Maria João Andrade Correia

Licenciada em Bioquímica

S-thiolated protein profile to assess the redox-modulation effects of antiretroviral drugs

Dissertação para obtenção do Grau de Mestre em

Genética Molecular e Biomedicina

Orientador: Sofia de Azeredo Pereira, Professora Auxiliar

NOVA Medical School, UNL

Co-orientador: Lucília Neves Diogo, Professora

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Júri:

Presidente: Prof. Doutora Ilda Maria Barros dos Santos Gomes Sanches

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Abstract

Efavirenz (EFV) is an anti-HIV drug that has been associated to neurotoxicity. The 8-hydroxy-efavirenz metabolite has been described as the responsible for its long-term neurotoxic effects. Upon bioactivation this metabolite is able to generate electrophilic species, forming protein adducts and eliciting oxidative stress. *S*-thiolation of proteins (e.g. *S*-glutathionylation) has been suggested as a mechanism of protein regulation by the redox status. The present study is aimed to investigate if *S*-thiolated proteins plasma profile (RSSP) is associated with EFV-neurotoxicity.

The RSSP was assessed in an animal model of EFV-induced neurotoxicity. The animal RSSP was compared to the one obtained in a cohort of HIV-infected patients on EFV. The dependence on 8-hydroxy-efavirenz generation and the influence of infection in RSSP profile were also assessed in the clinical study. This profile was also compared to the one obtained in patients on nevirapine (NVP), a non-neurotoxic antiretroviral of the same class of EFV.

The same RSSP was found in animal model and patients on EFV. This profile is characterized by increased *S*-glutathionylation and decreased *S*-cysteinylation of proteins. This is mainly observed in patients that form higher 8-hydroxy-efavirenz, which is in accordance with higher risk of neurotoxicity. RSSP of patients on NVP was the opposite of the one observed for EFV. This might explain why EFV is mostly metabolized by liver, but is barely hepatotoxic. No influence of HIV-infection in RSSP was found.

Summing up, we conclude that RSSP profile might be associated with EFV-induced neurotoxicity, suggesting that it is a suitable tool for therapeutic drug monitoring aimed at managing toxic events. Moreover, as EFV and NVP presented a different RSSP signature, this tool might be used for assessing antiretroviral redox-modulation effects and quantification of drug-induced oxidative stress. This knowledge might have important implications for toxicity risk assessment, antiretroviral prescription and drug development.

Keywords: efavirenz, neutoxicity, nevirapine, *S*-glutathionylation, *S*-thiolated proteins, oxidative stress.

Resumo

O efavirenz (EFV) é um fármaco antiretroviral, comummente usado na infecção por VIH, e cuja neurotoxicidade associada se encontra convenientemente documentada. Em concreto, o seu metabolito, 8-hidroxi-efavirenz, tem sido descrito como sendo responsável pelos seus efeitos neurotóxicos a longo prazo. Após bioactivação, este metabolito gera espécies electrofílicas, formando aductos de proteína e originando stress oxidativo. A *S*-tiolação de proteínas, de que é exemplo a *S*-glutationização, tem sido sugerida como um importante mecanismo de regulação de proteínas pelo estado *redox*. Com o presente estudo pretende-se investigar se o perfil plasmático das proteínas *S*-tioladas (PSSR) está associado com a neurotoxicidade associada ao EFV.

O perfil acima mencionado (PSSR) foi avaliado num modelo animal de neurotoxicidade induzida pelo EFV e, posteriormente, comparado com o perfil obtido num estudo de coorte de doentes infectados por VIH e tratados com EFV. O impacto da produção de 8-hidroxi-efavirenz, bem como, a influência da infecção no perfil PSSR foram também objeto de análise neste estudo clínico. Adicionalmente, este perfil plasmático foi comparado com o perfil obtido em doentes tratados com nevirapina (NVP), um antiretroviral da mesma classe do EFV, ao qual não é atribuído potencial neurotóxico.

Os nossos resultados revelaram que o perfil PSSR obtido no modelo animal é sobreponível ao observado nos doentes com EFV. Este perfil caracteriza-se por um aumento da *S*-glutationização e um decréscimo da *S*-cisteinilização de proteínas, maioritariamente observados em doentes com elevada formação de 8-hidroxi-efavirenz, o que é consistente com o seu maior risco de neurotoxicidade. O PSSR de doentes tratados com NVP foi o oposto do observado nos doentes com EFV, o que pode justificar o facto do EFV, não obstante de ser predominantemente metabolizado no fígado, ser pouco hepatotóxico. Por outro lado, a infecção por VIH não exerceu qualquer tipo de influência no PSSR.

Em conclusão, o perfil PSSR parece estar associado à neurotoxicidade inerente ao EFV, sugerindo a sua relevância enquanto ferramenta útil para a monitorização terapêutica de fármacos, tendo em vista a minimização de eventos tóxicos. Adicionalmente, o facto do EFV e da NVP terem evidenciado um perfil PSSR diferente possibilita a utilização desta ferramenta para a avaliação dos efeitos dos antiretrovirais na modulação do estado *redox*. Os dados resultantes do presente trabalho poderão revelar-se de extrema importância no contexto da avaliação do risco de toxicidade, na prescrição de antiretrovirais e no desenvolvimento de fármacos.

Palavras-Chave: efavirenz, neurotoxicidade, nevirapina, *S*-glutationização, *S*-tiolação de proteínas, stress oxidativo

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Abbreviations

8,14-diOH-EFV	8-dihydroxy-efavirenz
7-OH-EFV	7-hydroxy-efavirenz
8-OH-EFV	8-hydroxy-efavirenz
7-OH-EFV-gln	7-hydroxy-efavirenz-glucuronide
8-OH-EFV-gln	8-hydroxy-efavirenz-glucuronide
7-OH-EFV-sulf	7-hydroxy-efavirenz-sulfate
8-OH-EFV-sulf	8-hydroxy-efavirenz-sulfate
2-OH-NVP	2-hydroxy-nevirapine
3-OH-NVP	3-hydroxy-nevirapine
4-COOH-NVP	4-carboxy-nevirapine
8-OH-NVP	8-hydroxy-nevirapine
12-OH-NVP	12-hydroxy-nevirapine
12-sulfoxi-NVP	12-sulfoxi-nevirapine
AIDS	Acquired immunodeficiency syndrome
ARV	Antiretroviral drugs
BQL	Bellow quantification limit
cART	Combined antiretroviral therapy
CD4	Cluster differentiation 4
CNS	Central nervous system
CV	Variation coefficient
CYP450	Cytochrome P450
Cys	Cysteine
CysSSP	*S*-cysteinylated protein
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
EFV	Efavirenz
EFV-gln	Efavirenz-glucuronide
FDA	US Food and Drug Administration
GSH	Glutathione
GSSP	*S*-Glutathionylated protein
Hcy	Homocysteine
HDL	High-density lipoprotein
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-1A	Human immunodeficiency virus type 1 subtype A
HIV-1B	Human immunodeficiency virus type 1 subtype B
HIV-1C	Human immunodeficiency virus type 1 subtype C
HIV-1G	Human immunodeficiency virus type 1 subtype G
HPLC	High-performance liquid chromatography

HPLC-FD	High-performance liquid chromatography with fluorescence detection
HPLC-UV	High-performance liquid chromatography with ultraviolet detector
LLOQ	Lower limit of quantification
LMWT	Low-molecular weight thiols
na	not applicable
$Na_2B_4O_7$	Sodium tetraborate
NaOH	Sodium hydroxide
n-B	Human immunodeficiency virus type 1 non subtype B
nd	not determined
ns	not significant
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
PSSP	Protein disulfides
QC	Quality control
ROS	Reactive oxygen species
RSSP	*S*-thiolated protein
SD	Standard deviation
SH	Sulfhydryl group
SULT	Sulfotransferases
TCA	Trichloroacetic acid
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
UGT	UDP-glucoronosyltranferase
UV	Ultraviolet

1. Introduction

1.1 Human immunodeficiency virus-infection and the combined antiretroviral therapy

At the end of 2015, 33.3 million people are living with the human immunodeficiency virus (HIV) infection, the cause of immunodeficiency syndrome (AIDS) (WHO, 2016).

The HIV infects and depletes cells of immune system resulting in immunodeficiency, which if left untreated, has a high rate of morbidity and mortality (Tohyama *et al*., 2009). Nowadays, HIV-infected patients are treated with combined antiretroviral therapy (cART). The first-line cART comprises several alternatives of antiretroviral combinations, adapted to each patient, and includes at least three antiretroviral drugs (AIDS*info*, 2016). The cART was introduced in 1996, allowing a victorious control of the HIV-infection and leading to dramatic improvements in morbidity and lifespan (Heaton *et al*., 2011; Deeks, 2009). The success of cART changed dramatically the prognosis of this infection, which is currently perceived as a chronic disease (Mehellou and De Clercq, 2010). The number of people receiving cART has been expanded rapidly, with more than 17 million people on antiretroviral therapy by the end of 2015 (WHO, 2016). Consequently, the HIV-infected population now lives longer (Deeks, 2009). However, in treated patients the persistent infection/inflammation, residual viremia, compromised immune system, co-infections (e.g. hepatitis and tuberculosis), polimedication and drug-drug interactions, along with antiretroviral toxicity put HIV-infected patients at high risk of premature aging (Deeks and Phillips, 2009). In fact, treated patients are at increased risk of developing several complications, which are classically associated with the normal aging process, including cancer, cardiovascular, neurocognitive and bone diseases (Deeks and Phillips, 2009). However, they occur at an earlier age in HIV-infected persons (Deeks and Phillips, 2009) and antiretroviral toxicity has a role in this context.

There are about thirty approved antiretroviral drugs targeting the different steps of the HIV replication cycle (Zhan *et al*., 2016). These drugs are divided into six main classes, according to their mechanism of action in the HIV replication cycle. Despite the introduction of several antiretroviral drugs in the last three decades, the HIV therapies still have a wide range of problems, such as severe adverse reactions associated with long-term use of cART. For that reason, the search for new molecules with antiretroviral activity and novel therapeutic targets is still ongoing (Zhan *et al*., 2016). An alternative strategy is to understand the mechanisms of antiretroviral toxicity and find mechanistically-oriented biomarkers which allow preventive and management strategies to optimize the response to therapy (Grilo *et al*., 2016a). In this context, important advancement has been obtained for the class of non-nucleoside reverse transcriptase inhibitors (NNRTI) (Pereira *et al*. 2012a; Pereira *et al*. 2012b). NNRTIs, like efavirenz (EFV) and nevirapine (NVP), restrict viral replication by binding non-competitively with the HIV-1 reverse transcriptase (De Clercq, 2004), and are globally among the most prescribed antiretrovirals. The major drawbacks of these drugs are their adverse reactions, neurotoxicity in the case of EFV and hepatotoxicity for NVP.

