Clara Lúcia Gonçalves Dias

Licenciada em Genética e Biotecnologia

HDL in HIV-1 infection: a quality perspective through paraoxonase-1 activities

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: Alexandra Moita Antunes,
Investigadora Auxiliar, Instituto Superior Técnico, UL

Júri:

Presidente: Prof. Doutora Margarida Casal Ribeiro Castro Caldas Braga
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Abstract

Cardiovascular disease is highly prevalent on human immunodeficiency virus (HIV)-infected young adults, with serious implications on the choice of the most cardiovascular friendly antiretroviral regimen and its management. Nevirapine (NVP) is an antiretroviral drug that, although associated with hepatotoxicity and skin rash, is currently recognized as a high-density lipoprotein (HDL) booster in HIV-infected patients. This HDL booster effect is even more pronounced than the current available drugs for this purpose. However, besides HDL quantity, its quality is also essential. On this regard, the present study aims to give new insights into the effect of NVP on HDL quality, namely its antioxidant potential, which can be measured through the activities of its associated enzyme, paraoxonase-1 (PON1): paraoxonase (POase), arylesterase (AREase) and lactonase (LACase) activities. The role of PON1 as a protective player against the toxicity inherited to the use of NVP was also explored. Additionally, new methods were developed for the assessment of PON1 AREase and LACase activities.

The study protocol received prior approval from the ethics committees of the hospitals and a total of 54 HIV-infected patients were included.

The methods herein developed are reliable and suitable for monitoring PON1 AREase and LACase activities in human blood.

The negative effect of NVP on PON1 activities is dependent from the formation its phase-I metabolites, especially 12-OH-NVP. The 3-OH-NVP seemed to be the safest metabolite.

The current study gives new insights into the players on the mechanism of NVP-toxicity. Moreover, it provides evidence for the development of new NVP or its metabolites analogues, less toxic than NVP, which might in the future have a place in the border of HDL boosters.

Keywords: cardiovascular disease and human immunodeficiency virus infection, high-density lipoprotein booster, high-density lipoprotein quality, nevirapine, nevirapine toxicity, paraoxonase-1 activities.

Resumo

A doença cardiovascular é altamente prevalente em jovens adultos infectados pelo vírus da imunodeficiência humana (VIH), com implicações sérias na escolha e gestão da terapêutica antiretroviral combinada mais adequada para a comorbilidade cardiovascular. A nevirapina (NVP) é um antiretroviral que, apesar de estar associado a toxicidade hepática e cutânea, é actualmente reconhecido por aumentar os níveis da lipoproteína de elevada densidade (HDL). Este efeito é mais pronunciado que o conseguido com os fármacos actualmente disponíveis para esse fim. No entanto, além da sua quantidade, a qualidade da HDL é também essencial. Desta forma, este estudo tem como objectivo demonstrar o efeito da NVP na qualidade da HDL, nomeadamente no seu potencial antioxidante, que pode ser medido através das três actividades do enzima paraoxonase-1 (PON1) que circula associado à HDL: paraoxonase (POase), arilesterase (AREase) e lactonase (LACase). O papel protector do PON1 contra a toxicidade associada à toma da NVP também foi explorado. Adicionalmente, foram desenvolvidos e validados novos métodos para a monitorização das actividades AREase e LACase do PON1.

O protocolo do estudo foi previamente aprovado pela comissão de ética dos hospitais e foram incluídos um total de 54 doentes infectados pelo VIH positivo.

Os métodos desenvolvidos são válidos e adequados para a monitorização das actividades AREase e LACase do PON1 em sangue humano.

O efeito negativo da NVP nas actividades do PON1 é dependente da formação dos seus metabolitos de fase I, especialmente o 12-OH-NVP. O metabolito 3-OH-NVP foi o que menos influenciou a actividades do PON1.

Este estudo permitiu identificar novos protagonistas no mecanismo da toxicidade induzida pela NVP. Além disso, abre portas ao desenvolvimento de novos análogos da NVP ou dos seus metabolitos, que sejam menos tóxicos que a própria NVP e que possam futuramente ser usados como moduladores da HDL.

