with an increase in GSH concentration (p<0.001). In prefrontal cortex, both decreased CysSSP (p<0.01) and increased GSH synthesis (p<0.001) were observed. These variations were more modest than those found for hippocampus. The higher variation found in plasma was for GSSP (p<0.001), which increased over time in contrast to what was found for hippocampus. This increase was accompanied by a decrease in GSH (p<0.001).

Table 3: Evaluation of the *thiolomic* profile of control animals at 10 and 36 days.

µM/mg tissue	Liver			Prefrontal cortex			Hippocampus			
	10 days	**36 days**	p^a	**10 days**	**36 days**	p^a	**10 days**	**36 days**	p^a	p^b
GSH	8.80±1.48	10.19±1.80	ns	1.04±0.18	1.14±0.40	ns	1.05±0.22	1.03±0.30	ns	***
$_{LMW}$GSH	10.80±1.41	11.36±1.26	ns	1.83±0.27	1.91±0.47	ns	1.79±0.25	1.86±0.49	ns	***
GSSP	2.26±1.58	2.01±0.89	ns	0.50±0.32	0.38±0.18	ns	0.43±0.22	0.46±0.15	ns	***
CysSH	0.17±0.04	0.17±0.05	ns	0.08±0.02	0.08±0.03	ns	0.10±0.03	0.08±0.01	ns	***
$_{LMW}$Cys	0.25±0.05	0.26±0.04	ns	0.20±0.05	0.18±0.04	ns	0.23±0.04	0.20±0.03	ns	ns
CysSSP	0.22±0.09	0.21±0.05	ns	0.08±0.04	0.08±0.02	ns	0.09±0.03	0.09±0.02	ns	***
CysGlySH	0.03±0.002	0.03±0.005	ns	0.005±0.001	0.006±0.001	ns	0.004±0.001	0.005±0.001	ns	***
$_{LMW}$CysGly	0.03±0.01	0.04±0.01	ns	0.01±0.003	0.01±0.004	ns	0.01±0.001	0.01±0.003	ns	***
PSSCysGly	0.06±0.01	0.06±0.01	ns	0.01±0.003	0.004±0.003	ns	0.01±0.002	0.01±0.002	ns	***

Data are presented as mean ± standard error of the mean in µM/mg tissue. n = 8 animals per group. [a] Time-dependent differences for each tissue (*unpaired t-test*); [b]Comparison between liver, prefrontal cortex and hippocampus for the 10-day EFV exposure data point (one-way ANOVA ***p<0.001). **$_{LMW}$GSH**: total non-protein bound glutathione; **GSH**: reduced glutathione; **GSSP**: *S*-glutationylated proteins; **$_{LMW}$Cys**: total non-protein bound cysteine; **CysSH**: reduced cysteine; **CysSSP**: *S*-cysteinylated proteins; **$_{LMW}$CysGly**: total non-protein bound cysteinylglycine; **CysGlySH:** reduced cysteinylglycine**; PSSCysGly**: *S*-cysteinyl-glycinylated proteins. **ns**: not significant.

Table 4: Evaluation of the *thiolomic* profile in liver, prefrontal cortex, hippocampus, and plasma of efavirenz-exposed animals at 10 and 36-days

EFV/CTL Ratio	Liver			Prefrontal cortex			Hippocampus			Plasma		
	10 days	**36 days**	**Δ (%)**	**10 days**	**36 days**	**Δ (%)**	**10 days**	**36 days**	**Δ (%)**	**10 days**	**36 days**	**Δ (%)**
GSH	0.96±0.02	1.14±0.02	18[a]	1.13±0.04	1.31±0.07	18[a]	1.21±0.04	1.58±0.10	37[a]	1.11±0.05	0.96±0.02	-15[a]
$_{LMW}$GSH	1.01±0.02	1.22±0.02	21[a]	1.06±0.03	1.22±0.04	16[a]	1.04±0.02	1.17±0.05	13[a]	1.02±0.02	1.00±0.03	-2
GSSP	1.16±0.48	1.26±0.35	10	1.10±0.23	0.97±0.21	-13	1.23±0.27	0.89±0.11	-34[b]	0.82±0.06	1.16±0.07	34[a]
CysSH	0.83±0.06	0.93±0.04	10[b]	0.90±0.06	0.86±0.04	-4	0.92±0.05	0.93±0.03	1	1.05±0.05	0.95±0.03	-10[a]
$_{LMW}$Cys	0.89±0.04	1.09±0.06	21[a]	0.88±0.07	0.92±0.03	4	0.83±0.05	0.99±0.02	16[a]	1.00±0.01	1.05±0.01	5[a]
CysSSP	1.42±0.20	1.48±0.15	6	0.96±0.14	0.72±0.13	-24[b]	1.12±0.19	0.67±0.04	-45[a]	1.15±0.03	1.08±0.04	-7[b]
CysGlySH	0.90±0.03	1.12±0.03	22[a]	0.87±0.04	1.14±0.04	27[a]	0.95±0.02	0.91±0.05	-4	1.13±0.04	0.97±0.10	-16[a]
$_{LMW}$CysGly	1.00±0.02	1.22±0.02	23[a]	1.07±0.10	0.95±0.08	-12[c]	1.04±0.04	0.96±0.05	-8[b]	0.91±0.05	1.03±0.03	12[a]
PSSCysGly	0.98±0.01	1.17±0.01	19[a]	0.91±0.15	0.85±0.34	-6	0.87±0.14	0.86±0.19	1	1.03±0.05	1.08±0.01	5[b]

Data are presented as the EFV/CTL ratio (*unpaired t*-test, [a]$p<0.001$; [b]$p<0.01$; [c]$p<0.05$); **Δ**: variation in percentage of the EFV/CTL ratio between 10 and 36 days. n = 8 animals per group. **$_{LMW}$GSH**: total non-protein bound glutathione; **GSH**: reduced glutathione; **GSSP**: *S*-glutationylated proteins; **$_{LMW}$Cys**: total non-protein bound cysteine; **CysSH**: reduced cysteine; **CysSSP**: *S*-cysteinylated proteins; **$_{LMW}$CysGly**: total non-protein bound cysteinylglycine; **CysGlySH:** reduced cysteinylglycine**; PSSCysGly**: *S*-cysteinyl-glycinylated proteins. **ns**: not significant.

2.4 DISCUSSION

Our data suggest that EFV short- and long-term effects have different underlying mechanisms, which are related to the time-course of the drug's biotransformation and are also tissue-dependent. In particular, they show that the hippocampus responds differently to the electrophilic/oxidative stress generated upon long-term EFV exposure than the other tissues investigated in this study. The herein reported findings are consistent with our previous report that long-term neurotoxic effects are related with increased plasma levels of 8-OH-EFV (Grilo *et al*., 2016).

The formation of 8-OH-EFV is related with individual CYP metabolic capacity and with EFV-promoted auto-induction. We found major differences among tissues in the Cyp2b expression after EFV exposure. According to the higher mRNA levels, ECOD activity, and 8-OH-EFV plasma concentrations, the Cyp2b induction by EFV in the liver had a delayed onset and reached high levels after long-term exposure. Two non-mutually exclusive reasons may account for these results: i) a delay period is necessary to reach the steady-state plasma concentrations of EFV and of Cyp2b induction; and ii) the products of EFV metabolism may have the same pharmacological effect in Cyp2b expression as the parent drug.

The time required for a drug to maximally induce an enzyme is highly related with the half-life of the inducer, and the degradation half-life of the enzyme, which may be estimated from the clearance of the substrate after removal of the inducer (deinduction). In the case of inducers of their own metabolism, it is more difficult to predict this time course. EFV has a long half-life of 40-55 hours in healthy individuals (Vrouenraets *et al.*, 2007) and 19 hours in HIV-infected patients (Csajka *et al.*, 2003). After discontinuation, EFV can persist in plasma for 36-100 hours (Taylor *et al.*, 2004), which anticipates a late onset of EFV effects on Cyp2b expression and a long time to attain maximum enzyme induction. In man, it takes at least 4 months to reach steady-state EFV-CYP2B6 induction and this period is characterized by a progressive increase of 8-OH-EFV plasma concentrations (Ngaimisi *et al.*, 2010). The extent of auto-induction is not dependent on baseline EFV concentration (Zhu *et al.*, 2009). However, it is dependent on baseline EFV clearance and the intrinsic capacity of CYP2B6 (Zhu *et al.*, 2009; Ngaimisi *et al.*, 2010). In fact, the 8-OH-EFV/EFV ratio has been considered a metabolic phenotype for CYP2B6 activity (U.S. Food and Drug Administration, 2014). This could in turn implicate 8-OH-EFV or a metabolite generated from it (Bae *et al.*, 2011; Aouri *et al.*, 2016) as a maintainer of the induction period. Moreover, these observations suggest that the baseline 8-OH-EFV/EFV ratio may be relevant to predict the extension of the induction and deinduction periods. Our data support the hypothesis that there is a differential contribution of EFV and

8-OH-EFV to short- and long-term neurotoxic effects and that the Cyp2b induction period contributes to these differences.

Based on this last assumption, it is expectable that low CYP2B6 metabolizers or patients taking CYP2B6 inhibitors will have higher plasma EFV concentrations and lower baseline clearance, as well as shorter and lower induction, resulting in EFV accumulation that may induce direct toxicity. These patients are at higher risk of experiencing earlier neurologic adverse reactions than high metabolizers. This is in line with the earlier EFV discontinuation reported for low CYP2B6 metabolizers (Wyen *et al.*, 2011) and it is noteworthy that *in vitro* studies have associated EFV with mitochondrial dysfunction in hepatocytes, neurons and glia cells (Apostolova *et al.*, 2015b). By contrast with slow metabolizers, ultra-rapid CYP2B6 metabolizers present an increased plasma accumulation of 8-OH-EFV, along with a higher clearance of EFV.

While tissue availability restricts the study of CYP2B6 regulation in man, there are several similarities that make the rat a suitable model for the study of drug metabolism. For instance, the CYP2B family is similar between humans (CYP2B6) and rodents (Cyp2b1 and Cyp2b2), with high homology and sharing of substrates (Miksys *et al.*, 2000). This is also true for enzyme induction, as shown by studies involving phenobarbital (Ganem *et al.*, 1999). Moreover, CYP2B6 expression is regulated by the constitutive androstane receptor (CAR) (Sueyoshi *et al.*, 1999) and rat Cyp2b has also been reported to be regulated by the orthologous CAR (Muangmoonchai *et al.*, 2001). Nonetheless, our animal model does not mimic low metabolizers as rats have high metabolic capacity (Mutlib *et al.*, 2000). In fact, a high Cyp2b induction in liver was observed in the present study.

EFV is able to modulate 8-OH-EFV clearance by two mechanisms: i) inhibition of 8-OH-EFV-Glc formation (Ji *et al.*, 2012; Grilo *et al.*, 2016); and ii) CAR-dependent increase of phase II metabolism (Maglich *et al.*, 2003). The latter is more conceivable to happen in the liver than in brain tissues, which have lower activity of phase II enzymes (Ouzzine *et al.*, 2014). It is still to be demonstrated if 8-OH-EFV is able to cross the BBB. Nonetheless, the recently observed correlation between plasma 8-OH-EFV concentrations and mood changes in HIV-positive patients points in this direction (Grilo *et al.*, 2016). The likelihood of low 8-OH-EFV formation in the prefrontal cortex and hippocampus also suggests a contribution of the peripheral pool of the metabolite to the neurological effects of EFV. Moreover, the down-regulation of the Cyp2b expression and activity (ECOD) in hippocampus herein observed suggests a protective response of the tissue from exposure to *in situ* generated 8-OH-EFV by decreasing EFV conversion into 8-OH-EFV. It also suggests that 8-OH-EFV or its metabolites may behave as CAR inverse agonist in hippocampus. It is interesting to note that Cyp2b1 was mainly induced in the liver, while Cyp2b2 induction was predominant in the prefrontal cortex, which suggests a differential regulation of gene expression in these tissues. Cyp2b1 and

Cyp2b2 are known to be differentially regulated by several compounds. For instance, Cyp2b1 is more inducible by phenobarbital than Cyp2b2 (Christou *et al.*, 1987); on the other hand, 4-*n*-alkyl-methylenedioxybenzene-type derivatives induce Cyp2b2 to a larger extent than Cyp2b1 (Marcus *et al.*, 1990). We found the prefrontal cortex to exhibit higher expression of Cyp2b2, but this was not translated into higher Cyp2b activity, which may indicate that the contribution of this isoform to ECOD formation is minimal. The specificities of each compartment regarding the expression of enzymes and drug transporters (Miksys and Tyndale, 2002), along with EFV and 8-OH-EFV penetration and accumulation (Avery *et al.*, 2013b) will ultimately determine the exact concentrations of 8-OH-EFV *in loco*.