1.2 Efavirenz biotransformation, oxidative stress generation and its neurotoxicity mechanism

Efavirenz (EFV) was approved for the treatment of HIV-1 in 1998, by the U.S Food and Drug Administration (FDA) (WHO, 2010). EFV availability as generic drug and low cost (EMA, 2011) are its major advantages. Also, this antiretroviral is the first-line option during the treatment with rifampicin for tuberculosis (WHO, 2010). EFV has a long half-life, allowing once-daily administration and has a good penetration in HIV-*reservoirs* including brain tissue and testicles (Smith *et al*., 2001). In adults, 600 mg of EFV are used once-daily, which optimally results in steady state plasma concentrations within the therapeutic window 1-4 mg/L. This drug is highly protein bound (>99%), particularly to albumin (Usach *et al*., 2013).

Despite the benefits that arise from the use of EFV in HIV-1 therapy, this drug is associated to central nervous system (CNS) adverse reactions that are recognized as a main reason for discontinuing EFV use (Apostolova *et al*., 2015b; Fumaz *et al*., 2005). There is a panoply of neuro-adverse reactions associated to EFV that include among others dizziness, abnormal dreams, insomnia, mood changes, irritability, depression, suicidal ideation and hallucinations (Apostolova *et al*., 2015b; Naidoo *et al*., 2014). The majority of efavirenz-induced CNS effects appears soon after the beginning and disappears within the first 4 weeks of therapy. In some cases, these effects can persist for much longer periods and require a treatment switch (Apostolova *et al*., 2015b; Cespedes and Aberg, 2006). Importantly, there is a high prevalence of neurocognitive disorders associated with efavirenz use that emerge in apparently asymptomatic HIV+ individuals (Ciccarelli *et al*., 2011). These consequences of long-term efavirenz use represent a major concern, particularly in children.

Although, is a common clinical evidence, the knowledge of the mechanisms underlying EFV neurotoxicity is slowly becoming to be understood (Grilo *et al*., 2016a; Apostolova *et al*., 2015b; Funes *et al*., 2015) and related to EFV biotransformation. EFV is predominantly metabolized by cytochrome P450, primarily by CYP2B6 (Ward *et al*., 2003). The isoforms CYP3A4, CYP3A5, CYP1A2 and CYP2A6 have a minor participation (Ogburn *et al*., 2010; Bélanger *et al*., 2009; di Iulio *et al*., 2009; Ward *et al*., 2003). CYP2B6 is responsible for the hydroxylation of EFV to the metabolites 8-OH-EFV (8-hydroxy-efavirenz; major) and 8,14-dihydroxy-efavirenz (8,14-diOH-EFV; minor) (Ward *et al*., 2003). This isoform also mediates the metabolism of EFV to 7-hydroxy-efavirenz (7-OH-EFV), however this metabolic pathway has minor significance (Ogburn *et al*., 2010). The hydroxylated metabolites are subsequently glucuronidated by UGT2B7 (Bae *et al*., 2011; Bélanger *et al*., 2009) that seems to have a minimal influence on EFV clearance (Cho *et al*., 2011). Additionally, EFV can be directly conjugated to form efavirenz-N-glucuronide (Bélanger *et al*., 2009). Both 8-OH-EFV and 7-OH-EFV metabolites can also suffer another phase II reaction via sulfatation (Aouri *et al*., 2016). The metabolites of EFV (mainly 8-OH-EFV) are excreted predominantly in urine and bile (Ward *et al*., 2003; Mouly *et al*., 2002). Less than 1% of EFV is excreted unchanged in urine.

EFV is thought to enhance its own metabolism, via induction of CYP2B6 and CYP3A4 (Ngaimisi *et al*., 2010). This auto-induction is known to be concentration and time dependent (Ngaimisi *et al*., 2010).

Recent non-clinical studies (Harjivan *et al*., 2014; Brandmann *et al*., 2013; Tovar-y-Romo *et al*., 2012; Ward *et al*., 2003) have implied EFV biotransformation, namely the generation of 8-hydroxy-efavirenz (8-OH-EFV), in this context (**Figure 1.1**). This metabolite is able to damage neurons and is known to be, at least an order of magnitude, more toxic than EFV itself and other of EFV phase I metabolites, the 7-hydroxy-efavirenz (Tovar-y-Romo *et al*., 2012). Furthermore, 8-OH-EFV has the ability to damage astrocytes and neurons in a dose-dependent manner (Brandmann *et al*., 2013; Tovar-y-Romo *et al*., 2012). Our group has recently given the first clinical evidence of the association of 8-OH-EFV concentrations and mood changes in HIV-infected patients (Grilo *et al*., 2016a) (**Figure 1.1**).

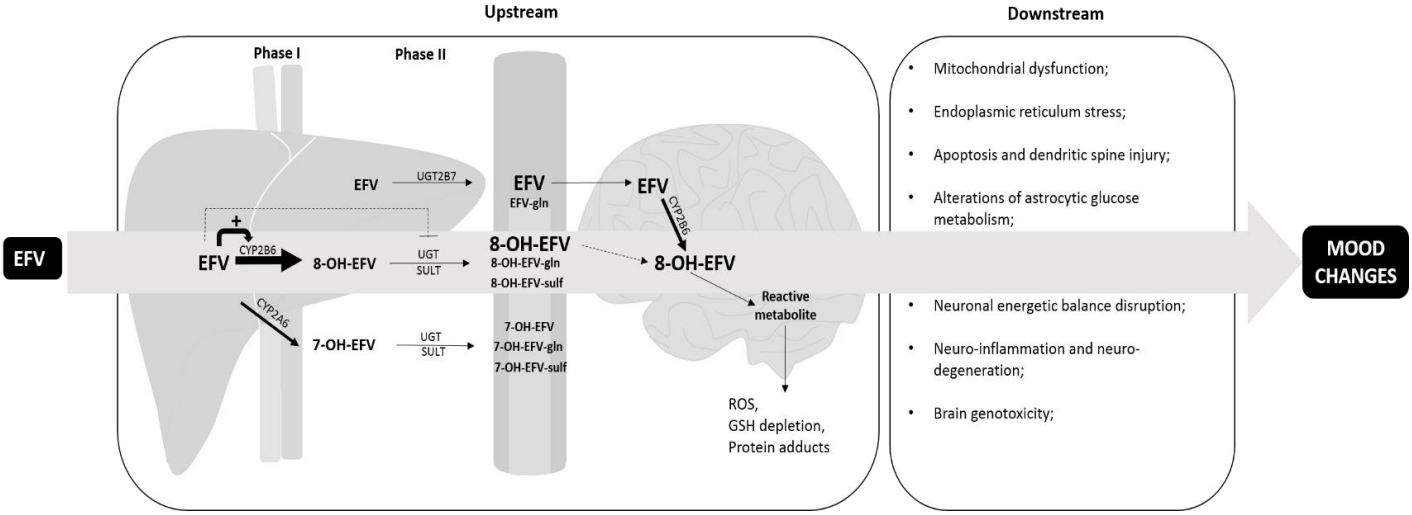

Figure 1.1 Schematic representation of the events preceding mood changes upon EFV exposure (in Grilo *et al.,* 2016a).

Upstream events: in the liver, EFV is mainly metabolized via CYP2B6 to 8-OH-EFV, to a slight degree to 7-OH-EFV via CYP2A6 and barely conjugated by UGT2B7 to EFV-N-gln. The 8-OH-EFV and 7-OH-EFV are subjected to phase II metabolism through glucuronidation (major) and sulfatation (minor). EFV is an inducer of CYP2B6 and a strong inhibitor of several UGT. Comparatively to the liver, in brain CYP2B6 is expressed into lesser extent and CYP 2A6 expression as well as phase II reactions are quite lower. Thus, an accumulation of 8-OH-EFV is expected in this organ. Further oxidation of 8-OH-EFV originates a reactive metabolite that might be involved in the genesis of mood changes and other CNS side effects observed in patients receiving EFV.

EFV: efavirenz; **8-OH-EFV**: 8-hydroxy-efavirenz; **7-OH-EFV:** 7-hydroxy-efavirenz; **EFV-gln:** efavirenz-glucuronide; **8-OH-EFV-gln:** 8-hydroxy-efavirenz-glucuronide; **7-OH-EFV-gln:** 7-hydroxy-efavirenz-glucuronide; **8-OH-EFV-sulf**: 8-hydroxy-efavirenz-sulfate; **7-OH-EFV-sulf:** 7-hydroxy-efavirenz-sulfate; **UGT:** UDP-glucuronosyltransferases; **SULT:** sulfotransferases.

The bioactivation upon oxidation of 8-OH-EFV generates highly reactive electrophilic species, such as quinone and quinoneimine (Harjivan *et al*., 2014). The toxicological value of quinone and quinoneimine is widely recognized, and is assigned to their ability to generate reactive oxygen species and capacity of yielding covalent adducts with proteins (Monks and Jones, 2002; Bolton *et al*., 2000). In fact, EFV has been associated to enhanced oxidative stress (Weiß *et al*., 2016; Gomez-Sucerquia *et al*., 2012; Apostolova *et al*., 2010; Hulgan *et al*., 2003) which underlies the mechanisms of its neuro-adverse reactions (Brown *et al*., 2014). In addition, the formation of adducts could in turn adversely impact the function of proteins, interfering with the energy metabolism, axonal transport or pre-synaptic neurotransmitter release (Lopachin and Decaprio, 2005; Asanuma *et al*., 2003).

1.3 Nevirapine biotransformation, oxidative stress generation and its hepatotoxicity mechanism

Nevirapine (NVP) was the first NNRTI approved by the American Food and Drug Administration (FDA) in 1996 for the treatment of HIV-1 infection, as part of cART (FDA, 1996). Partly due to its low cost, NVP remains the most prescribed NNRTI worldwide (Lockman *et al*., 2007; Ades *et al*., 2000). Actually, NVP presents several advantages when compared with other antiretroviral drugs. One of the most significant benefits of this drug is its efficacy in the prevention of mother-to-child transmission of the virus, being NVP one of the most commonly prescribed antiretroviral drug to pregnant women and their children (Medrano *et al*., 2008; Ades *et al*., 2000). Unlike EFV, NVP presents low incidence of adverse drug reactions in central nervous system (Medrano *et al*., 2008), allowing its use in the context of psychiatric disorders or drug dependence. The favorable metabolic profile is another important advantage of NVP (Srivanich *et al*., 2010; Clotet *et al*., 2003; Ruiz *et al*., 2001) rendering it suitable for use in patients with diabetes, dyslipidemia or metabolic syndrome.