Once in the brain, the formation of electrophilic species upon oxidation of 8-OH-EFV is a plausible event for EFV toxicity (Harjivan *et al.*, 2014), similarly to what happens with endogenous neurotoxins (Baumgarten and Lachenmayer, 2004). The brain is highly susceptible to oxidation, due to its high oxygen consumption rate and its poor antioxidant defenses, when compared to other tissues (Dringen *et al.*, 2005; Ballatori *et al.*, 2009). Additionally, the oxidation of 8-OH-EFV might be confined to a few compartments since it depends on the availability of oxidases (Meyer zu Schwabedissen *et al.*, 2012). Thus, 8-OH-EFV oxidation is likely related to a change in the subtle balance among redox forms of thiols. In fact, the time-dependent thiol redox changes that we found do not seem to be linked to the initiation of the toxicity pathway or the formation of a toxic metabolite. They may rather be associated with a response to a toxic insult triggered by increased 8-OH-EFV concentrations. In the same way, the changes observed in the tissue-dependent *thiolomic* redox code suggest a tissue-specific response to the electrophilic/oxidative stress generated by 8-OH-EFV oxidation.

There is increasing awareness of the ubiquitous role of oxidative stress in neurotoxicity (Sayre *et al*., 2008). The oxidation of thiol groups is one of the first events during oxidative stress-mediated damage (Grant *et al*., 1999), $_{LMW}$GSH is the most abundant thiol/disulfide in cells, being present mainly in its reduced form (Rossi *et al*., 2009. Upon exposure to oxidative stress, a tissue response involving GSH increase is expected. This may happen in four different ways: increase of $_{LMW}$GSH synthesis, decrease of $_{LMW}$GSH degradation, regeneration of GSSR, or displacement from *S*-thiolated proteins.

The RSH and RSSP profile of the liver was completely distinct from that of brain tissues. The increase in PSSCysGly and all RSH analytes observed in the liver may represent an adaptive response to 8-OH-EFV insult, which was not observed in prefrontal cortex or hippocampus. Recently, GSSP formation was proposed as a mechanism of resistance to paracetamol-induced hepatotoxicity (McGarry *et al.*, 2015), by conferring protection against electrophilic stress *in vivo*. A reasonable explanation is that *S*-thiolation protects the protein thiol groups from acting

as nucleophiles, thereby preventing covalent protein modification by electrophilic metabolites. Moreover, *S*-thiolation has a key role in regulation of macromolecular interactions, directional trafficking proteasomal degradation, folding and activity of proteins (McGarry *et al.*, 2015). This helps to explain why, despite mostly metabolized by the liver, EFV has rarely been associated with hepatotoxicity (Rivero *et al.*, 2007). Additionally, it also highlights that the evaluation of genetic variability of crucial thiol redox enzymes (Hayes *et al.*, 2005; Townsend *et al.*, 2009; Lok *et al.*, 2012) might be useful to identify patients at higher risk for EFV-induced toxicity.

While intracellular proteins are predominantly S-glutathionylated, plasma is poor in $_{LMW}$GSH content; as such, plasma proteins are mainly *S*-cysteinylated (Rossi *et al*. 2009). In plasma, GSSP and PSSCysGly vary inversely with CysSSP (Rossi *et al*. 2009). In the current study, the major variation observed in plasma was an increase in GSSP. This is consistent with increased *S*-glutathionylation at higher 8-OH-EFV concentrations, to replace the displacement of CysSH from CysSSP. The released CysSH can then be oxidized to CysSSCys. CysSSCys can penetrate the CNS, being the main source of CysSH, and consequently of GSH, in the brain (Wang and Cynader, 2000). Similarly, small dipeptides such as CysGly can also penetrate the CNS and contribute to the CysSH pool (Dringen *et al.*, 1997; Dringen *et al.*, 1998). Thus, our data point towards a higher efflux of cystine and CysGly from plasma into brain tissues, to increase the pool of intra-tissue reduced thiols. However, this peripheral contribution may not be sufficient, requiring intracellular displacement of CysSH and GSH from the CysSSP and GSSP brain pool. This was particularly evident for hippocampus compared to prefrontal cortex, suggesting that hippocampus is more vulnerable to oxidative/electrophilic stress at higher 8-OH-EFV concentrations. This might explain the down-regulation of Cyp2b in this tissue, in order to decrease 8-OH-EFV formation.

It is noteworthy that the majority of RSSP in plasma are formed with albumin (Di Simplicio *et al.*, 2005) and that EFV is highly bound to this protein (Wanke *et al.*, 2013). However, it is not known to what extent these changes in plasma RSSP modify the fraction of EFV available to cross the BBB, or its intra-tissue accumulation. Moreover, the information available on 8-OH-EFV affinity for plasma proteins is scarce.

Interestingly, the EFV-exposed animals presented a significant slowing of body growth, similarly to what was described before (Aïssi *et al.*, 2015). This might be related to an increase in the metabolism through hepatic CAR activation. It is known that CAR activation markedly improves fatty liver, by inhibition of hepatic lipogenesis and induction of β-oxidation (Dong *et al.*, 2009). Also, in an *in vitro* study performed by El Hadri and collaborators, EFV prevented pre-adipocytes from accumulating lipids during the differentiation process and altered

adipocyte differentiation (El Hadri *et al*., 2004). In accordance with these reports, EFV use is associated to fat loss in HIV-infected patients (Perez-Molina *et al.*, 2008).

CHAPTER 3

Long-term exposure to efavirenz impairs hippocampus-dependent learning memory

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3.1 SUMMARY

EFV is an anti-HIV drug which is chronically used and prescribed to both adults and children. Long-term exposure to EFV has been associated with higher concentration of its main metabolite 8-OH-EFV and to increased electrophilic/oxidative stress in brain. Nonetheless, whether this translates into neurological impairments is still unknown. The aim of the present work was to evaluate the neurological phenotype and brain molecular changes in rats long-term exposed to EFV.

Male *Wistar* rats were orally exposed to EFV during 36 days and their memory and emotional performance were evaluated. Brain tissues were histologically evaluated and markers of brain function were assessed by immunofluorescence. The EFV group displayed a slower learning curve during the acquisition phase of the MWM test (p=0.004) and a normal behavior during the probe test, suggesting an impairment in memory acquisition without memory retrieval disturbance. The short-spatial memory was also compromised in the EFV -treated group, as assessed by the Y Maze test (p=0.02). Additionally, the EFV group spent more time in the open arms in the Elevated Plus Maze test (p = 0.003), thus revealing lower anxiety levels. Finally, no differences were identified between groups either in depressive-like or locomotion behavior, as assessed by Forced Swim and Open Field tests, respectively. Histopathological analysis of the brains showed any evidence of neuronal cell death. However, a decrease in neuronal dendrites and in markers of neuronal function, were found in the hippocampus of EFV group.

These data suggest that EFV impairs hippocampus-dependent learning memory, mimicking EFV chronic neurotoxic effects in man. This is seemingly a suitable model for the assessment of EFV pharmacokinetic/neurotoxicity and the evaluation of strategies aimed to its prevention/management.

3.2 MATERIALS AND METHODS

3.2.1 Drugs and Chemicals

EFV was kindly provided by Dr. Frederick A. Beland (National Center for Toxicological Research, Arkansas, USA). The primary antibodies used were the following: microtubule-associated protein 2 (MAP2) and synaptosomal-associated protein 25 (SNAP-25) from *Sigma-Aldrich* (M4403 and S9684, respectively); FosB from *Santa Cruz Biotechnologies* (SC-515210) and glial fibrillary acidic protein (GFAP) from *Millipore* (MAB3402). The secondary antibodies were Alexa Fluor® 488 goat anti-rabbit (A-11034) and Alexa Fluor® 594 goat anti-mouse (A-11005) and were from *Invitrogen*. The VECTASHIELD media with 4'-6-diamidino-2-phenylindole (DAPI) was purchased from Vector Labs.

3.2.2 Animals

Sixteen male *Wistar* rats (6 weeks old, with mean body weight 230 ± 21 g) were obtained from the *NOVA Medical School* animal facility and were housed two *per* cage in polycarbonate cages with wire lids (*Tecniplast*, Italy), and maintained under controlled environmental conditions (12-h light/dark schedule [8 am-8 pm] at 22 ± 2.0 °C, 60 ± 10% humidity, with food (SDS RM1, *Special Diets Services*, UK)) and reverse osmosis water supplied *ad libitum*. Animals were Specific Pathogen Free (SPF) according to FELASA recommendations (Nicklas *et al.*, 2010).

Applicable institutional and governmental regulations concerning ethical use of animals were followed, according to the NIH Principles of Laboratory Animal Care (NIH Publication 85-23, revised 1985), the European guidelines for the protection of animals used for scientific purposes (European Union Directive 2010/63/EU) and the Portuguese Law nº 113/2013. Experimental procedures were previously approved by the Institutional Ethics Committee of the *NOVA Medical School* for animal care and use in research.

3.2.3 Experimental Protocol

Rats were randomly assigned into two groups of 8 animals each: EFV group (administered with EFV 9 mg/Kg/day suspended in reverse osmosis water – 1.5 mL) and CTL group (administered only with vehicle – 1.5 mL).

EFV or vehicle gavage administration was performed using a sterile polypropylene feeding tube (15 gauge; tip diameter: 3 mm; length: 78 mm; *Instech Laboratories*, Inc., USA) to reduce the risk of trauma, perforation and cross contamination and lasted 36 days. All animals underwent a 7 days' period of handling acclimatization. Rats were handled daily for a period of 2 minutes each, by the same individual and accustomed to the gavage position, in a different animal facility procedures room.

Animals were weighted at baseline and twice a week throughout the entire study. The amount of EFV was adjusted accordingly in order to ensure a daily dose of 9 mg/Kg. EFV was weighed immediately before administration, suspended in the vehicle and labeled individually for each rat on a daily basis. EFV or vehicle was administered daily to the animals at approximately the same schedule (9:00 to 9:30 am).

After 36 days of EFV administration, five consolidated behavioral tests were performed during one week. EFV administration was continued throughout this period.

At the end of the behavioral tests, approximately 2 hours after EFV delivery, rats were anesthetized by intraperitoneal injection with medetomidine (0.5mg/kg body weight; Domitor®, *Pfizer Animal Health*) and ketamine (75mg/kg body weight; Imalgene 1000®, *Merial*). Four animals from each group were perfused transcardially with phosphate buffered saline followed by 10% neutral buffered formalin (NBF), under deep anaesthesia, for histological and immunohistochemical analysis. Thereafter, the head of the animal was immersed and fixed for 24 hours in 10% NBF before brain removal.

3.2.4 Behavioural tests

The animals were routinely tested during the first half of the light phase of their light/dark cycle, in a quiet room intended only for this purpose. The following behavioural tests were used:

3.2.4.1 Elevated Plus Maze (EPM) test

EPM test is one of the most commonly used in animal models for the evaluation of anxiety (Pellow *et al.*, 1985). This test consists in two 'open' (no walls; 5 x 29 cm) and two 'closed' arms with 29 cm high walls, arranged perpendicularly, and elevated 50 cm above the floor (Pellow *et al.*, 1985).

Each animal was placed at the center of the apparatus, facing one of the open arms. Each test lasted 5 minutes and all testing sessions were performed between 10:00 am and 12:00 pm in a sound-attenuated room. The maze was cleaned with a 70% ethanol solution and rinsed with water after each test to avoid odor cues. The total time spent in the open arms and the total arms entries (number of entries in open and closed arms) were used as anxiety and locomotor measures (Pellow S., 1985). All experiments were conducted by the same individual blinded to experimental groups.