Concerning its pharmacokinetic properties, NVP is rapidly absorbed after oral administration, being its bioavailability higher than 90% (Usach *et al*., 2013). NVP binds approximately 60% to plasma proteins, easily crosses the blood-brain barrier and placenta and has been found in breast milk (Elias *et al*., 2013; Mirochnick *et al*., 2000).

Nevirapine is extensively metabolized by cytochrome P450 (CYP) into five phase I metabolites: 2-hydroxy-nevirapine (2-OH-NVP), 3-hydroxy-nevirapine (3-OH-NVP), 8-hydroxy-nevirapine (8-OH-NVP), 12-hydroxy-nevirapine (12-OH-NVP) and 4-carboxynevirapine (4-COOH-NVP) (Erickson *et al*., 1999; Riska *et al*., 1999a; Riska *et al*., 1999b). The phase I reactions of NVP involves several CYP isoforms (Erickson *et al*., 1999). The hydroxylation into 2-OH-NVP is mediated by CYP3A, while 3-OH-NVP formation is attributed to CYP2B6. CYP3A4, CYP2B6, and CYP2D6 are implicated in 8-OH-NVP generation. The formation of the main NVP phase I metabolite, 12-OH-NVP, is mediated by CYP2A6, CYP3A4, CYP3A5, CYP2D6, CYP2C9 and also CYP2C19. Finally, 4-COOH-NVP is formed

by secondary oxidation of 12-OH-NVP (Chen *et al*., 2008; Erickson *et al*., 1999; Riska *et al*., 1999b; Riska *et al*., 1999a). These hydroxylated metabolites suffer subsequent phase II biotransformation, mainly by glucuronidation (Riska *et al*., 1999a).

Similarly to EFV, NVP induces its own metabolism, through an inductor effect on CYP3A4 and CYP2B6 (Lamson *et al*., 1999; Riska *et al*., 1999a).

The treatment with NVP has been associated with idiosyncratic hepatotoxicity and/or skin rash (De Lazzari *et al*., 2008; Medrano *et al*., 2008; Taiwo, 2006). Women seem to be at higher risk than men of developing these adverse reactions (De Lazzari *et al*., 2008; Medrano *et al*., 2008; Antinori *et al*., 2001; Ho *et al*., 1998), which justifies the recommendation that the drug should be initiated in cART-naïve-women with a $CD4^+$ cell count below 250 cells/mm^3 and below 400 cells/mm^3 in men (Thompson *et al*., 2010; Clotet, 2008). Despite the molecular mechanisms of NVP-induced toxic events are not yet fully understood, current evidences are unanimous in attribute to NVP bioactivation a key role in this context. Several approaches involving in vitro (Antunes *et al*., 2008; Antunes *et al*., 2010a; Antunes *et al*., 2010b), ex vivo (Pinheiro *et al*., 2016; Pinheiro *et al*., 2015), and animal models (Chen *et al*., 2008; Shenton *et al*., 2003), as well as, clinical trials (Marinho *et al*., 2014b; Meng *et al*., 2013; Caixas *et al*., 2012) have suggested that the bioactivation of phase-I NVP metabolite 12-OH-NVP to 12-sulfoxy-NVP and the formation of NVP quinone methide from NVP, 12-OH-NVP and 12-sulfoxy-NVP are closely involved in the mechanism of NVP-related hepatotoxicity (**Figure 1.2**). Concretely, in the last years, Antunes and colleagues have extensively showed the *in vitro* reactivity of 12-sulfoxy-NVP with several amino acids (Antunes *et al*., 2010a), human serum albumin, hemoglobin (Antunes *et al*., 2010b) and also with nucleosides and DNA (Antunes *et al*., 2008). Furthermore, while 12-sulfoxy-NVP has been proposed to be involved in NVP-induced hepatotoxicity (Sharma *et al*., 2012), NVP quinone methide has been associated with skin rash (Sharma *et al*., 2013).

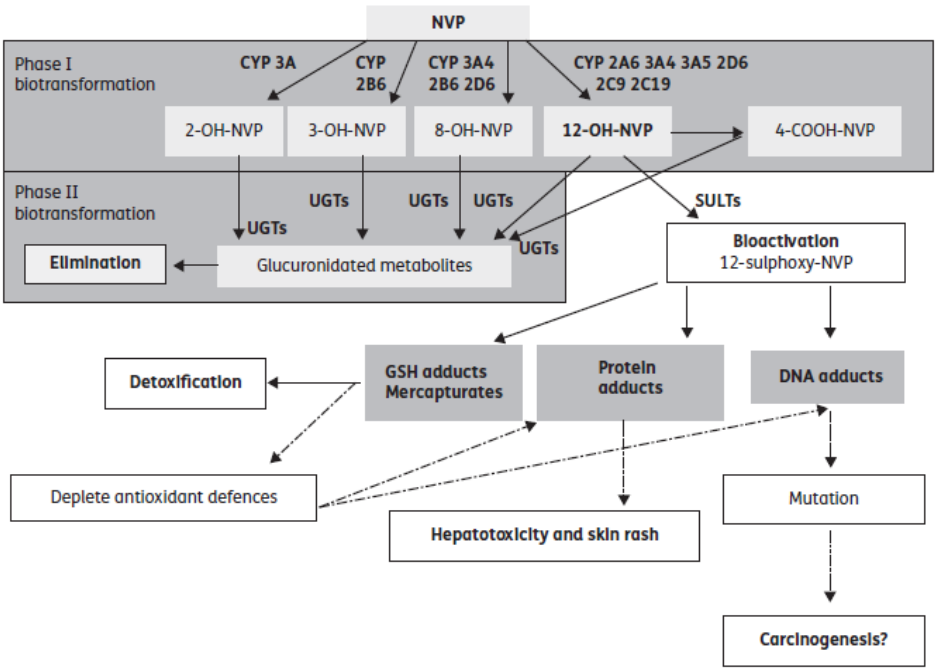

Figure 1.2 Nevirapine biotransformation, disposition and proposed bioactivation pathways (in

(Marinho *et al*., 2014b).

NVP is metabolized by several isoforms of cytochrome P450 (CYP) generating several phase I metabolites: 2-OH-NVP, 3-OH-NVP, 8-OH-NVP and 12- OH-NVP. The 12-OH-NVP metabolite is further oxidized by CYP450 yielding 4-carboxynevirapine (4-COOH-NVP). The phase I NVP metabolites undergo extensive glucuronidation, which represents a major pathway of NVP elimination. The bioactivation of 12-OH-NVP by SULTs can generate 12-sulfoxy-NVP, which is a reactive metabolite that can bind covalently to proteins and DNA. The formation of adducts with proteins could explain the NVP-associated adverse reactions, hepatotoxicity and skin rash.
NVP: nevirapine; **2-OH-NVP:** 2-hydroxy-nevirapine; **3-OH-NVP**: 3-hydroxy-nevirapine; **8-OH-NVP:** 8-hydroxy-nevirapine; **12- OH-NVP:** 12-hydroxy-nevirapine; **4-COOH-NVP:** 4-carboxynevirapine; **UGT:** UDP-glucuronosyltransferase; **SULT**: sulfotransferase.

Nevirapine also induces oxidative stress (Pinheiro *et al*., 2016; Adaramoye *et al*., 2012), which is a common feature of hepatotoxicity (Elias *et al*., 2013; Kashou and Agarwal, 2011; Yamamoto *et al*., 2005).

All together these findings suggest that the formation of adducts with key proteins and generation of oxidative stress might be a plausible explanation for the onset of NVP-related toxicity (Russmann *et al*., 2009).

1.4 Plasma *S*-thiolated proteins as a tool for oxidative stress and neurotoxicity risk biomonitoring

Neurotoxicity studies in man are limited by the inaccessibility to the target tissues. Hence, there is a demand for suitable models to obtain further insights into the mechanisms of toxicity and to find molecular biomarkers in peripheral tissues that can be easily and ethically obtained in humans. EFV clearly illustrates these circumstances. It is infeasible to routinely measure 8-OH-EFV concentrations in cerebrospinal fluid, as a surrogate of brain tissue concentration. Until now, the pharmacokinetic targets that are upstream events of EFV neurotoxicity have been explored through CYP2B6 activity and pharmacogenetics or 8-OH-EFV plasma concentrations. A plausible way for the identification of biomarkers is to evaluate markers of response.

There is increasing awareness of the ubiquitous role of oxidative stress in neurotoxicty (Sayre *et al*., 2008). The perturbations in antioxidant defense systems and the subsequent redox-imbalance are present in many tissues of HIV-infected patients, including the brain (Sharma, 2014; Aquaro *et al*., 2008).

The oxidation of thiol -SH groups is one of the first observable events during reactive oxygen species (ROS)-mediated damage (Grant *et al*., 1999). Among the major roles in metabolism and homeostasis of the biological systems, thiols are known for maintaining the control of the antioxidant system and for the detoxification of several molecules and xenobiotics (Bald *et al*., 2004). Blood levels

of proteins' and low molecular weight thiols (LMWT) are frequently measured as biomarkers of oxidative stress (Colombo *et al*., 2015; Giustarini *et al*., 2012). Many proteins and enzymes contain cysteine residues which have a sulfhydryl group (-SH) (Lepedda *et al*., 2013). The redox status of cysteine residues is involved in both structure and function of several enzymes, receptors and transcription factors (Grant *et al*., 1999). However, cysteine residues are one of the most easily oxidized residues in proteins (Dean *et al*., 1997; Coan *et al*., 1992). The oxidation of -SH groups can lead to the formation of protein disulfides (PSSP) or mixed disulfides, by binding between protein-SH groups and LMWT, as a whole referred to as *S*-thiolated proteins (RSSP) (Rossi *et al*., 2009; Giustarini *et al*., 2006; Seres *et al*., 1996). The largest pool of LMWT in human plasma is the cysteine/cystine redox couple followed by cysteinylglycine, being glutathione the minor component (Rossi *et al*., 2009). Glutathione is the thiol most abundant in cells and its present mainly in the reduced form (Rossi *et al*., 2009; Moriarty-Craige and Jones, 2004). Thus, intracellular proteins are predominantly *S*-glutathionylated, while extracellular proteins are predominantly *S*-cysteinylated (Rossi *et al*., 2009; Dalle-Donne *et al*., 2008). Protein *S*-glutathionylation arises from the glutathione reaction with the free thiol group in certain cysteine residues of proteins (Xiong *et al*., 2011). This post-translational reaction represents an important redox signaling mechanism regulating the normal function of proteins (Xiong *et al*., 2011). Moreover, deregulated protein *S*-glutathionylation was associated with chronic diseases, such as diabetes mellitus, neurodegeneration or cancer (Hill and Bhatnagar, 2012).

Plasma is poor in glutathione content and the extent of protein *S*-glutathionylation (GSSP, ranges from 0.8 to 1.4 μM) is low comparatively to thiolation by compounds such as *S*-cysteinylation (CysSSP, from 150 to 180 μM) (Rossi *et al*., 2009; Dalle-Donne *et al*., 2008; Giustarini *et al*., 2006). The pool of plasmatic thiols is mainly constituted by free thiol groups of proteins, whose concentration is mostly due to the single free thiol at Cys34 of albumin (Oettl and Stauber, 2007). Reduced albumin is a very abundant and important circulating antioxidant (Roche *et al*., 2008; Gutteridge, 1986), representing an important defense against oxidative stress. At physiological pH, the sulfhydryl group of Cys34 of albumin exists is highly reactive (Turell *et al*., 2013; Roche *et al*., 2008). Together with the lack of thiol reducing systems in plasma, this might explain the fact that Cys34 is *S*-thiolated in about one-half of the plasma albumin molecules, forming RSSP (Oettl and Stauber, 2007). Thus, albumin is a regulator of thiol levels and this feature seems to compensate the lack of GSH recycling in plasma (Di Simplicio *et al*., 2005), showing the relevance of *S*-glutathionylation of plasma proteins.

Plasma protein *S*-thiolation has been considered as an adaptive response to protect protein thiol groups from losing their biological activity by irreversible oxidation (Colombo *et al*., 2015; Lepedda *et al*., 2013; Biswas *et al*., 2006). Additionally, this post-translational modification modulates the activity of many enzymes, structural proteins and nuclear factors (Di Simplicio *et al*., 2005) that contain cysteine residues in their catalytic centers or as part of protein-protein interaction interfaces (Dalle-Donne *et al*., 2008). Despite protein thiolation is considered as a defensive mechanism, it can also be pathological in particular cases. For instance, homocysteine and *S*-homocysteinylation of proteins is a recognized risk factor for cardiovascular diseases (Di Simplicio *et al*., 2005; Chrestensen *et al*., 2000).

As described above, both EFV and NVP after bioactivation of their phase I metabolites originate reactive electrophilic species and generates oxidative stress. The main targets of these metabolites are

nucleophilic protein cysteine thiol group (Harjivan *et al*., 2014). Much of the current knowledge about *S*-thiolation of proteins and its role against oxidative protein modifications and protein regulation has been gleaned from studies focused on oxidative stress situations other than drug toxicity. While many are known to be associated to oxidative stress and organ toxicity, the RSSP profile generated upon drug exposition remains to be identified.

1.5 Rational, working hypothesis and specific aims

What is already known?

1. EFV is an antiretroviral drug that has been associated with neurotoxicity.

2. EFV biotransformation into 8-OH-EFV is an up-stream event of this neurotoxicity. Upon bioactivation of its main phase-I metabolites, 8-OH-EFV is able to form electrophilic metabolites and form adducts with proteins.

3. Clinical trials have shown that there is a high variability in the manifestation and severity of EFV-induced neuro-adverse reactions.

4. EFV and NVP share the characteristics mentioned above (**Table 1.1**). However, NVP is hepatotoxic despite its penetration CNS.

5. Protein *S*-thiolation is a defense mechanism against irreversible oxidation of proteins.

Table 1.1 Efavirenz and nevirapine comparison: oxidative stress and toxicity mechanism.

	EFV	**NVP**
NNRTI	✓	✓
CYP450 metabolism	✓	✓
Auto-induction of metabolism	✓	✓
Bioactivation of phase I metabolites	✓	✓
Oxidative stress	✓	✓
Protein Adducts	✓	✓
Neurotoxicity	✓	**X**
Hepatotoxicity	Barely	✓

EFV, efavirenz; **NNRTI**, non-nucleoside reverse transcriptase inhibitor; **NVP**, nevirapine.

What is still unknown and should be addressed?

1. What is the RSSP profile of long-term EFV exposure that is associated to neurotoxicity?
2. What is the influence of 8-OH-EFV concentrations in this RSSP profile?
3. What is the impact of HIV-infection in this profile?
4. Do EFV and NVP share the same profile?

Working hypothesis:

Efavirenz is a mostly prescribed (NNRTI) that induces oxidative/electrophilic stress and it is associated to neurotoxicity. We firstly hypothesized that long-term EFV exposure modulate the RSSP profile of HIV-infected patients. Secondly, this individual modulation allows the definition if there will be a toxic progression response or an adaptive response. Lastly, the RSSP profile of HIV-infected patients on nevirapine differs from efavirenz.

Accordingly, we further hypothesized that plasma RSSP profile would be a useful tool to assess antiretroviral redox modulation and quantification of oxidative stress.

In order to test our hypothesis the following specific aims were defined:

1. To study the impact of EFV exposure on RSSP profile in an animal model of EFV-related to memory impairment.
2. To find out the RSSP profile of HIV-infected patients on EFV and further compare it with the RSSP profile associated to the neurotoxicity observed in the animal model.
3. To assess the dependence of RSSP on EFV and 8-OH-EFV concentrations.
4. To understand the dependence of this profile on HIV-infection.
5. To compare EFV-RSSP with NVP-RSSP.

2. Materials and Methods

2.1 Study design

2.1.1 Animal study

Experiments were performed in male Wistar rats (Rattus norvegicus L.) that were obtained from the NOVA Medical School animal facility. Animals were housed two *per* cage, in polycarbonate cages with wire lids (Tecniplast, Buguggiate, Varese, Italy), under 12 hours 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 standard laboratory diet (SDS diets RM1) and reverse osmosis water, given *ad libitum*. Corncob bedding (Probiológica, Lisbon, Portugal) was used and changed once a week. Animals were Specific Pathogen Free (SPF) according to FELASA recommendations.

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. Experimental procedures were previously approved by the *Institutional Ethics Committee* of the *NOVA Medical School* for animal care and use in research.

On total 32 rats were randomly assigned and divided into four groups of 8 animals each according to exposure time and treatment. Group 1 and 2 were administered with EFV 9 mg/Kg/day suspended in reverse osmosis water – 1.5 mL (EFV groups) during 10 and 36 days, respectively. Group 3 and 4 were administered only with vehicle – 1.5 mL (control groups) during a period of 10 and 36 days, respectively.

EFV or vehicle-only 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. 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 amounts of EFV were adjusted accordingly to weight variation in order to ensure a constant daily dose. EFV or vehicle was administered daily to the animals in the morning period at approximately the same schedule.

At day 10 or 36, approximately 1-2 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®, Mérial, Lyon, France). Cardiac puncture was performed to blood sampling and the plasma samples obtained were stored at -80 °C until use.

2.1.2 Clinical study

Two groups of adult patients were included in this work: a group of non-HIV volunteers and a group of HIV-infected patients on NVP- or EFV-therapy for at least 6 moths. The current work was conducted in accordance with the Declaration of Helsinki. All patients were fully informed about the study and gave their written informed consent.

The study protocol for HIV-infected patients received prior approval from Hospitals Ethics Committee of *Centro Hospitalar de Lisboa Central, EPE* (process number 115/2013) and *Hospital Prof. Doutor Fernando Fonseca*, *EPE* (process number CA 21/2011). Patients' compliance was controlled by the clinician. HIV-infected patients were stratified according to cART use: naive, on NVP-cART and on EFV-cART. Exclusion criteria included kidney and hepatic dysfunction. Patients with detectable viral load in cART groups were also excluded. Anthropometric and clinical data (age, $CD4^+$ T-cell count, viral load, kidney and liver function parameters, hepatite B and C co-infection) were recorded for each patient. For EFV-group, HIV-subtype was also recorded.

The study protocol for non-HIV volunteers had been approved by the National Institute of Health Ethics Committee.

Blood samples (2 mL) were collected in EDTA (ethylenediaminetetraacetic acid) - containing tubes. The samples were centrifuged at 3 000*g* for 10 min, 4 ˚C. Plasma samples were stored at -80 ˚C until use. All patients' samples were heated for 60 min, at 60 ˚C, for HIV inactivation, just before handling at room temperature.

2.2 Quantification of *S-thiolated proteins* profile in plasma

The *S*-thiolated protein profile (RSSP) was herein defined as the pool of plasmatic *S*-glutathionylated (GSSP) and *S*-cysteinylated (CysSSP) proteins. For their quantification the total and free plasma fractions of glutathione and cysteine were measured by an HPLC method with fluorescence detection (HPLC-FD), adapted from Nolin and co-authors (Nolin *et al*., 2007) and previously validated by our group (Lemos, 2015). For each thiol, the subtraction of non-protein bound from the total fraction allowed the quantification of the *S*-thioled protein fraction, GSSP and CysSSP respectively.

Total fraction (non-protein bound + protein bound) of cysteine and glutathione was obtained by reducing the sulfhydryl groups with Tris (2-carboxyethyl) phosphine hydrochloride (TCEP, *Sigma*-aldrich) reagent (100g/L). After a 30 min incubation step at room temperature, the samples were treated with trichloroacetic acid (TCA, *Roth*) (100 g/L) containing ethylenediaminetetra-acetic acid (EDTA; 1 mM) (*Sigma*-aldrich), for protein precipitation. The obtained mix was centrifuged (13000 *g*, 10 min, 4°C) and the supernatant collected to a new tube containing sodium hydroxide (NaOH; 1.55 M) (*VWR,* USA), sodium tetraborate buffer ($Na_2B_4O_7$;

0.125 M) (*Sigma*-aldrich) with EDTA (4 mM) and 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid ammonium (SBD-F; 1 g/L) (*Sigma*-aldrich) in $Na_2B_4O_7$ buffer. The final mix was vortexed and incubated in the dark, at 60 ºC for 1 h, to complete the derivatization of free sulfhydryl groups. Finally, a volume of 30 µL were injected into the HLPC.

For quantification of the glutathione and cysteine non-protein bound fraction, 50 µL from the same sample were submitted to protein precipitation with TCA, with subsequent centrifugation (13000 *g*, 10 min, at 4ºC), based on Przemyslaw and co-workers methodology (Przemysław *et al*., 2011). The supernatant was reduced with TCEP reagent. After incubation at room temperature, for 30 min, the previously described protocol was followed.

HPLC analysis was performed 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. The detector was set at excitation and emission wavelengths of 385 and 515 nm, respectively. The mobile phase consisted of acetate buffer 0.1 M (pH 4.5, *VWR,* USA) and methanol (*VWR,* USA) [99:1 (v/v)]. The analytes were separated in an isocratic elution mode for 20 minutes, at a flow rate of 0.8 mL/min.