3.2.4.2 Open Field (OF) test

The OF test allows a simple and rapid measurement of animal locomotor activity and anxiety (Seibenhener and Wooten, 2015). A square arena (66 × 66 x 66 cm) that was surrounded by vertical walls was used herein. Three different zones were defined for analysis (Choleris *et al.*, 2001): (1) the area adjacent to the wall (1896 cm^2; "arena periphery"); (2) the central area of the arena (552 cm^2; "arena center"); (3) the intermediary area between the two previous ones (1908 cm^2). The percentage of time spent in each zone, the total distance travelled (cm) and the average speed (cm/s) were recorded. Rearings (episodes of animals standing on their hind legs) and the number of fecal boluses were also manually monitored. The animal was placed at the arena center and was allowed to explore the maze for 5 minutes. At the end of the 5 minutes test, the rat was placed into its home cage, and the maze was cleaned with a 70% ethanol solution and rinsed with water after every test, to prevent avoid odor cues. All experiments were conducted by the same individual blinded to experimental groups. Rat's movements were recorded and analysed using the video-tracking software – SMART® 2.5 (*PanLab,* Barcelona, Spain). The reference point used by the software to determine the position of the animal was the center of the rat's dorsum.

3.2.4.2 Y–Maze (YM) test

YM test is aimed to evaluate the short-term spatial memory of the rodent, and is based on its disposition to explore new environments (Conrad *et al.*, 1996). The apparatus consisted in a Y shape maze, as described by (Salamone *et al.*, 1994). The animal was placed at the end of the 'start' arm and allowed to explore the 'start' arm and the 'other' arm for 10 minutes (beginning from the time that the rat first left the start arm). An opaque door blocked the access to the third arm of the maze ('novel' arm). The rat was then removed from the maze and returned to its home cage for 1 hour. In the test phase the rat was placed again in the 'start' arm of the maze, the door of the 'novel' arm was removed and the rat was allowed to explore the maze for 5 minutes (from the time that the rat first left the start arm). The number of entries and amount of time (s) the animal spent in each arm of the Y-maze was recorded to assess short-term memory.

Between each rat, the maze was cleaned with a 70% ethanol solution and rinsed with water to avoid odor cues. All experiments were conducted by the same individual blinded to experimental groups. Rat movements were recorded and analysed using the video-tracking software – SMART® 2.5 (PanLab, Barcelona, Spain). The reference point used by the software to determine the position of the animal was the centre of the rat's dorsum.

3.2.4.3 Morris Water Maze test

To evaluate the existence of long-term spatial memory deficits in EFV vs. control animals, the animals were submitted to spatial reference memory test described by Morris and co-authors (1982). The maze consisted of a large circular tank (1.8 m in diameter, 0.6 m in height) of water (23 ± 1 ^{o}C) that was made opaque with the addition of non-toxic water-based black paint. An escape platform (10 cm in diameter) was submerged 1 cm below the water. Visual cues were placed on the walls of the testing room, to be used as spatial references by the rats. An automated tracking system (Smart 2.5, *PanLab,* Barcelona, Spain) monitored the following parameters: escape latency (s); swim path length (cm); average speed (cm/s); and time spent in each quadrant (%).

Rats were given spatial (acquisition) training consisting of four trials per day for 4 days, in which the platform was placed at a fixed position in the center of one of the four quadrants of the tank (platform Q, left, right and across). The starting position, at which subjects were placed in the tank facing the wall, was randomly chosen across trials. Inter-trial interval period was 15 minutes, during which animals were towel-dried and placed in a heated incubator (25 ^{o}C) to

prevent hypothermia. The maximum trial duration was 60 s, after which animals were manually guided to the platform if they failed to locate it. Once animals reached the platform, they were allowed to remain there for 20 s. On the 5th day, animals were subjected to a probe test in which the platform was removed and animals were allowed to swim freely for 60 seconds, while recording the percentage of time spent on each quadrant. All experiments were carried out by the same individual blinded to experimental groups. Rat movements were recorded and analysed using the video-tracking software – SMART® 2.5 (PanLab, Barcelona, Spain). The reference point used by the software to determine the position of the animal was the center of the rat's dorsum.

3.2.4.5 Forced Swim (FS) test

The FS test is used to infer depressive-like behavior in rats, by evaluating their latency to immobility (or to despair; LTD) and the time spent swimming versus the time spent floating (percentage of time spent immobile) (Porsolt *et al.*, 1978a). The test involves two exposures to a vertical Plexiglas cylinder (height: 45 cm, diameter: 19 cm) filled with water (23 ^{o}C) at a depth that makes it impossible to reach the bottom with hind paws (28 – 30 cm).
On the first day of the two test days, all animals were gently placed individually in the cylinder during 10 minutes. Before animals return to their home cages, they were dried and exposed to infrared light, in order to prevent hypothermia. On the second day, the animals were placed in the same cylinders for 5 minutes. The water was changed after each session. The rat was judged to be immobile when it floated passively, making only small movements to keep its nose above the surface. All experiments were carried out by the same individual blinded to experimental groups.

3.2.5 Histological and Immunofluorescence assays

For histological analysis, animals were deeply anesthetized for transcardial perfusion with PBS, followed with 10% NBF; and after perfusion, the head was further immersed into neutral buffered formalin for 72h. Brain was then removed from the skull, trimmed (10-level trimming, according to (Bolon *et al.*, 2013), and routinely processed for paraffin embedding and sectioning. Hematoxylin and eosin-stained and cresyl violet-stained 4 µm sections were screened by a pathologist blinded to experimental groups, in a Leica DM2500 microscope coupled to a Leica MC170 HD microscope camera.

The following procedure was applied for immunofluorescence analysis of the frontal cortex and hippocampus: 4 μm sections, which were previously subjected to heat-induced epitope-retrieval (HIER) using DAKO PT Link (Dako, Glostrup, Denmark) and to quenching of endogenous peroxidase activity with 3% hydrogen peroxide, were blocked with goat serum diluted in glycine 0.3M (1:50), for 30 minutes at room temperature. There was an ensuing incubation with the following primary antibodies [diluted in 0.1% (w/v) BSA in PBS 1X] for 6o minutes at room temperature: anti-MAP2 (1:1500), anti-GFAP (1:1500), anti-FosB (1:200) and anti-SNAP-25 (1:1500). Slides were then rinsed with PBS 1X and incubated with secondary antibody [diluted in 0.1% (w/v) BSA in PBS 1X, 1:1000] for 120 minutes, at room temperature Sections were mounted in VECTASHIELD media with DAPI and examined by standard fluorescence microscopy using a Nikon Instruments Eclipse Ti-S Inverted Microscope (Hamamatsu digital camera C10600 ORCA-R^2). Images were acquired with NIS-Elements AR-3.2 and analyzed with ImageJ software. Images were taken on the same areas of the prefrontal cortex or hippocampus from each brain section, using identical microscopy parameters, including exposure time.

3.2.6 Statistical Analysis

Two-way ANOVA with *Bonferroni* post-test was used to compare CTL and EFV groups in the MWM acquisition curve. The Unpaired *Student t* test was used for EPM, OP, YM and FS tests. Data are expressed as mean ± SEM. One-way ANOVA was used for immunofluorescence. Data are presented as EFV/CTL ratio. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., version 5.01, San Diego, CA). Statistical significance for all tests was set at the level of $p<0.05$.

3.3 RESULTS

3.3.1 Animal data

While daily food intake was similar for both animal groups (26 ± 1 g for both), the EFV-exposed rats (34 ± 1 mL) consumed significantly less ($p=0.022$) water *per* day than the CTL group (39 ± 1 mL). EFV-exposed rats showed a lower ($p=0.049$) body weight gain (130 ± 8 g) than control animals (157 ± 9 g).

3.3.2 Behavioural tests

On the MWM, EFV-exposed animals showed an impaired learning ability to swim to the scape platform when compared to CTL animals (Figure 10A). The onset of this impairment in the EFV group was on day 2 (p=0.004). On the other hand, the retention ability of EFV animals was not compromised, once in the probe test there was no difference in the time spent in the platform quadrant between EFV and the CTL groups (Figure 10B). The average speed (cm/s) and total swimming distance (cm) on probe test were similar between groups (data not shown).

The YM test revealed short-term spatial memory deficits in EFV-treated rats as indicated by a significant reduction of the percentage of time and entries in the 'novel arm in comparison to CTL group (Fig. 10C: 24 ± 2 versus 18 ± 1%; p=0.038; Fig. 10D: 46 ± 1 versus 51 ± 3%; p<0.001). No differences were found to the total number of entries between EFV and CTL rats (Fig. 10E). The reduced novelty-seeking of EFV-treated rats indicates that these animals had significant short-term spatial memory impairment.

In the EPM test, EFV group presented a lower anxious-related behavior since these rats had more percentage of entries in the open arms and spent more time (%) in these arms than the control group (Figure 10F: 53 ± 1% versus 49 ± 1%; p=0.02; Figure 10G: 26 ± 1.4% versus 19 ± 1.3%; p=0.003). Additionally, EFV did not alter the locomotor performance, since there was no difference in the total number of arms entries between groups (data not shown).

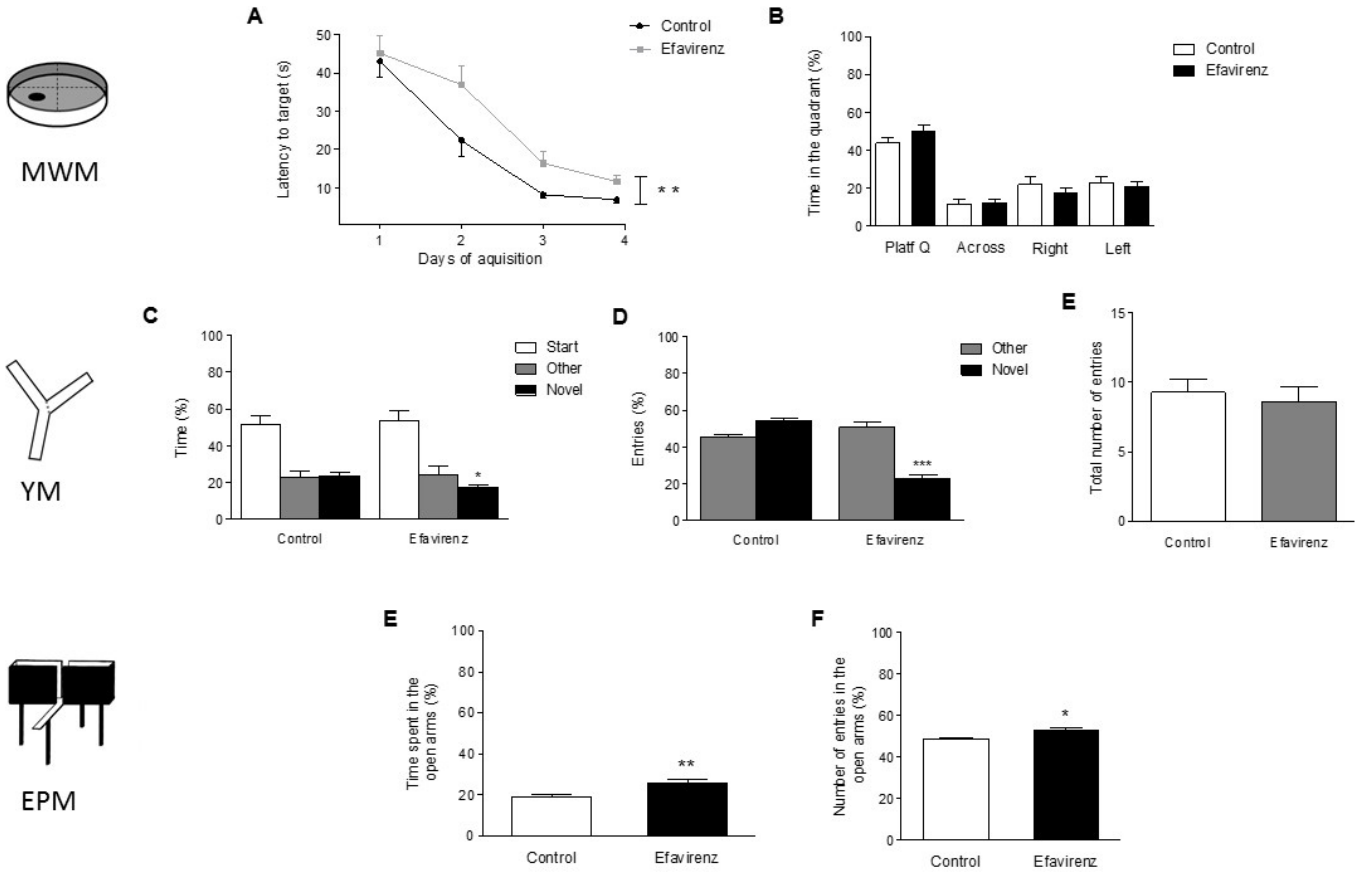

Figure 10. **Behavior evaluation of long-term efavirenz-exposed *Wistar* rats.** (A) Morris Water Maze (MWM) protocol (n=7 for each group): Learning curve of escape latencies over 4 days of acquisition. Two-way ANOVA, ***p*=0.004. (B) Probe Test: Time spent in each quadrant in hidden platform test. ns, ANOVA. Y Maze (YM) protocol (n=6 for each group): (C) Time (%) spent in the start, other and novel arm of the maze. (D) entries (%) in the other and novel arms; (E) total number of entries. Unpaired student´s *t*- test, **p*=0.04, ***p<0.001. Elevated Plus Maze protocol (n=7 for each group): (F) Time (%) spent in open arms; entries (%) into the open arms over 5 minutes. Unpaired student´s *t*- test, ** *p*= 0.003, * *p*= 0.015. Data from all tests are expressed as the mean ± SEM.