2.3. Quantification of efavirenz, nevirapine and their main phase I metabolites plasma concentration

EFV and NVP were kindly provided by Dr. F.A. Beland from *National Center for Toxicological Research* (Jefferson, AR, USA). The phase I metabolites of EFV and NVP were synthesized at *Centro de Química Estrutural, Instituto Superior Técnico* (Lisboa, Portugal) as previously described (Harjivan *et al*. 2014).

2.3.1 Efavirenz and 8-hidroxy-efavirenz

2.3.1.1 Method validation

EFV and 8-OH-EFV plasma concentrations were quantified by a HPLC method that was herein validated. The validation criteria were defined according to guidebooks, regarding the validation of bioanalytical assays (U.S. Department of Health and Human Services, 2013; European Medicines Agency, 2011). The parameters validated were linearity, lower limit of quantification, accuracy, precision, stability and recovery (Grilo *et al*., 2016a).

2.3.1.2 Standard operating procedure

As described by Grilo *et al*, aliquots of patients' plasma samples (600 µL) were added to an extraction tube with 2 mL sodium acetate (0.2 M, pH 6.8) (*VWR,* USA). For the free fraction measurement the aliquot was treated with 60 µL of desionized ultrapure water (instead of the enzyme). All samples were then incubated at 37˚C, for 60 minutes. Analytes were then extracted from plasma with 7.5 mL of tert-butyl methyl ether (*VWR,* USA). The samples were stirred by inversion for 5 minutes and after centrifugation (3 000 rpm, 4 ºC, 10 min), the organic phase was evaporated to dryness at 60 ºC in a Speed-Vac concentrator (Labconco, Kansas City, MO, USA). The dried residue was reconstituted in 200 µL of mobile phase (acetonitrile: 0.1% formic acid buffer (1:1)) and 2 mL n-hexane (*Merck*, USA). Thereafter, the samples were stirring by inversion for 5 minutes and centrifuge at 3000 g, at 4 ºC, for 10 minutes. The aqueous phase was recovered for a vial and 100 µL were injected into HPLC.

HPLC analysis was performed on an Agilent 1100 Series equipment (Agilent Technologies, Santa Clara, CA, USA), using a LiChrospher 100 RP-18 (250 x 4 mm; 5 µm) column protected by a LiChrospher 100 RP-18e (4 x 4mm; 5 µm) guard-column, both from Merck. The mobile phase consisted of a mixture of 65% of 0.1% formic acid buffer (pH 2.65) *(VWR,* USA) (A) and 35% of acetonitrile (*VWR,* USA) (B), initially delivered at a flow rate of 1.2 mL/min for the first 5 minutes. Then the percentage of solution A gradually decreased to 55% for 10 minutes and maintained at this value for a period of 16 minutes. Finally, this percentage gradually decreased to 49% during 5 minutes and maintained at this proportion for 7 minutes. The post-run time was 6 minutes and the injection volume 100 µL. The analytical run was performed with a mobile phase flow rate 1.2 ml/min, at 30º C and detection wavelength of 246 nm. The chromatographic peaks occurs at 30 minutes to 8-OH-EFV and 41 minutes to EFV (**Figure 2.1**).

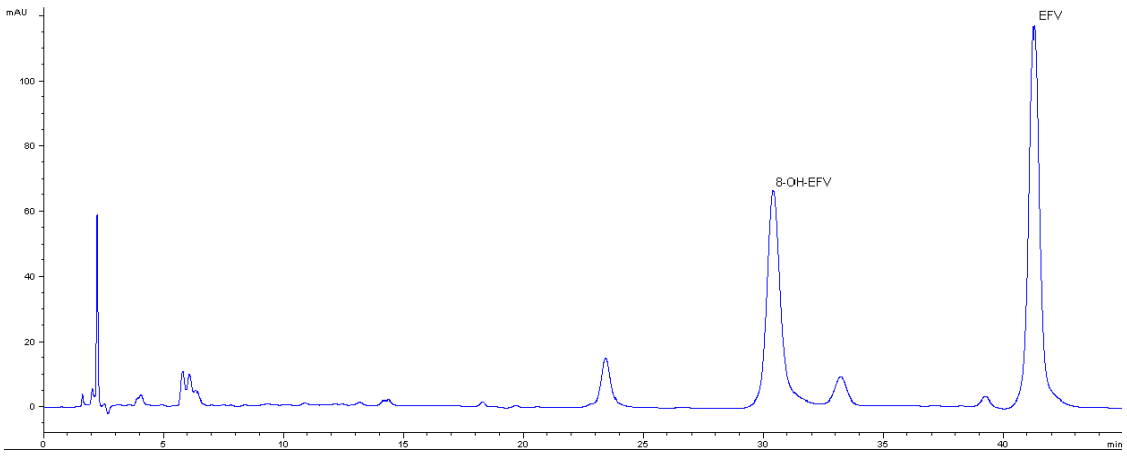

Figure 2.1 Representative chromatogram of a standard sample with 10 mg/L of EFV (41 min) and 8-OH-EFV (30 min).

2.3.2 Nevirapine and 12-Hidroxy-Nevirapine

NVP and 12-OH-NVP plasma concentrations were determined by HPLC method, previously developed and validated by Marinho and co-authors (Marinho *et al*., 2014a). Briefly, analytes were extracted from plasma samples (900 µL) with dichloromethane (*VWR,* USA). The organic phase was evaporated to dryness at 60 ºC in a Speed-Vac concentrator (Labconco, Kansas City, MO, USA) and the dried residue was reconstituted in 150 µL of a 1:1 methanol:water solution.

Separation of NVP and its phase I metabolites by HPLC was performed in an Agilent 1100 Series system (Agilent Technologies, Santa Clara, CA, USA) using a reverse-phase Luna C18 column (250 mm × 4.6 mm; 5 µm; 100 Å; Phenomenex, Torrance, CA, USA). The mobile phase – 10% acetonitrile in 15 mM ammonium acetate buffer, pH 4 (*Merck,* USA) – was delivered at a flow rate of 0.8 mL/min for 90 minutes; the flow rate was then increased to 1.5 mL/min in 5 minutes and maintained at this value for an additional period of 19 minutes. The column temperature was 40 ºC, the injection volume was 100 µL, and UV absorbance was monitored at 254 nm.

2.4 Statistical Analysis

Statistical analysis was performed using GraphPad Prism® version 5.0 (GraphPad Software Inc., CA). Tests results were considered significant at $P<0.05$. Normality of groups was checked using *Kolmogorov–Smirnov* or *Shapiro-Wilk* tests whenever applicable. Comparisons among groups were performed based on the results of the normality test: *Unpaired t-test* or *Mann-Whitney test* and *ANOVA or Kruskal-Wallis* test for two and more than two groups, respectively. The *Chi-Square* test was used to assess the independence of categorical data. *Spearman rank correlation* was used to explore the association between two ranked variables.

3. Results

3.1 Validation of the method for efavirenz and 8-hydroxy-efavirenz quantification

3.1.1 Linearity

The evaluation of linearity was performed using calibration standards ranging from 0.25 to 10 mg/L for the 8-OH-EFV metabolite and 0.1 to 10 mg/L for EFV, using eight standard samples. The linear regression model showed to be the most suitable for fitting a function to the points obtained (*Run Test p*> 0.05). The concentration of standard samples significantly influenced the chromatographic signal area (*F tests p*<0.0001) for the two analytes. The correlation coefficient was higher than 0.99 for 8-OH-EFV and EFV, which is good indicator of adjustment of the calibration curves. For the calibrations curves of both analytes, the 95% confidence interval for the intercept contained zero. The average back-calculated concentrations for EFV and its metabolite 8-OH-EFV were also evaluated. The obtained results were close to the expected theoretical values at each tested concentration and presented differences lower than 12% for both analytes.

3.1.2 Lower limit of quantification (LLOQ)

The LLOQ of the current method was 0.1 mg/L for EFV and 0.25 mg/L for its main metabolite. Although the same lower limit of EFV was tested for 8-OH-EFV, the chromatographic peaks were not efficiently distinguished from background noise at this concentration.

3.1.3 Accuracy

The accuracy for QC samples ranged between 89-99% for EFV and 91-101% for 8-OH-EFV.

3.1.4 Intra- and inter-assay precision

The results of intra- and inter-assay precision for all QC ranged between 3-11% for EFV and 2-11% for 8-OH-EFV.

3.1.5 Carryover

No carryover effect was observed when two blank samples were injected after the analysis of the higher calibration standard sample.

3.1.6 Stability

Regarding the stability performance after two freezing cycles, no significant deviation from the nominal concentration tested of 1.5 mg/L was found.

3.1.7 Recovery

A recovery of 99% for EFV and 92% for its metabolite from plasma supported the workability of liquid-liquid extraction protocol.

3.2 Animal study

3.2.1 Animals

A total of 32 male *Wistar* rats were used. There were no differences regarding animal weight or age in the beginning of the experiments (**Table 3.1**).

Table 3.1 Animal weight and age at the beginning of the experiments

	10 days			**36 days**		
Variable	Control group	EFV group	*p*-value	Control group	EFV group	*p*-value
n	8	8	-	8	8	-
Weight (g)[a]	304 ± 67	299 ± 75	ns	271 ± 49	274 ± 41	ns
Age (weeks)[a]	13 ± 1.6	13 ± 1.4	ns	12 ± 0.5	12 ± 0.5	ns

[a] Mean±SD; **EFV**, efavirenz; **ns**, not significant.

3.2.2 *S*-thiolated proteins plasma profile upon animal's exposure to efavirenz

RSSP profile was assessed in plasma samples of male *Wistar* rats by HPLC-FD analysis and data are present in **Table 3.2.** There was a time-dependent variation of RSSP profile upon EFV exposure. CysSSP and GSSP changed inversely along with EFV exposure time.

Table 3.2 *S*-thiolated proteins plasma profile at 10 and 36 days of efavirenz exposure

	Fold change[a]		Variation (%)[b]	*p-value*[c]
	10 days	36 days		
CysSSP	1.15 ± 0.03	1.03 ± 0.03	-12	<0.001
GSSP	0.82 ± 0.06	1.16 ± 0.07	34	<0.001

[a] Mean±SD Fold change: ratio efavirenz-group/control-group; [b] % variation between 10 and 36 days; [c]*Unpaired t*-test; **CysSSP**, *S*-cysteinylated proteins; **GSSP**, *S*-glutathionylated proteins.

3.3 Clinical study

3.3.1 Anthropometric and clinical data of study groups

Anthropometric and clinical data of all groups of patients are presented on **Table 3.3**. A total of 235 patients were included and 171 were HIV-infected patients. The EFV group had more male patients included (*Chi-square* test, $p<0.001$) as well as higher $CD4^+$ T-cell count (One-way ANOVA analysis of variance with *Bonferroni's* multiple comparison test, $p = 0.022$), in comparison with *naïve* and NVP groups.

Table 3.3 Anthropometric and clinical data of the study groups

Variable	non-HIV	*naïve*	NVP	EFV	*p*-value[b]
N	64	22	66	83	-
Men (%)	48	41	48	83	<0.001[c]
Age (years)	47 ± 14	42 ± 10	46 ± 11	43 ± 11	*ns*
CD4⁺ T-cell count (cell/µL)	nd	479 ± 192	589 ± 272	642 ± 239	0.022[d]
Viral load (cps/mL)	na	43096 ± 55670	BQL	BQL	-

[a] mean ± SD; [b] between groups; [c] *Chi-square* test, [d] One-way ANOVA with *Bonferroni's* post test for HIV-groups; **BQL**, bellow quantification limit; **EFV,** efavirenz; **na,** not applicable; **nd** not determined; **ns,** not significant; **NVP**, nevirapine.

3.3.2 *S*-thiolated proteins plasma profile of patients on efavirenz

RSSP profile of EFV group is presented in **Figure 3.1.** The CysSSP levels (146 ± 36 µM) were much higher than GSSP levels (2.7 ± 1.5 µM) (*unpaired* t-test, p<0.001) (**Figure 3.1**). The coefficient of variation of GSSP was twofold the CysSSP (**Figure 3.1**). Patients with higher GSSP levels had lower CysSSP levels (**Figure 3.2**).

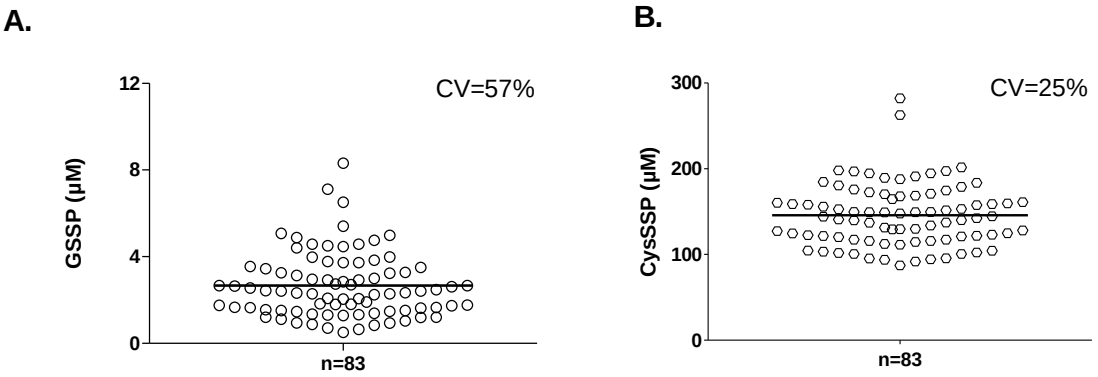

Figure 3.1 A. *S*-glutathionylated (GSSP) and B. *S*-cysteinylated (CysSSP) proteins plasma concentrations in patients on cART-containing EFV. CV; coefficient of variation**. CV;** coefficient of variation

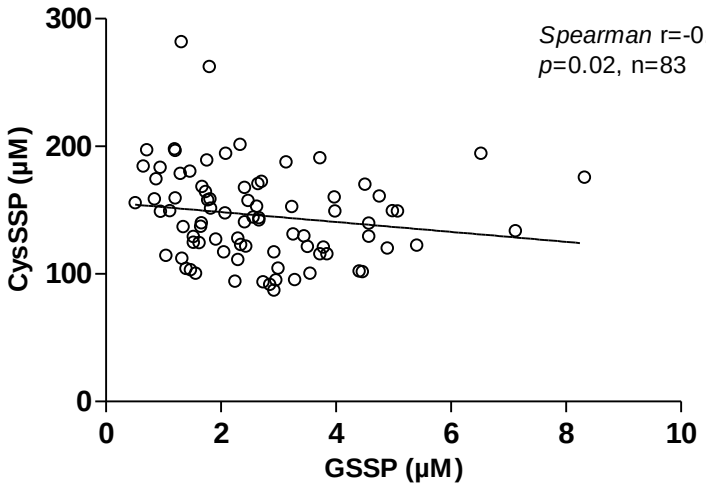

Figure 3.2 Correlation between *S*-glutathionylated (GSSP) and *S*-cysteinylated (CysSSP) proteins in patients on efavirenz

3.3.3 Effect of 8-hydroxy-efavirenz on *S*-thiolated proteins plasma profile

EFV and 8-OH-EFV concentrations and their coefficients of variation are presented in **Table 3.4**.

Table 3.4 Efavirenz and 8-hydroxy-efavirenz concentrations

Analyte	Concentration (mg/L)	CV (%)
EFV[a]	2.0 ± 1.1	53
8-OH-EFV[a]	1.8 ± 1.4	81
8-OH-EFV/EFV[a]	1.1 ± 1.5	129

[a] Mean±SD, **CV**, coefficient of variation; **EFV**, Efavirenz; **8-OH-EFV**, 8-hydroxy-efavirenz.

GSSP was positively associated with 8-OH-EFV/EFV (*Spearman* r=0.254, p=0.03) (**Figure 3.3A**). This association was contrary for CysSSP (*Spearman* r=-0.335, p=0.003) (**Figure 3.3B**). Patients with high 8-OH-EFV/EFV ratio have higher GSSP and lower CysSSP levels.

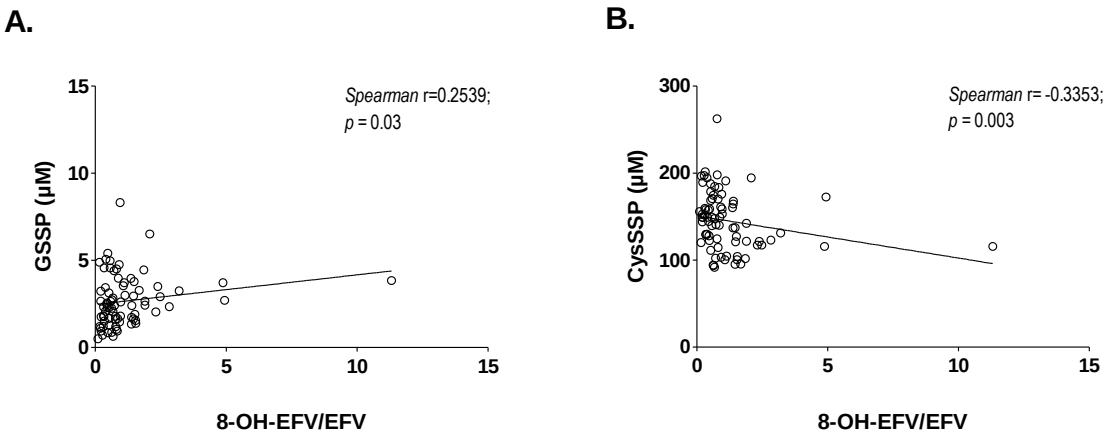

Figure 3.3 Association between the ratio 8-hydroxy-efavirenz/efavirenz (8-OH-EFV/EFV) on A. *S*-glutathionylated (GSSP) and B. *S*-cysteinylated (CysSSP) proteins profile. (n=77).

3.3.4 Effect of HIV-infection on *S*-thiolated proteins plasma profile

The RSSP profile of EFV group was compared with those of a non-HIV-group and a naïve (non-treated) HIV-group, which are presented in **Figure 3.4**.

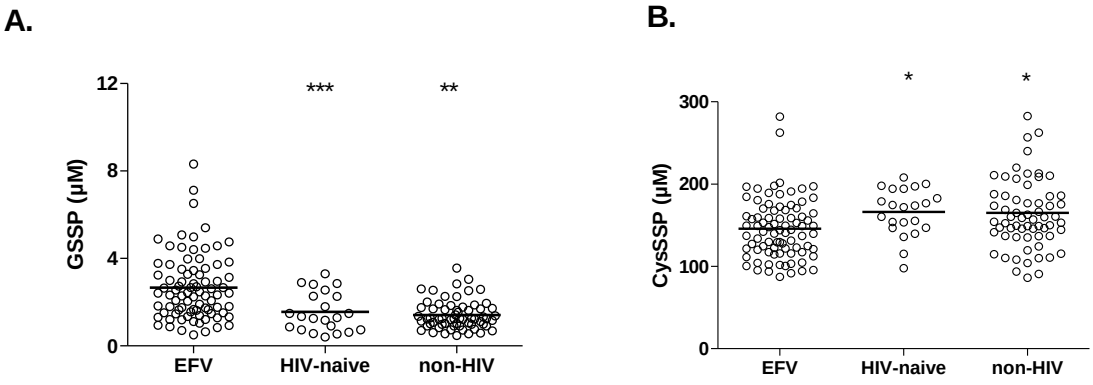

Figure 3.4 Comparison between efavirenz (n=83), *naïve* (n=22) and non-HIV (n= 64) groups of A. *S*-glutathionylated (GSSP) and B. *S*-cysteinylated (CysSSP) proteins profile. (*Kruskal-Wallis* with *Dunn's* multiple comparison test) *p<0.05; **p<0.01; **p<0.001.

EFV patients had higher GSSP levels (2.7 ± 1.5 µM) than naïve (1.6 ± 0.9 µM) and non-HIV individuals (1.4 ± 0.7 µM) (*Kruskal-Wallis test with Dunn's multiple comparison test, p<0.001*). CysSSP were lower (*Kruskal-Wallis test with Dunn's multiple comparison test, p<0.01*) in EFV-group (146 ± 36 µM) comparatively to non-HIV group (165 ± 46 µM) and HIV-naïve patients (166 ± 29 µM). Naïve patients and non-HIV group had similar levels of both GSSP and CysSSP.

The subtype of HIV in EFV-group was available for 31 patients. Approximately 60% of patients were B subtype, with non-B groups including 3 patients HIV-1A, 3 patients HIV-1C and 4 patients HIV-1G No differences was found between B and non-B subtypes on GSSP and CysSSP profile (**Figure 3.5**).