Likewise, it was also performed the OF arena test to further analyze rodent anxiety-like behavior and their locomotor activity. No differences between the time spent in periphery or in the center were found (Fig.11), further confirming that EFV-treated rats do not show an anxiety-like behavior. Also, the EFV treatment did not induce changes in the locomotor activity of the animals. This is in line with the data obtained from MWM test.

Finally, latency-to-despair (LTD) was evaluated by FS test. No differences were found between CTL and EFV-treated groups regarding depression evaluation (Fig.12).

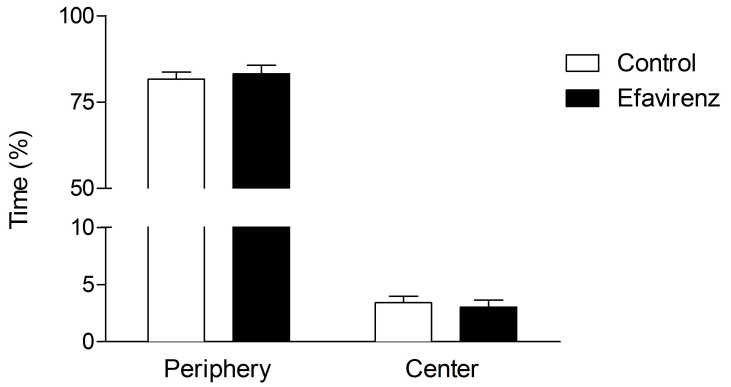

Figure 11. Open Field test. Time spent in the arena center and in arena periphery by control (n=8) and EFV-exposed animals (n=8), Unpaired student´s *t*- test. Data are expressed as the mean ± SEM.

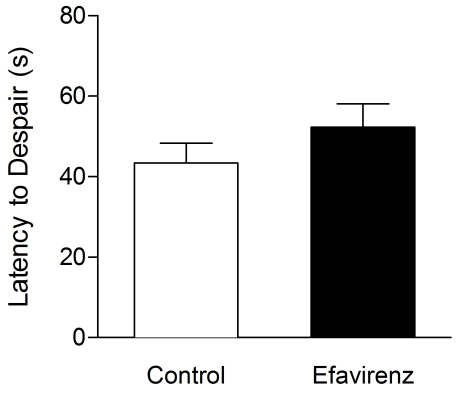

Figure 12. Forced swim test. The latency to despair (time before immobility) over 5 minutes for control (n = 8) and EFV-exposed animals (n = 7), Unpaired student´s *t*- test. Data are expressed as mean ± SEM.

3.3.3 Histological and immunofluorescence analysis

An in-depth histopathological analysis of the brains from EFV animals is provided herein. In particular, the frontal, parietal, temporal and occipital lobes of the cerebral cortex and cingulate gyrus, the caudate putamen, amygdala and thalamus, piriform cortex, cerebellar Purkinje cell layer and hippocampus showed no changes, including no evidence for neuronal cell death (Fig. 13). White matter tracts in the cortex were also unaltered.

Concerning the immunofluorescence analysis, major differences were found in hippocampus and prefrontal cortex, between CTL and EFV-treated rats. Rats exposed to EFV had a significant decrease in Map2 expression in the gyrus dentate and in CA1 region of the hippocampus, in comparison with the prefrontal cortex ($p<0.001$) (Fig. 14A and B); and the same pattern was found for FosB, as this protein was markedly decrease in the gyrus dentate of EFV group, followed by CA1 region, resulting in a bigger difference for prefrontal cortex ($p<0.001$) (Fig. 14A and C). No differences were found for GFAP expression (Fig. 16). For SNAP-25, differences were only found in the gyrus dentate of the hippocampus, which showed a slight decrease in expression when compared with the CA1 region ($p<0.05$) and prefrontal cortex ($p<0.05$) (Fig. 15). However, when comparing SNAP-25 between control and EFV groups no differences were found.

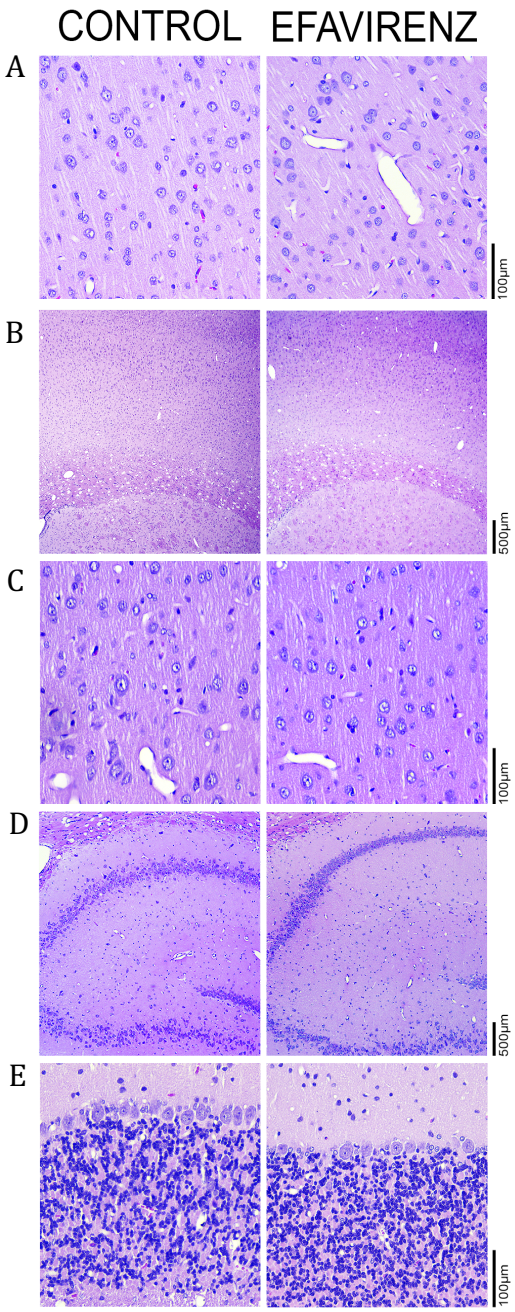

Figure 13. Representative microphotographs of prefrontal cortex (A), frontal cortex (B and C), hippocampus (D) and cerebellum (E) from control and efavirenz-exposed rats. No changes or evidence of neuronal cell death were seen in any of the areas analyzed. *Hematoxylin and eosin staining.*

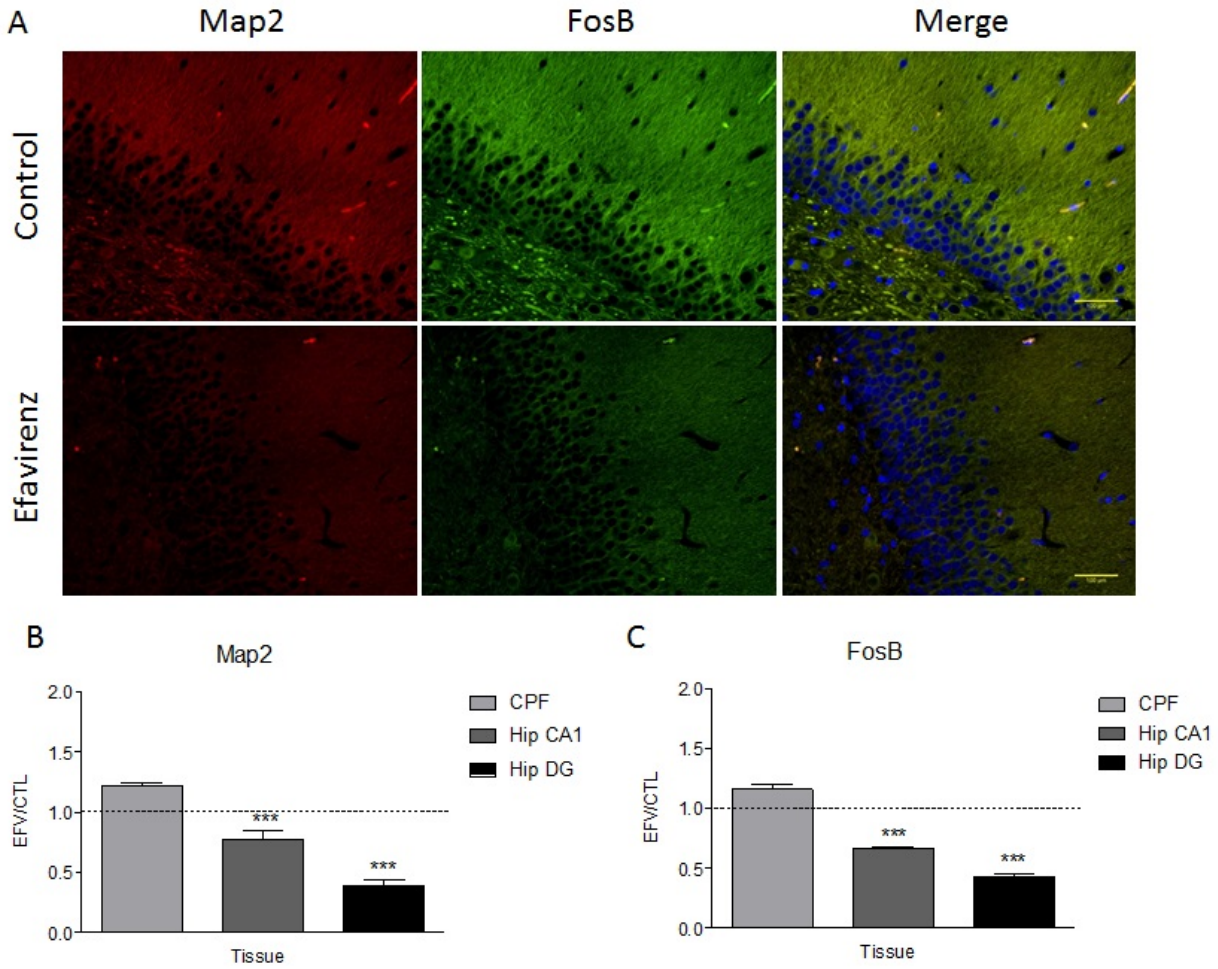

Figure 14. (A) Representative immunofluorescence analysis of hippocampus stained for Map2 (red) and FosB (green). (B) Quantification of Map2 and (C) FosB immunofluorescence intensity in prefrontal cortex, gyrus dentate and hippocampus CA1. One-way ANOVA, ***p<0.001. Data are presented as EFV/CTL ratio.

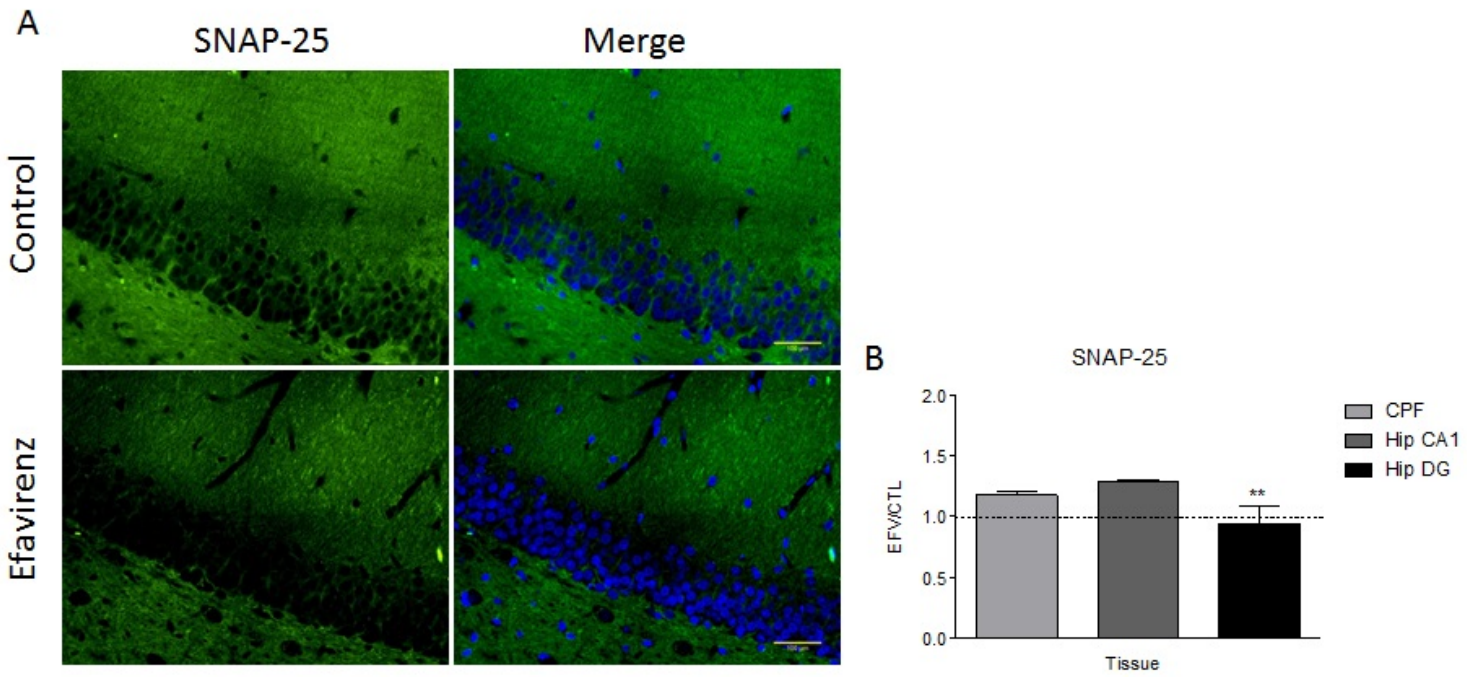

Figure 15. (A) Representative immunofluorescence analysis of hippocampus stained for SNAP-25. (B) Quantification of SNAP-25 immunofluorescence intensity in prefrontal cortex, gyrus dentate and hippocampus CA1. One-way ANOVA, **p=0.001. Data are presented as EFV/CTL ratio.

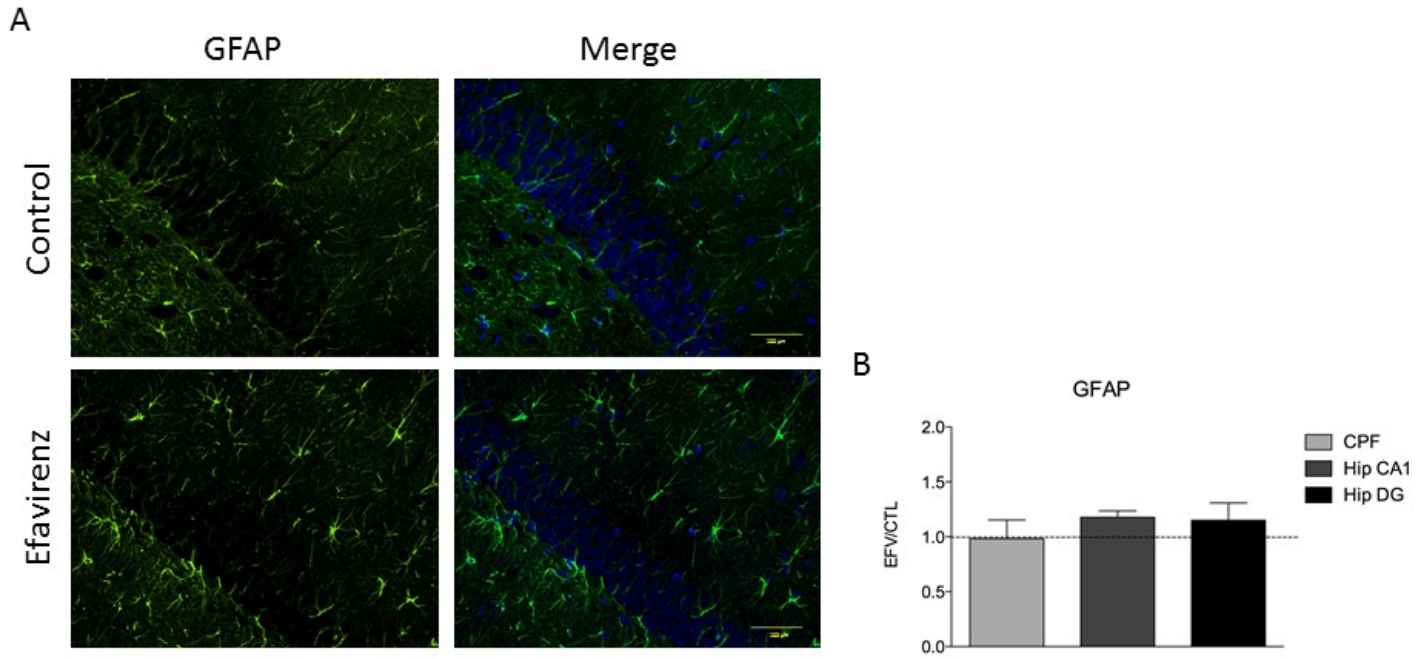

Figure 16. (A) Representative immunofluorescence analysis of hippocampus stained for GFAP. (B) Quantification of GFAP immunofluorescence intensity in prefrontal cortex, gyrus dentate and hippocampus CA1. One-way ANOVA, **p=0.001. Data are presented as EFV/CTL ratio.

3.4 DISCUSSION

As most CNS adverse reactions of EFV occur in the first weeks of treatment, the knowledge about the mechanisms underlying them, even if it is very limited, is related to short-term EFV exposure. We have previously reported that long-term EFV exposure of *Wistar* rats lead to significant changes in biotransformation of EFV via Cyp2b in liver, prefrontal cortex and hippocampus, that differs from short-term exposition. These changes were accompanied by an accumulation of 8-OH-EFV in plasma and with major tissue differences in electrophilic/oxidative stress induced upon EFV exposure as evaluated by thiol redox code, with marked decrease of glutathionylation of proteins in hippocampus, a decrease of lower magnitude in pre-frontal cortex and no variation in the liver. Herein, we have evaluated the molecular alterations in neurons, astrocytes and synaptic function of prefrontal cortex and hippocampus induced by long-term exposure to EFV and their relation to a behavior profile of anxiety, depression and cognitive impairment.

Our results show for the first time that long-term EFV exposure in rats triggers short- and long-term spatial memory deficits observed in YM and MWM tasks, respectively, that go along with a decrease in neuronal activity. In both tests, the animals need to make associations among the spatial environment clues to perform a cognitive map that helps them to find out the platform localization (Morris *et al.,* 1982) or to recognize the unvisited arm (Dellu *et al.*, 1997). EFV exposure led to a slower learning curve during the acquisition of MWM, without affecting memory retrieval. Also, the reduced novelty-seeking of EFV-treated rats found in the YM indicates that these animals had significant spatial memory impairment. Usually, animals prefer to investigate a new arm (novel arm) of the maze than returning to one that was previously visited. These results mean that EFV-exposed rats have memory impairment, particularly linked to the hippocampus (Morris, 1984). Notably, this test non-aversive as it does not require food deprivation as the radial maze or electric foot-shook as inhibitory avoidance test. Therefore, this test is less prone to modify the motivational and emotional status of the animals (Bekker *et al.*, 2006), avoiding confounding factors in the spatial memory parameters evaluated.

There are only three studies with behavior evaluation upon EFV exposure (O'Mahony *et al.*, 2005; Romao *et al.*, 2011; Gatch *et al.*, 2013). These studies differ strongly in study aims, in EFV exposure time (1-36 days) and EFV administration route (intravenous or oral gavage). Our study is the first associating EFV with learning memory impairment by using MWN and YM, suggesting that our model is the one that best fits for studies on this purpose. Moreover, the *Wistar* rat is a suitable model for the study of EFV biotransformation into 8-OH-EFV as an up-stream event of its neurotoxicity as the Cyp2b family is similar between humans (CYP2B6)

and rat (Cyp2b1 and Cyp2b2) and as they share inducible mechanisms (Ganem *et al.*, 1999; Sueyoshi *et al.*, 1999; Miksys *et al.*, 2000; Muangmoonchai *et al.*, 2001).

In our model, EFV did not induce anxiety. The animals spent more time in the open arms, presenting lower anxious-related behavior. In accordance with the results from the EPM, the locomotion from both groups was not affected in the *Open Filed* test, neither was it found an anxiety-like behavior. Our data differs from what was observed in mice exposed to the same EFV dose, which presented an elevated anxious behavior (Romao *et al.*, 2011). In that study, similar results were found for another anti-HIV drug, the nevirapine (NVP). NVP and EFV share more than the mechanism of action. Both are inducers of their metabolism via CYP2B6 through CAR activation (Faucette *et al.*, 2007). Also, their toxicity is associated with reactive metabolites generated by bioactivation of phase I metabolites (Caixas *et al.*, 2012; Harjivan *et al.*, 2014). In man, NVP is not associated to neurotoxicity but to hepatotoxicity (Sanne *et al.*, 2005). Plausible contributors to explain this difference are, the selective accumulation of the phase I metabolites in tissues, the capacity of each tissue to respond to electrophilic/oxidative stress as well as the availability of the enzymes that generate electrophile metabolites, the sulfotransferase in the case of NVP (Antunes *et al.*, 2013) and the oxidases in the case of EFV (Harjivan *et al.*, 2014). Despite sulfotransferases are present in brain, their activity is 300-fold lower than in liver (Rajkowski *et al.*, 1997), which might mean that brain is not so capable of producing NVP toxic metabolite (12-sulfoxy-nevirapine). Thus, the effect found by Romão and collaborators (2011) might be related to the capability of NVP to be a CAR ligand, rather than the formation of toxic metabolites. Interestingly, EFV was shown to be genotoxic at the brain, but not at the liver (de Oliveira *et al.*, 2014). Our previous data showed lower Cy2b mRNA levels in hippocampus are lower upon long-term EFV exposure, differently to what happens at the liver or prefrontal cortex. It is difficult to compare our results as these authors used mice and there are species-related differences on Cyp2b induction (Pustylnyak *et al.*, 2007). and behavior tests can induce different fear/stress in the species, resulting in a potentially different neurotransmitter and brain area stimulation (Porsolt *et al.*, 1978b).