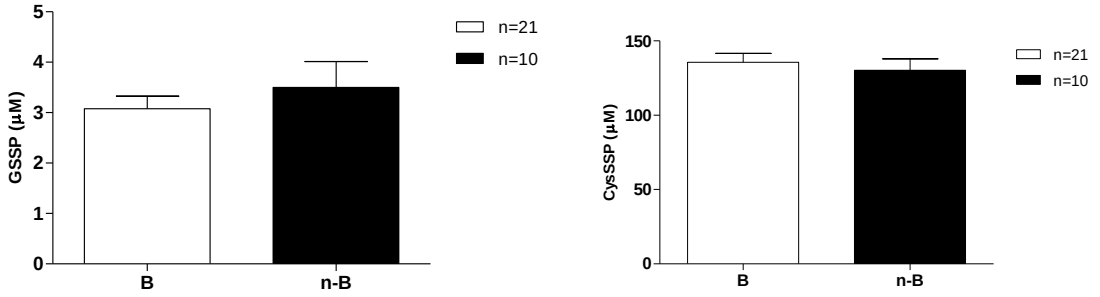

Figure 3.5 A. *S*-glutathionylated (GSSP) and B. *S*-cysteinylated (CysSSP) proteins on cART-containing EFV patients with B vs. non-B HIV-subtype. (Unpaired t-test).

There was no association between RSSP profile in naïve patients and $CD4^+$ T-cell count (**Figure 3.6**) or HIV viral load (**Figure 3.7**).

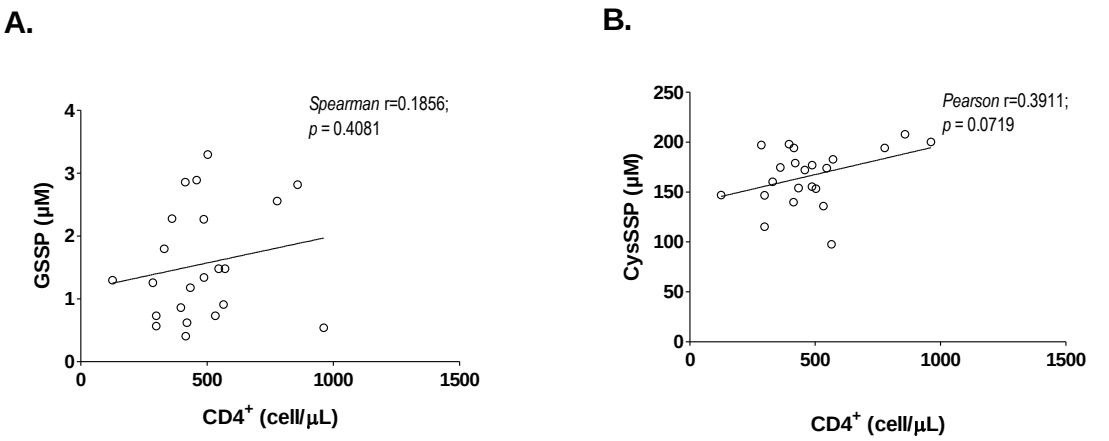

Figure 3.6 Association between CD4⁺ T-cell count in *naïve* patients (n=22) and A. *S*-glutathionylated (GSSP) and B. *S*-cysteinylated (CysSSP) proteins profile. (*Spearman* test to GSSP and *Pearson* test to CysSSP).

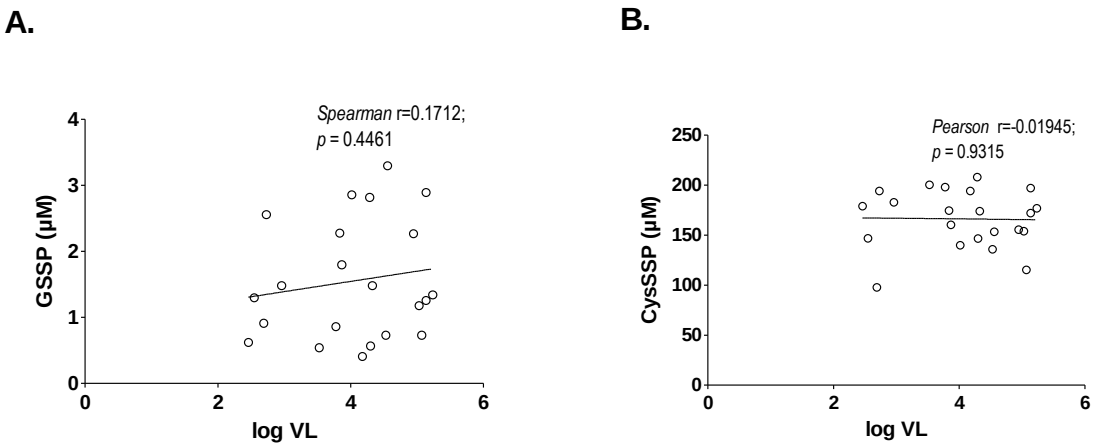

Figure 3.7 Association between viral load in *naïve* patients (n=22) and A. *S*-glutathionylated (GSSP) and B. *S*-cysteinylated (CysSSP) proteins profile.

3.3.5 *S*-thiolated proteins plasma profile of efavirenz vs. nevirapine

EFV-group had higher GSSP levels and lower CysSSP levels (respectively, 2.7 ± 1.5 µM and 146 ± 36 µM) than NVP-group (respectively, 1.0 ± 0.6 µM and 174 ± 39 µM) (*Unpaired* t-test, *p<0.001*) (**Figure 3.8**).

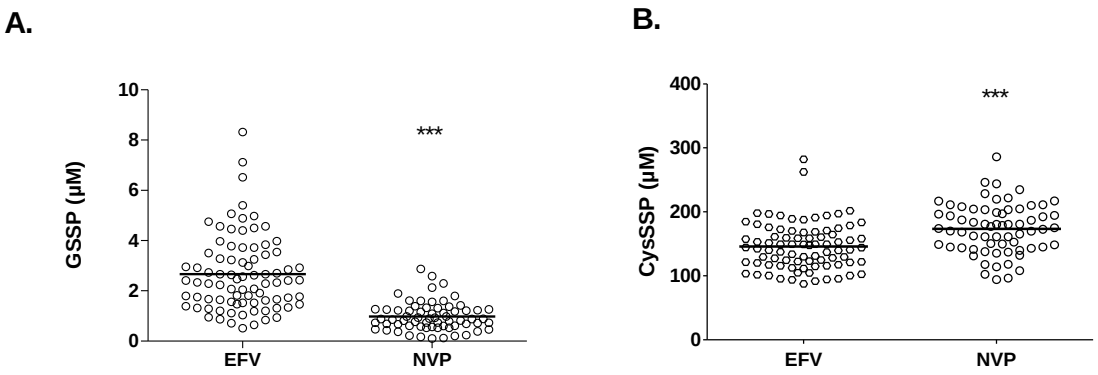

Figure 3.8 Comparison between efavirenz (EFV) (n=83) and nevirapine (NVP) (n=66) groups on A. *S*-glutathionylated (GSSP) and B. *S*-cysteinylated (CysSSP) proteins profile. (Unpaired t-test); ***p<0.001.

3.3.6 Effect of 12-hydroxy-nevirapine on *S*-thiolated proteins plasma profile

A total of 42 patients were included for the quantification of NVP and 12-OH-NVP by HLPC-UV (**Table 3.5**). There was no relation between CysSSP (**Figure 3.9A**) or GSSP (**Figure 3.9B**) and 12-OH-NVP/NVP ratio.

Table 3.5 Nevirapine and 12-hydroxy-nevirapine concentrations

Analytes	Concentrations (mg/L)	CV (%)
NVP[a]	5.0 ± 2.1	41
12-OH-NVP[a]	0.5 ± 0.3	63

[a] Mean±SD, **CV**, coefficient of variation; **NVP**, Nevirapine; **12-OH-NVP**, 12-hydroxy-nevirapine

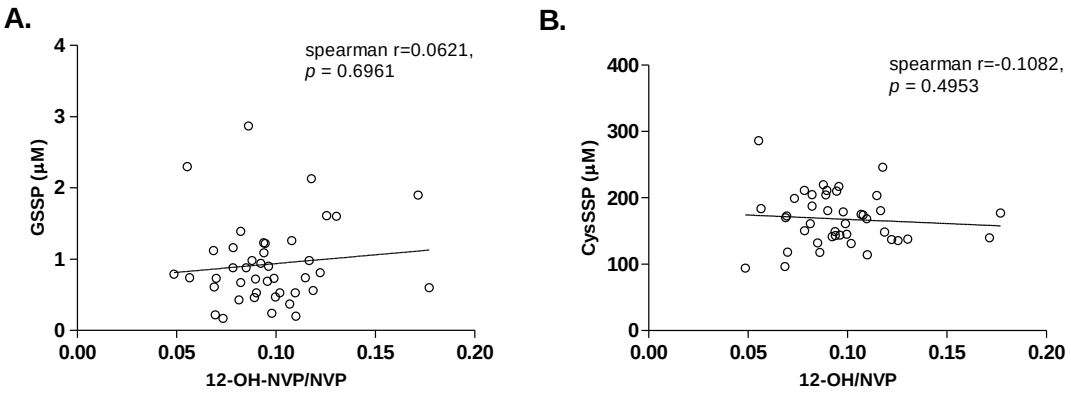

Figure 3.9 Influence of the ratio 12-hydroxy-nevirapine/nevirapine (12-OH-NVP/NVP) on A. *S*-glutathionylated (GSSP) and B. *S*-cysteinylated (CysSSP) proteins profile. (n=42).

Multivariable analysis including all HIV patients showed that GSSP levels were associated with age (B:-0.03, 95% CI (-0.05; -0.009), p=0.001), cART type (B:0.63, 95% CI (0.34; 0.93), p<0.001), as well as $CD4^+$ T-cell count (B:0.001, 95% CI (0.00; 0.002). CysSSP levels were only influenced by age (B: 1.2, 95% CI (0.72; 1.73), p<0.001).

4. Discussion

This study was part of an ongoing project aimed at unravel short and long-term mechanisms of EFV neurotoxicity. Previous data from this project showed that male *Wistar* rats upon 36 days of EFV exposure present memory impairment, as it was evaluated by behavioral tests performance (Grilo *et al.* 2016b). A decrease in neuronal activity and impairment in synaptic function were also found in the hippocampus of the animals (Grilo *et al.* 2016b). Our current knowledge about proteins *S*-thiolation and its relation with drug-induced toxicity is scarce. Although *S*-thiolation of plasma proteins has been associated with the aging, oxidative stress and chronic disease (Rossi *et al*., 2009; Giustarini *et al*., 2006; Perna *et al*., 2006; Andriollo-Sanchez *et al*., 2005). Herein, we have firstly identified the RSSP plasma signature in this animal model of long-term EFV-induced neurotoxicity. We found an increase in GSSP along with time of EFV exposure, which was accompanied by a decrease in CysSSP. This suggest that there might be an association between RSSP profile and EFV-induced memory deficits.