We have not explored if this low-anxiety behavior was related to an absence of fear or to a hallucinogenic effect. Patients on EFV complain of hallucinations, vivid dreaming and psychosis (Gutierrez *et al.*, 2005; Cespedes and Aberg, 2006). There are reports on the use of EFV by non-infected teens who crush the pills and smoke the powder for its psychoactive effects (Marwaha, 2008; Sciutto, 2009). EFV has been describe as having an LSD-like effect, in the manner that it can be a partial agonist of the 5-HT_{2A} receptor an it can block dopamine and serotonine uptake (Gatch *et al.*, 2013), similar to other recreational drugs like cocaine,

methamphetamine and MDMA (Han and Gu, 2006). EFV might also have effects on serotonin as they compete for CYP2B6 metabolism (Fradette *et al.*, 2004) and EFV is an inducer of this CYP450 isoform.

Data obtained in the *Forced swim* test suggested no depression upon EFV exposure. Depression has been reported in a few clinical studies with EFV-treated patients, but the relation between EFV use and higher risk of depression is not easy defensible (O'Mahony *et al.*, 2005; Journot, 2006).

EFV and 8-OH-EFV were shown to induce cell death in primary hippocampus cell cultures (Tovar-y-Romo *et al.*, 2012). To evaluate the effect of EFV in our model, histopathological analysis of the brain was performed and no significant changes or signs of neuronal cell death were seen in any of experimental groups or areas of the brain analysed. Also, considering that MWM test has been introduced as an instrument with particular sensitivity to the effects of hippocampal lesions in rats (Morris *et al.*, 1982), we have explored if any alterations regarding neuronal dendrites (Map2) (Lewis *et al.*, 1989) and neuronal activity (FosB) (Flavell and Greenberg, 2008) were present in our animal model. Levels of Map2 and FosB were significantly decreased in hippocampus, both gyrus dentate and CA1 region, and no alterations were found in prefrontal cortex. At the dentate gyrus, neurogenesis has an important role in hippocampus-dependent learning and memory (Eriksson *et al.*, 1998; Shors *et al.*, 2002; Snyder *et al.*, 2005). Accordantly with our data, a decrease in the proliferation of neural stem cells induced by EFV was observed *in vitro* and in the subventricular zone of C57BL/6 mice (Jin *et al.*, 2016). The decrease found in Map2 indicates a loss of dendritic spines (Bernhardt and Matus, 1984). This suggests that this effect is mediated by the accumulation of 8-OH-EFV associated with long-term EFV exposure. This corroborates with the previous *in vitro* studies of Tovar-y-Romo showing that the metabolite 8-OH-EFV is one order of magnitude more potent in inducing dendritic spines injury than EFV itself (Tovar-y-Romo *et al.*, 2012). In rodent models of aging and AD, decreased Map2 levels has been shown to be coincident with an increase of Aβ peptides in CA1 dendrites (Takahashi *et al.*, 2013), early dendritic degeneration and reduction in total dendritic area (Moolman *et al.*, 2004). Also, an *in vitro* study has revealed an association between EFV exposure and an increased production of Aß plaques due to increased oxidative stress conditions (Brown *et al.*, 2014). Soluble Aβ oligomers represent the most toxic form of Aβ peptide, which are mediators of inflammation (Bradt *et al.*, 1998; Murakami *et al.*, 2005) and oxidative stress (Murakami *et al.*, 2005), and are critical in inducing cognitive impairment and synaptic dysfunction (Lue *et al.*, 1999). Corroborating the results found for Map2, a lower FosB levels within the hippocampus revealed a decrease in

neuronal activity. FosB has been associated with impairment in spatial memory (Solecki *et al.*, 2008).

SNAP-25 was analysed as a surrogate of synaptic activity. Our data showed a slight decrease at the dentate gyrus. This protein is a component of SNARE complexes that mediate the release of neurotransmitters (Sollner *et al.*, 1993; Tafoya *et al.*, 2006) and has been associated to cognitive deficit (Braida *et al.*, 2015).

Recent evidence support that 8-OH-EFV can be bioactivated (Harjivan *et al.*, 2014). Several authors have been shown that electrophiles and free radicals generation could negatively impact the function of proteins and thereby interfere with axonal transport or pre-synaptic neurotransmitter release (Asanuma *et al.*, 2003; Lopachin and Decaprio, 2005). This has been described for several toxins as acrolein or acrylamide (Lopachin and Decaprio, 2005), to L-DOPA and dopamine (Asanuma *et al.*, 2003). The 8-OH-EFV has also been shown to induce an alteration in the astrocytes glucose metabolism (Brandmann *et al.*, 2013), which lead us to evaluate astrocytes population in hippocampus and prefrontal cortex. As no changes in GFAP expression were seen, we can speculate that astrocytes may be more protected or not so vulnerable to EFV-toxicity, compared to neurons.

A slower weight gain was observed for EFV-exposed animals when compared with CTL rats, which is in accordance to ours and others (Aïssi *et al.*, 2015) previous observations. This was not associated with reduced food consumption, since no differences were found between the two groups. A possible explanation could be related with a decrease in growth hormone production within the hippocampus, where this hormone responds to several factors including exposure to environmental stimuli (Vander Weele *et al.*, 2013). A slower weight gain was also demonstrated in other exposure conditions associated with hippocampus injury and oxidative stress as chronic intermittent hypoxia (Diogo *et al.*, 2015) or methotrexate (Seigers *et al.*, 2008).

The data herein presented suggest that EFV significantly impairs neurological performance in animals, mimicking long-term neurotoxic effects observed in HIV-patients. This effect was particularly evident for hippocampus-dependent learning and memory. In clinical setting, approximately 40 - 50% of patients receiving EFV suffered from cognitive disorders, whereas 23 - 29% are related to memory (Lochet *et al.*, 2003; Ma *et al.*, 2016). Evidently some important aspects of human pathology will probably never be accessible in animal models, but these treatment conditions in *Wistar* rats represent a suitable model that replicates human phenotypical (behavioural) features. We believe that this will permit to get further insights into

the molecular mechanism of EFV-induced neurotoxicity and explore the effect of electrophile species, CYP2B6 activity and CAR activation in hippocampus-dependent learning and memory (Fig. 17).

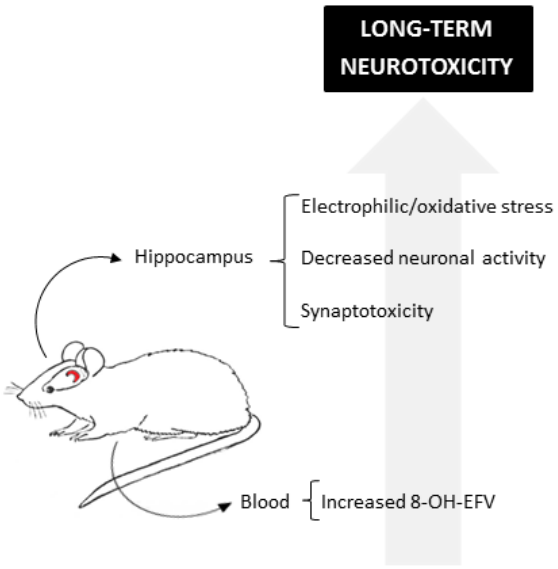

Figure 17. Schematic representation of the toxic outcomes induced by long-term efavirenz exposure. 8-OH-EFV, 8-hydroxy-efavirenz.

FINAL CONSIDERATIONS

FINAL CONSIDERATIONS

The starting premise for this research work was the contradictory data in literature regarding the dependency of CNS adverse reactions on EFV concentrations (Ngaimisi *et al.*, 2010; Cho *et al.*, 2011; Habtewold *et al.*, 2011; Aouri *et al.*, 2016).

Data (unpublished) from our lab TDM unit (Fig. 18) and from others (Kwara *et al.*, 2010), show that among patients on the recommended dose of EFV, 600 mg/day, there are a great percentage of patients that manifest CNS adverse reactions within therapeutic concentrations (1 – 4 mg/L, Marzolini *et al*., 2001). Additionally, there are patients on 800 mg daily dose that had lower EFV concentrations than patients on 600 mg daily (Manosuthi *et al.*, 2006).

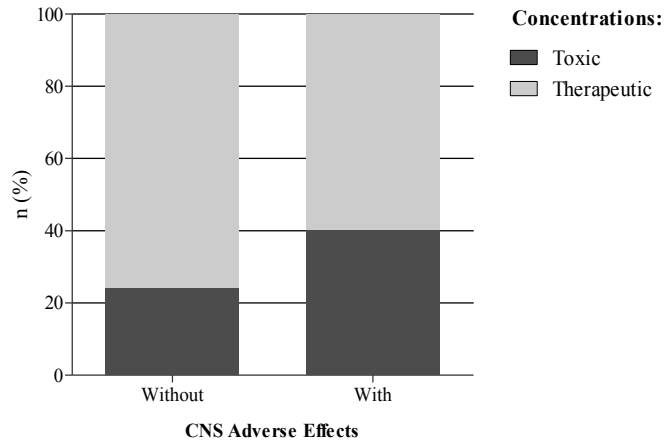

Figure 18. Data from the Therapeutic Drug Monitoring Unit of CEDOC-NMS (Unpublished data). Concentrations of efavirenz (C_{though}) were measured in 43 patients on 600 mg of EFV and central nervous system adverse reactions were reported by the clinician. Therapeutic (1-4 mg/L) and toxic concentrations (above 4 mg/L) were defined according to Marzolini and co-authors (2001). ***p*=0.015, Chi-square.**

Data reflects the high inter-individual variability of EFV concentrations (Marzolini *et al.*, 2001; Pereira *et al.*, 2008), which is mainly attributed to its biotransformation. Additionally, as many neuro-adverse reactions have a delayed onset, we hypothesized the role of EFV metabolite(s) accumulation in the genesis of neurotoxic effects. Therefore, the work herein presented was aimed at unraveling the role of EFV biotransformation in EFV-induced CNS adverse reactions in order to find mechanistically-oriented tools for its monitoring and for evaluating strategies with the ultimate goal of optimizing EFV use.

The top five studies that strongly supported our findings and influenced our choices in the progression of this thesis are herein enounced.

One published in 2012 by Tovar-y-Romo and collaborators in *Sprague-Dawley* hippocampal neuronal culture, showing that 8-OH-EFV was a more potent neurotoxin than EFV itself (Tovar-y-Romo *et al.*, 2012). The minimal toxic concentration used was three times lower than what is found in the CSF of HIV-infected patients (Tovar-y-Romo *et al.*, 2012). This study placed 8-OH-EFV in the roadmap of EFV neurotoxicity mechanisms. However, this is an *in vitro* study and there was a need for the translation of this finding.

The second, from 2011 by Khokhar and collaborators, showed that by inhibiting Cyp2b specifically in brain, the pharmacological effect of the anesthetic propofol is changed (Khokhar and Tyndale, 2011). The authors observed an increase in the sleep time that was related with propofol concentration in brain, but not with the concentration in plasma. Additionally, seven days of nicotine treatment can induce the expression of brain Cyp2b but not hepatic isoform, and this induction reduced propofol sleep time by 2.5-fold. This work elegantly showed the importance of studying CYP activity and regulation in brain and the need to find peripheral markers that reflect this brain activity.

The third was published by Harjivan and colleagues in 2014, and showed that upon *in vitro* oxidation of 8-OH-EFV, this metabolite was prone to yield a toxic quinoid derivative (Harjivan *et al.*, 2014), paving the way for a new and unexplored mechanism of EFV neurotoxicity. This was later corroborated by Oliveira *et al* (2014) that found a selective genotoxicity in brain but not in liver of mice exposed to EFV (de Oliveira *et al.*, 2014).

Finally, Ngaimisi and collaborators showed in 2010 that the metabolites profile changed over at least 16 weeks since EFV beginning and it is characterized by a decrease in EFV and increase in 8-OH-EFV concentrations (Ngaimisi *et al.*, 2010). This long-term auto-induction effect is dose-, duration of treatment- and genetically-dependent. These data lead us to hypothesize that 8-OH-EFV contributes differently to long- and short-term toxicity mechanisms.

Herein are presented our final considerations, divided into four components. The first one summarizes the relevant findings of both clinical and animal studies. Then, it is described the added value of this work to the field. The next sections outline the main limitations of our work and explore some future perspectives concerning the management of EFV-CNS toxicity. It is

therefore unavoidable that there is some content overlap between these sections and the discussion section of each chapter.

1. Summary of relevant findings

In our opinion, to justify the discrepant data reported in literature about the correlation between EFV concentration and the occurrence of neuro-adverse drug reactions, we should not only considerer the inter-individual PK variability of EFV, but also the broad type of symptoms and the time for their onset. It is highly plausible that the panoply of described EFV-adverse reactions have different underlying mechanisms. For this reason, in the clinical study of the present work, we focused on mood changes. We choose to start with this adverse effect because it was the most observed in patients on long-term EFV exposure in our cohort. In short-term EFV use, sleep disorders are the major complaint. Our study showed that patients on long-term EFV exposure with mood changes complaints have higher plasmatic concentrations of 8-OH-EFV than those without CNS complaints. To minimize confounding factors, we have limited the study to male patients with normal hepatic function and controlled viremia. One additional factor for this choice was the fact that the number of woman included on EFV is much lower than man, as EFV was classified as Pregnancy Category D by FDA clinicians avoid its prescription in woman in childbearing age. Differently to previous studies, we took in consideration the type of CNS adverse reaction, the time on EFV, the PK parameter and the concentration of non-conjugated 8-OH-EFV and 8-OH-EFV-Glc, which we consider a plus in our study design.

In conclusion, with the results obtained in the first part of the present study, is possible to suggest the plasmatic concentration of 8-OH-EFV as a suitable parameter for TDM, particularly aimed at controlling mood changes.

In the animal studies, we evaluated the time-course of EFV biotransformation and profiled Cyp2b induction by EFV in brain and liver as well as tissue *thiolomic* signatures. We observed that changes in these endpoints were particularly evident in brain at 36 days of EFV exposure and we ascertained that these molecular changes are related to an animal neurologic phenotype (memory impairment), similar to what is observed in patients.

Long-term EFV exposure was associated to induction of Cyp2b at the liver, higher 8-OH-EFV plasma concentrations, memory and learning impairment, neuronal dysfunction in hippocampus, a brain area with unquestionable roles in cognition and emotional experiences

(Sigurdsson and Duvarci, 2016). Similarly to man, the Cyp2b induction by EFV had a delayed onset and persisted after long-term exposure, occurring a progressive increase in 8-OH-EFV formation. This effect is opposed in the hippocampus, showing a different regulation of Cyp2b in brain and liver upon long-term EFV exposure. According to our data interpretation, 8-OH-EFV should be barely formed in the hippocampus, suggesting a contribution of a peripheral pool of this metabolite, which is in accordance with our previous result in man and with the possibility of 8-OH-EFV to be able to cross the BBB. Other observation was that hippocampus is the tissue more susceptible to 8-OH-EFV toxicity (where a decrease in GSSP and CysSSP was observed) and this was coincident with a decrease in GSH and an increase of GSSP in plasma, suggesting the suitability of plasma *thiolomic* profile, together with 8-OH-EFV concentrations in evaluating what happens in brain. New data from our group aiming at the translation of this data showed a similar thiolation plasma profile in patients on EFV (Correia *et al*. 2016). Moreover, patients with higher plasmatic GSSP had higher 8-OH-EFV. This profile was not related to viral load or $CD4^+$ T-cell count of the patients and it was independent on viral subtype (subtype B vs non-B).

2. What is the added value and the impact of the present work to the field?

Contribution to the knowledge of the mechanisms of EFV neurotoxicity

We propose that short-term neurotoxic effects would probably be more dependent on EFV concentration, while those at long-term will most likely be related to 8-OH-EFV accumulation. This issue is pertinent for further evaluation as CNS-adverse reactions occurring in the first weeks of treatment (short-term) are normally transient, or if not the patient usually switches to other cART options. Thus the long-term effects of EFV exposition in brain are a main concern and much more difficult to predict and to manage. This is particularly worrying in children, which start EFV at 3 years of age, ideally through their entire life.

Identification of peripheral markers to monitor the risk of long-term mood changes and memory impairment and to evaluate strategies for its management

Our findings suggest for the first time the importance of monitoring 8-OH-EFV, besides EFV, to predict and manage EFV-CNS toxicity. Together with evaluation of *thiolomic* signature as a measure of electrophilic stress they can be components of a better index for the risk of long-term EFV associated mood changes and memory impairment.

New input in the interpretation of pharmacogenetic data on CYB2B6 and in the identification of new targets for pharmacogenetic studies on EFV neurotoxicity

These data are also relevant for the interpretation of pharmacogenetic data. Most of pharmacogenetic studies are related to CYP2B6 and consider EFV itself as the responsible for its toxicity. Consequently, low metabolizers are assumed to be at greater risk for adverse reactions. However, it has been not evaluated the correlation between polymorphisms with a specific type of adverse reaction, with EFV biotransformation into 8-OH-EFV or with time of exposure. Due to this lack of information and considering our results, we suggest that low metabolizers are at higher risk of short-term toxicity (due to EFV), while ultra-rapid and extensive metabolizers are more prone to long-term toxicity (as mood changes and memory impairment). It should be also considered other pharmacogenetic targets as, other CYP isoforms, UGTs, the nuclear receptors CAR and PXR, which have been related with EFV PK alterations (Swart *et al.*, 2012b) and in glutathione-S-transferase (responsible for catalyze the conjugation of electrophiles to GSH for their detoxification), which have been associated with increase susceptibility to toxic compounds (Wormhoudt *et al.*, 1999).

Identification of drug interactions and their mechanisms that increase long-term EFV neurotoxicity risk

It is important to consider drug interactions in EFV prescription, since EFV has a narrow therapeutic window and HIV-infected patients might develop comorbidities and are often polymedicated. Possible targets of drug interactions in the EFV neurotoxicity are the enzymes involved in EFV metabolism that contribute to 8-OH-EFV increased concentration, e.g., CYP3A4 inhibitors (e.g., ritonavir (Eagling *et al.*, 1997)), CYP2B6 inducers (e.g., rifampicin (Faucette *et al.*, 2004)) and UGT2B7 inhibitors (e.g., valproic acid (Trapnell *et al.*, 1998)). It should be given attention to the prescription of other inducers of CYP2B6, as the antiepileptic drugs phenytoin and carbamazepine, which are inducers of CYP2B6 (Faucette *et al.*, 2004) and deplete GSH (Raya *et al.*, 1995). Depletion of GSH is involved in toxicity outcomes/susceptibility to toxicity and is present in the plasma, PBMCs and brain lysates of HIV-infected patients (de Quay *et al.*, 1992; Saing *et al.*, 2016). For these reasons, attention should be also given to drugs and pathologic conditions with associated reduction of GSH content. One particular case on drug interactions with EFV is rifampicin, used for tuberculosis. Rifampicin is a potent inducer of CYP3A4 and a moderate of CYP2B6 (U. S. Food and Drug Administration, 2016), which might lead to sub-therapeutic EFV concentrations, particularly in patients with bodyweight above 50 kg (Lopez-Cortes *et al.*, 2002). In 2012, FDA recommended the increase of EFV dose from 600 to 800 mg/daily when co-administrated with rifampicin (AIDS*info*, 2012). However, several studies do not support this recommendation

due to the inter-variability of EFV concentrations (Manosuthi *et al.*, 2006) and genetic factors (Manosuthi *et al.*, 2014). Nevertheless, the effect of this co-administration in 8-OH-EFV concentrations was not assessed yet.

Contribute to the evaluation of drug bioactivation risk and justify the unquestionable need of animal studies in neurotoxicity assessments

The treatment conditions used in *Wistar* rat represent a suitable model for the assessment of EFV PK/neurotoxicity relationships and to ascertain the mechanisms underlying it. Therefore, we believe that this model will permit to get further insights into the molecular mechanism of EFV-induced neurotoxicity, and explore not only more possible targets but also uncover drug metabolism/bioactivation in brain. Also, we consider that the evaluation in different compartments is of unquestionable importance to understand the likelihood of a drug to be toxic. Our findings in prefrontal cortex and hippocampus highlight the importance of not study the complete brain, since as we and others (Miksys *et al.*, 2000) have observed that Cyp2b has tissue- and cell-specificities. Hence, it is important to not assume that the regulation of CYPs is the same in all compartments. The same rational must be applied for tissue capability to bioactivate 8-OH-EFV and tissue response to electrophilic stress.

The use of an animal model was unavoidable for our research approach, mainly since our study involved long-term drug exposure, liver-blood-brain tissues analysis and importantly its relation to behavior evaluation. Therefore, the use of *in vitro* alternatives were not found to be suitable. Importantly, during the entire project the 3Rs policy (replacement, reduction and refinement) was always followed. The number of animals used was kept to the minimum and, whenever possible, the same tissue was used for more than one assay. Also, *NIH Principles of Laboratory Animal Care* (NIH Publication 85-23, revised 1985), the European guidelines for the protection of animals used for scientific purposes (European Union Directive 2010/63/EU) and the Portuguese Law nº 113/2013 concerning ethical use of animals were followed.

If translated into clinical practice, the evidence herein provided could be helpful on the interpretation and clinical understanding of pharmacogenomics and TDM data in both short- and long-term EFV neurotoxicity, as well as on the identification of patients prone to toxicity. Moreover, it highlights the awareness of not only of drug-drug interactions with chronically used enzyme inducers that cross BBB, but also of drugs that are extensively metabolized by the liver and whose metabolites penetrate the CNS.

3. Which were the limitations of the present work?

We are conscious about some limitations of our clinical study. Our sample size should ideally be larger; however, in order to avoid bias in our results, we have implemented restricted inclusion and exclusion criteria, which decreased significantly our study population. Another limitation was the low number for women in our cohort, which did not allow us to evaluate sex-differences in the toxicity markers herein explored, despite the reported sex-differences in EFV PK (Burger *et al.*, 2006). Additionally, our CNS adverse effects were based on self-reported complaints made by the patients. Ideally, these adverse effects would be assessed by validated cognitive tests and interviews.

Moreover, we have not quantified 8-OH-EFV-sulfate that was recently identified by Aouri and co-authors (2016) as a new phase II metabolite. This metabolite occurs approximately 10 times lower than the glucuronide conjugate (Aouri *et al.*, 2016). With this knowledge, not only 8-OH-EFV-Glc but also 8-OH-EFV-sulfate should have been considered. Currently, it is unknown if 8-OH-EFV-Glc and 8-OH-EFV-sulfate pass through BBB. Usually, phase II metabolites are unable to cross this barrier; however, there are reported exceptions (D. Wu *et al.*, 1997). Particularly in the case of 8-OH-EFV, both phase II metabolites were found in the CSF (Aouri *et al.*, 2016) and considering that phase II enzymes content in brain is low (Ouzzine *et al.*, 2014), it is possible to postulate that BBB is permeable to EFV phase II metabolites.

In animals, it was only possible to quantify 8-OH-EFV when glucuronidase enzyme was use, thus the quantification reflects both the sum of non-conjugated and 8-OH-EFV-Glc fractions. Again, 8-OH-EFV-sulfate was not measured despite its identification in rat urine (Mutlib *et al.*, 1999). As the rat metabolism is more rapid than man, makes it difficult to quantify the free metabolite with the use of HPLC methodology. The identification of the phase II metabolite, 8-OH-EFV-sulfate in man has implications for the use of EFV as a probe to phenotype the CYP2B6 activity. Thus, in the future, sulfatase hydrolysis has also to be performed. Also, other limitation arising from the factors mentioned above was the lack of 8-OH-EFV quantification in tissues, especially in CNS compartments.

Furthermore, it was not possible to evaluate the formation of covalent adducts between 8-OH-EFV-derived reactive metabolites and proteins. Despite the oxidative bioactivation pathway was demonstrated *in vitro* (Harjivan *et al*., 2014; Wanke *et al*., 2012), to attest the role of 8-OH-EFV bioactivation in the onset of EFV-induced neurotoxic events, the identification of these protein adducts should have been performed *in vivo*. Nonetheless, the identification of

drug-protein adducts *in vivo* constitutes still a great analytical challenge, due to the low concentration of adducted protein when compared with the concentration of non-modified protein. In fact, despite the huge developments in terms of bottom-up mass spectrometry-based proteomics approaches towards the identification of covalent drug-protein adducts, this still constitutes a time-consuming task. Considerable work is needed, not only for the optimization of protein isolation and digestion steps, but also for the optimization of chromatographic and mass spectrometry methodologies for the separation and identification of adducted peptide and for the subsequent data analysis step. For these reasons, it was not possible to achieve this goal within the time-period of this thesis.