The clinical study was designed in order to translate the results previously obtained in animals. We have found that long-term EFV-induced plasma RSSP profile in animals is similar to the observed in HIV-infected patients on long-term EFV-containing cART. Clinical trials have consistently reported a high inter-patient variability for type, duration and severity of the CNS complaints as well as for EFV and its metabolites concentration profile (Aouri *et al*., 2016; Winston *et al*., 2015; Ngaimisi *et al*., 2010; Fumaz *et al*., 2005). Accordingly, we herein show that GSSP is highly variable among patients (57%).

Protein thiolation by glutathione is a reversible post-translational modification that is increased in response to oxidants (Popov, 2014). A common feature of organ toxicity is oxidative stress, which arises when reactive oxygen and/or nitrogen species exceed amounts required for normal redox signaling. An imbalance in reactive species impacts functionality of cysteines and perturbs thiol homeostasis. *S*-glutathionylation acts in protein regulation and serves as a protective mechanism against irreversible cysteine oxidation. Dysregulation of redox signaling and sulfhydryl homeostasis are important considerations in the context of brain diseases (Sabens Liedhegner *et al*., 2012).

GSSP levels were much lower than CysSSP, which is in agreement to what is found in literature (Rossi *et al*., 2009; Giustarini *et al*., 2006; Di Simplicio *et al*., 2005). This probably explains the higher inter-individual variability found in GSSP comparatively to CysSSP. Also, the GSSP might represent the fine-tuning of protein *S*-thiolation upon EFV exposure. The albumin represents almost exclusively the protein thiol pool existent in plasma and it functions as a regulator of thiols levels (Di Simplicio *et al*., 2005). Plasma proteins are mainly *S*-cysteinylated (Dalle-Donne *et al*., 2008; Ogasawara *et al*., 2007). The cysteine/cystine redox couple represents the major pool of low-molecular-mass thiol in plasma, while in cells this pool is mainly represented by GSH/GSSG (Rossi *et al*., 2009; Moriarty-Craige and Jones, 2004). Under oxidative stress occurs a decrease of GSH content and an increase of GSSG in the affected cells (Dalle-Donne *et al*., 2008). As a result, GSH redox status in plasma pool is altered. Due to the higher export of GSSG into plasma, the Cys residues of plasma proteins can suffer *S*-thiolation to reduce the excess of GSSG and to regenerate GSH as well as to protect protein sulfhydryl groups against

irreversible oxidation (Di Simplicio *et al*., 2005). The results obtained suggest that EFV leads to an increase in export of GSSG into plasma, increasing thereby the *S*-glutathionylation of plasma proteins. Accordingly, in our animal model there is an increase of GSSG in hippocampus of animal exposed to EFV (Grilo *et al*. 2016b). Patients with higher GSSP had lower CysSSP. The decrease of *S*-cysteinylation observed suggest that Cys is interchangeable with GSH in bound to plasma proteins. These data suggest that as higher is the concentrations of 8-OH-EFV, the greater is the tendency for the free fraction cysteine to be disconnect from proteins. Since the cysteine is an essential precursor to the synthesis of glutathione, it can be recruited for its biosynthesis in cells. This interchange of GSSP and CysSSP was previously observed in aging in a group of volunteers (Rossi *et al*., 2009).

As we (Grilo *et al*., 2016a) and others (Aouri *et al*., 2016; Ngaimisi *et al*., 2010) have been demonstrating, the patients' individual metabolic capacity is at the genesis of EFV-induced neuro-adverse reactions. In particular, evidence support the cytochrome P450 2B6 isoenzyme (CYP2B6)-mediated biotransformation of EFV into its main phase I metabolite, 8-hydroxy-efavirenz (8-OH-EFV) (Harjivan *et al*., 2014; Brandmann *et al*., 2013; Tovar-y-Romo *et al*., 2012), as an upstream event of the neuro-adverse reactions profile of this NNRTI. While this data comes majorly from non-clinical studies, in the present year we showed the first clinical evidence on the association between the 8-OH-EFV plasma concentrations and mood changes in HIV-population (Grilo *et al*., 2016a). The ratio 8-OH-EFV/EFV is considered a metabolic phenotype for CYP2B6 activity assessment in clinical studies (U.S. Food and Drug Administration, 2014). Herein, we validated a method for the measurement of 8-OH-EFV/EFV. The validation of this method supports the possibility to monitoring of EFV and 8-OH- EFV by this cheaper and generally available technique, in detriment of liquid chromatography-mass spectrometry (LC-MS) methodologies (Aouri *et al*., 2016; Cho *et al*., 2011; Ngaimisi *et al*., 2010; Winston *et al*., 2015). The concentrations of EFV and 8-OH-EFV varied strongly among individuals (up to 81%) as well as the ratio 8-OH-EFV/EFV (129%). Patients with higher 8-OH-EFV/EFV ratio have higher GSSP. Accordingly CYP2B6 ultra-rapid metabolizers are at higher risk developing neurotoxicity (Grilo *et al*. 2016b).

There is expanding evidence that redox-imbalance plays a role on viral, inflammatory and immunological response of HIV-infection (Aquaro *et al*., 2008). HIV-infected patients have been found to have a disturbance in glutathione metabolism, enhanced spontaneous generation of ROS and a decreased antioxidant concentrations (Müller *et al*., 2000). To ascertain if this RSSP profile is characteristic of EFV exposure or due to HIV-infection we performed the comparison of this profile between EFV-group, a naïve-patients group and a group of non-HIV volunteers. We found that the RSSP profile of untreated infected HIV-patients is similar to that of non-HIV individuals. The RSSP profile in patients on EFV-containing cART was not associated to the main markers of HIV-infection, which are $CD4^+$ T-cell count and HIV viral load, nor with HIV subtype B.

In multivariable analyses both cART type and $CD4^+$ T-cell count were associated to GSSP. Nevirapine is prescribed according with patients $CD4^+$ T-cell count. That is why patients on NVP

presented lower $CD4^+$ T-cell count than EFV. High $CD4^+$ T-cell count is a risk factor for NVP toxicity. HIV is classified into nine different subtypes from A to K based on phylogenetic analysis (Robertson *et al*., 2000). The large majority of circulating HIV-1 are B and C subtype (Buonaguro *et al*., 2007). In EFV-group most of patients were from B subtype. This is the predominant subtype of HIV-1 infection that occur in North America, Western Europe and Australia (Buonaguro *et al*., 2007; Rambaut *et al*., 2001). During infection, HIV-1 subtypes B and non-B differentially contribute to the neuropathogenesis and development of HIV-associated neurocognitive disorders, in which the generation of reactive oxygen species play a significant role (Samikkannu *et al*., 2014). HIV-1B potentially induces oxidative stress, leading to more immunoneuropathogenesis than infection with HIV-1 non-B (Samikkannu *et al*., 2014). In the present work we were not able to distinguish the RSSP profile between HIV-1B and HIV-1 non-B groups. Although, it might be highlighted that these patients are receiving treatment and had no detectable viral load (this was an exclusion criteria), so this result was somehow expected. We concluded that EFV use is associated an increase in GSSP and a decrease in CysSSP, which was dependent on 8-OH-EFV generated and independent on HIV-infection.

EFV and NVP belong to the same class of antiretroviral and despite the common features on their mechanism of action, biotransformation routes and toxicity mechanisms through bioactiation, EFV is associated to neurotoxicity, while NVP is related to hepatotoxicity. So we compared the RSSP profile of EFV and NVP and we found that NVP exposure modulates differently GSSP. Recently, GSSP has been proposed as a mechanism of resistance to paracetamol-induced hepatotoxicity (McGarry *et al*., 2015), demonstrating that it can confer protection against electrophilic stress *in vivo*. A reasonable explanation is that *S*-thiolation confers protection of protein thiol groups, preventing irreversible inactivation by electrophiles. Moreover, *S*-thiolation has key roles in regulation of macromolecular interactions, directional trafficking proteasomal degradation, folding and activity of proteins (McGarry *et al*., 2015). This explains why, despite mostly metabolized by the liver, EFV is barely hepatotoxic (Rivero *et al*., 2007). Additionally, this indicates that genetic variability of crucial redox enzymes and transport systems might identify patients at higher risk for EFV-induced hepatotoxicity. For instance, glutathione *S*-transferases (GST) participates in the conjugation of GSH with reactive electrophiles (Hayes *et al*., 2005), in GSSP formation (Townsend *et al*., 2009) and in transport of nitric oxide (Lok *et al*., 2012). Nitric oxide implication in EFV toxicity has been steadily described (Apostolova *et al*., 2015a). In addition, dysregulation of redox signaling and sulfhydryl homeostasis contributes to neurodegeneration (Sabens Liedhegner *et al*., 2012) and EFV exposure has been associated to an increased production of soluble amyloid beta (Aß), due to increased oxidative stress conditions (Brown *et al*., 2014).

The lower GSSP found in patients on NVP suggest that NVP modulation of thiol redox status does not allow an adaptive response as it is possible for EFV. We concluded that patients have higher GSSP upon EFV exposure are at higher risk of neurotoxicity and lower risk of hepatotoxicity.

Finally, we must highlight that the majority of RSSP in plasma are from albumin (Rossi *et al*., 2009) and EFV is highly bound to this protein (Wanke *et al*., 2013). However, it is not known if these changes in plasma RSSP modify the fraction of EFV available to cross the BBB, or its intra-tissue accumulation. Furthermore, the information available on 8-OH-EFV affinity for plasma proteins is scarce.

The data herein obtained supports that the RSSP profile allows the assessment of antiretroviral redox-modulation effects. This gives future perspectives for strategies against electrophilic stress prior to or early after onset of clinical symptoms upon EFV and NVP exposure.

As main weakness of our study the absence of information on adverse reactions. Also, we have not evaluate the effect of the other components of the cART and we do not know other drugs that patients were doing.

In the future, it will be interesting to explore this profile in a higher number of patients with the following endpoints: a) neuro-adverse reactions b) severity of neuro-adverse reactions. It will be also interesting to find out the relation between RSSP profile and a) CYP2B6 polymorphism b) sex c) age d) comorbidities. Moreover it will to perform a clinical study in order to evaluate the benefit of antioxidant supplements in RSSP profile and neurotoxicity.

Other possibilities would be expand this tool application to other antiretrovirals and evaluate the effect of antiretrovirals on the enzymes that modulate GSSP levels and to evaluate this enzymes polymorphisms in RSSP profile and antiretroviral adverse reactions. Other RSSP as cysteinylglicinylation or homocysteinylation might be also addressed.

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