Finally, we have not considered the effects of the other drugs of cART regimen, which are not exempt of toxicity and of changing EFV PK (Carr and Cooper, 2000). Moreover, our animal model does not explore the contribution of HIV-infection and its associated immunosuppression, residual inflammation and viremia to neurotoxicity. Nowadays, it is possible to develop humanized mouse models with HIV (Denton and García, 2011). These models are generated by transplanting human cells or tissues into genetically modified immunodeficient mice. These animals are then susceptible to infection through the natural routes by which humans are. With these models, it would be possible to study cART toxicity outcomes in parallel with the effects of HIV-infection. Regarding CNS status, this is of particular importance, since HIV also generates neurocognitive disorders (Letendre, 2011). This association has also been linked to a higher oxidative stress status.

4. What is still unknown and should be addressed?

Despite the significant progress in recent years, there are still large gaps in the current knowledge of the effects of drug biotransformation in adverse neurological events. Further studies must be conducted to elucidate the importance of 8-OH-EFV concentrations towards personalized and safer prescription of EFV. We believe that it is necessary to define an 8-OH-EFV concentration threshold above which the risk of toxicity increases, similar to what exists for EFV (> 4 mg/L). Pharmacogenetic results with short- and long-term toxicity appear to be of interest too.

The hypothesis of adducts formation in brain, particularly between bioactivated 8-OH-EFV and synaptic proteins in these times of exposure to the drug should also be addressed. Another interesting question still to be answered: Is the administration of *N*-acetylcysteine useful? Or if

not, would it be useful to perform structural modifications of EFV molecule in order to avoid 8-OH-EFV formation? The proximity of the OH group at position 8 to the nitrogen group in the benzoxazine structure appears to be critical for the generation of neurotoxic metabolites. This is demonstrated by the fact that 7-OH-EFV, which possesses a OH group at the carbon-7 position, is not neurotoxic. Consequently, is it possible to hypothesize that the substitution of the carbon at position 8 (*e.g.* with a fluorine), avoiding EFV hydroxylation at this position, should produce a compound with decreased neurotoxicity.

Future work should also explore *thiolomic* profile monitoring to assess the usefulness of intervention strategies against electrophilic stress prior to or early after the onset of clinical symptoms upon EFV exposure. For example, it will be interesting to evaluate the contribution of polymorphisms in GSH-related genes or thiolation profiles, not only for the evaluation EFV neurotoxicity but also to interpret its resistance to hepatotoxicity.

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ATTACHMENTS

ATTACHMENT #1

Informação ao participante voluntário no estudo intitulado *Deteção de Efavirenz e de adutos de proteínas em indivíduos infetados pelo VIH*

É seguido nesta consulta por estar infetado VIH e está no momento a ser tratado com medicamentos para controlo da infeção. À luz dos atuais conhecimentos, os medicamentos que utiliza são os mais indicados para si. Como sabe são medicamentos não isentos de efeitos adversos que podem manifestar-se a curto ou longo prazo e para os quais o seu médico está sempre atento.

Numa tentativa de podermos identificar possíveis efeitos adversos não esperados dos medicamentos, agradecemos a sua colaboração na colheita de uma amostra do seu sangue para realização de testes laboratoriais específicos que detetem eventuais toxicidades. Esta amostra de sangue será colhida em consulta e serão apenas colhidos 3 ml de sangue. A sua participação será voluntária, não sendo paga qualquer quantia pela sua contribuição no estudo. O seu médico assistente também não receberá nenhuma remuneração pela realização deste estudo. Os dados relativos à sua colheita estarão protegidos pela lei da confidencialidade e proteção de dados.

O presente estudo não trará qualquer benefício direto para si ou para a evolução da sua infeção. Com este estudo pretendemos saber mais sobre os medicamentos que utiliza e com os dados colhidos esperamos chegar a conclusões que no futuro possam contribuir para o melhor conhecimento dos medicamentos para controlo da infeção VIH e com isso, eventualmente, prevenir outras patologias e melhorar a qualidade de vida dos doentes infetados.

Obrigado.

Consentimento informado

Li ou foi-me lido e explicado pelo meu médico o texto acima e consinto na colheita da amostra de sangue

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(o doente)

(o médico)

Lisboa, _____/_____/_____

ATTACHMENT #2

Patient data notebook

FACULDADE DE CIÊNCIAS MÉDICAS | UNIVERSIDADE NOVA DE LISBOA

INFORMAÇÃO DO HOSPITAL			
Médico		Telefone	
Hospital		E-mail	

CRITÉRIOS DE INCLUSÃO E EXCLUSÃO		
	Não	**Sim**
≥ 18 anos de idade	☐	☐
Consentimento informado	☐	☐
Suspeita de não adesão *Adesão - cumprir pelo menos 95% da terapêutica antiretroviral*	☐	☐

INFORMAÇÃO DO DOENTE

Doente (n) |___|___|___|___|___|___|___|___|

Data de nascimento |DD|MM|AA|

Sexo F ☐ M ☐

Peso (Kg) |___|___| Altura (*cm*) |___|___|___|

Etnia: Caucasiana ☐ Negra ☐ Outra

Subtipo HIV: _____

Consumo de álcool N ☐ S ☐ ESPECIFICAR

Uso de drogas injectáveis N ☐ S ☐ ESPECIFICAR Fumador(a) N ☐ Y ☐ ESPECIFICAR

Reacções adversas N ☐ S ☐ VER ANEXO I

Patologias associadas N ☐ HCV ☐ HBV ☐ Diabetes ☐ Outra ☐ ESPECIFICAR

Infecções oportunistas N ☐ Tuberculose ☐ Outra ☐ ESPECIFICAR

Primeira terapêutica antiretroviral N ☐ S ☐

Terapêuticas antiretrovirais anteriores

(data de início/fármaco)

|___|___|___|___|___|___|___|___|___|___|___|___|

|___|___|___|___|___|___|___|___|___|___|___|___|

|___|___|___|___|___|___|___|___|___|___|___|___|

|___|___|___|___|___|___|___|___|___|___|___|___|

Por favor, enviar o formulário completo acompanhado das amostras para:

Departamento de Farmacologia, Faculdade de Ciências Médicas
Campo dos Mártires da Pátria, 130, 1169-056 Lisboa
Tel.: 218 803 035 **Fax**: 218 803 083 **E-mail:** farmacologia@fcm.unl.pt, sofia.pereira@fcm.unl.pt **telemóvel** Sofia Pereira 96 4243174

	|__|__||__|__||__|__|		
Efavirenz	Data de início *(dia/mês/ano)*	mg por toma	Nº de tomas
	|__|__||__|__||__|__|	|__|__|__|__|	|__|
Co-terapêutica antiretroviral	Data de início *(dia/mês/ano)*	mg por toma	Nº de tomas
NOME DO FÁRMACO	|__|__||__|__||__|__|	|__|__|__|__|	|__|
NOME DO FÁRMACO	|__|__||__|__||__|__|	|__|__|__|__|	|__|
NOME DO FÁRMACO	|__|__||__|__||__|__|	|__|__|__|__|	|__|
NOME DO FÁRMACO	|__|__||__|__||__|__|	|__|__|__|__|	|__|
NOME DO FÁRMACO	|__|__||__|__||__|__|	|__|__|__|__|	|__|
Outros fármacos Não ☐ Sim ☐ (incluir todos os que tomou no último mês) (incluir fitoterápicos, medicamentos de venda livre, etc.)			
NOME DO FÁRMACO	|__|__||__|__||__|__|	|__|__|__|__|	|__|
NOME DO FÁRMACO	|__|__||__|__||__|__|	|__|__|__|__|	|__|
NOME DO FÁRMACO	|__|__||__|__||__|__|	|__|__|__|__|	|__|
NOME DO FÁRMACO	|__|__||__|__||__|__|	|__|__|__|__|	|__|

Parâmetro	Valor	Unidade 1	Unidade 2	Data
Subpopulações de CD_4	|__|__|__|__|__|__|	☐ *cel/mm³*	☐ ________	|DD|MM|AA|
Subpopulações de CD_8	|__|__|__|__|__|__|	☐ *cel/mm³*	☐ ________	|DD|MM|AA|
Carga viral	|__|__|__|__|__|__|	☐ *cópias/mL*	☐ ________	|DD|MM|AA|
Hemoglobina	|__|__|__|__|__|__|	☐ *g/dL*	☐ ________	|DD|MM|AA|
Plaquetas	|__|__|__|__|__|__|	☐ *cel/mm³*	☐ ________	|DD|MM|AA|
Leucócitos	|__|__|__|__|__|__|	☐ *g/L*	☐ ________	|DD|MM|AA|
Neutrófilos	|__|__|__|__|__|__|	☐ *cel/mm³*	☐ ________	|DD|MM|AA|
Linfócitos	|__|__|__|__|__|__|	☐ *cel/mm³*	☐ ________	|DD|MM|AA|
Monócitos	|__|__|__|__|__|__|	☐ *cel/mm³*	☐ ________	|DD|MM|AA|
Tempo de protrombina (INR)	|__|__|__|__|__|__|	☐ *%*	☐ ________	|DD|MM|AA|

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Campo dos Mártires da Pátria, 130, 1169-056 Lisboa
Tel.: 218 803 035 **Fax**: 218 803 083 **E-mail:** farmacologia@fcm.unl.pt, sofia.pereira@fcm.unl.pt **telemóvel** Sofia Pereira 96 4243174

α_1-glicoproteína ácida	|__|__|__|__|__|__|__|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Glicémia	|__|__|__|__|__|__|__|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Creatinina	|__|__|__|__|__|__|__|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Ureia	|__|__|__|__|__|__|__|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Albumina	|__|__|__|__|__|__|__|	☐ *g/L*	☐ _____	|DD|MM|AA|
AST	|__|__|__|__|__|__|__|	☐ *U/L*	☐ _____	|DD|MM|AA|
ALT	|__|__|__|__|__|__|__|	☐ *U/L*	☐ _____	|DD|MM|AA|
γ–GT	|__|__|__|__|__|__|__|	☐ *U/L*	☐ _____	|DD|MM|AA|
Fosfatase alcalina	|__|__|__|__|__|__|__|	☐ *U/L*	☐ _____	|DD|MM|AA|
LDH	|__|__|__|__|__|__|__|	☐ *U/L*	☐ _____	|DD|MM|AA|
Bilirrubina total	|__|__|__|__|__|__|__|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Bilirrubina directa	|__|__|__|__|__|__|__|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
PCR	|__|__|__|__|__|__|__|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Ácido hialurónico	|__|__|__|__|__|__|__|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Haptoglobina	|__|__|__|__|__|__|__|	☐ *µg/dL*	☐ _____	|DD|MM|AA|
Alfa2-macroglobulina	|__|__|__|__|__|__|__|	☐ *g/dL*	☐ _____	|DD|MM|AA|
Apolipoproteina A1	|__|__|__|__|__|__|__|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Colesterol total	|__|__|__|__|__|__|__|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
LDL – col	|__|__|__|__|__|__|__|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
HDL - col	|__|__|__|__|__|__|__|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Triglicéridos	|__|__|__|__|__|__|__|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Transferrina	|__|__|__|__|__|__|__|	☐ *g/L*	☐ _____	|DD|MM|AA|
Ferro	|__|__|__|__|__|__|__|	☐ *µg/dL*	☐ _____	|DD|MM|AA|
Sódio	|__|__|__|__|__|__|__|	☐ *mmol/L*	☐ _____	|DD|MM|AA|
Potássio	|__|__|__|__|__|__|__|	☐ *mmol/L*	☐ _____	|DD|MM|AA|
MPKA	|__|__|__|__|__|__|__|	☐	☐ _____	|DD|MM|AA|
CPK	|__|__|__|__|__|__|__|	☐ *U/L*	☐ _____	|DD|MM|AA|

História Familiar Não ☐ Sim ☐	*Notas*

Data |DD|MM|YY|

Responsável pela recolha de dados _____

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