Palavras-Chave: actividades do paraoxonase-1, doença cardiovascular e a infecção pelo vírus da imunodeficiência humana, modulador da lipoproteína de elevada densidade, nevirapina, qualidade da lipoproteína de elevada densidade, toxicidade da nevirapina.

Table of contents

Index of figures

Index of tables

Abbreviations

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
ABC	Abacavir
ABCA1	ATP-binding cassette transporter
AIDS	Acquired immunodeficiency syndrome
Apo A-1	Apolipoprotein A-1
AREase	Arylesterase
ARV	Antiretroviral
BMI	Body mass index
BSA	Albumin from bovine serum
CAD	Coronary artery disease
cART	Combined antiretroviral therapy
CD4	Cluster differentiation 4
CETP	Cholesterylester transfer protein
CRI	Co-receptors inhibitor
CV	Variation coefficient
CVD	Cardiovascular disease
CYP450	Cytochrome P450
DAD	The Data Collection on Anti-HIV Drugs
DHC	Dihydrocoumarin
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
EFV	Efavirenz
FDA	US Food and Drug Administration
FI	Fusion inhibitor
GSH	Glutathione
Hcy	Homocysteine
HcyTL	Homocysteine thiolactone
HDL	High-density lipoprotein
HIV	Human immunodeficiency virus
HLOQ	Higher limit of quantification
HPLC	High-performance liquid chromatography
INI	Integrase inhibitor
IQR	Interquartile range
LACase	Lactonase

LCAT	Lecithin-cholesterol acyltransferase
LDL	Low-density lipoprotein
LLOQ	Lower limit of quantification
MI	Myocardial infarction
Nef	Negative regulatory factor
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NtRTI	Nucleotide reverse transcriptase inhibitor
NVP	Nevirapine
o-HPPA	3-(o-hydroxyphenyl) proprionic acid
ox-LDL	Oxidized LDL
PI	Protease inhibitor
POase	Paraoxonase
PON1	Paraoxonase-1
PON2	Paraoxonase-2
PON3	Paraoxonase-3
PROCAM	Prospective Cardiovascular Münster study
QC1	Quality control 1
QC2	Quality control 2
RCT	Reverse cholesterol transport
SEM	Standard error of the mean
TC	Total cholesterol
TG	Triglycerides
UGT	UDP-glucoronosyltranferase
UV	Ultraviolet
Y0	Y-intercept

1. Introduction

1.1 Cardiovascular disease in human immunodeficiency virus infection: the virus and the antiretroviral drugs

Nearly 34 million people are currently living with the human immunodeficiency virus (HIV)-infection, the cause of acquired immunodeficiency syndrome (AIDS) (WHO, 2013). HIV-infection is a chronic condition characterized by persistent infection and inflammation. The virus infects and depletes cluster of differentiation 4 (CD4) lymphocyte cells, resulting in immunodeficiency and on a slowly progressive disease (Shor-Posner *et al.*, 1993; Riddler *et al.*, 2003) that, if left untreated, has a high rate of morbidity and mortality (Tohyama *et al.*, 2009).

Though HIV-infection continues to spread, the survival of these patients is considerably extended by the combined antiretroviral therapy (cART). As so, the HIV population is getting older and it is likely that long-term consequences of both HIV-treatment and infection will become increasingly common (Parra *et al.*, 2010a). The persistent infection/inflammation, residual viremia, compromised immune system, co-infections (*e.g.* tuberculosis and hepatitis), polimedication and drug-drug interactions, potentially along with antiretroviral (ARV)-toxicity, could place these patients at a particular increased risk of developing several complications, many of which are commonly associated with ageing. For instance, liver disease is often present in these patients, partly due to the high rates of chronic viral hepatitis and alcohol misuse, as well as long-term exposure to potentially hepatotoxic ARVs (Deeks and Phillips, 2009). Compelling data suggest that other diseases are more prevalent in HIV-infected patients than in age-matched uninfected people, such as cancer (Kirk *et al.*, 2007) and bone disease (Arnsten *et al.*, 2007). Also, neurological disease persists or even progresses during long-term treatment (McCutchan *et al.*, 2007). Currently, cardiovascular disease (CVD) is turning into a preponderant condition in HIV-infected patients, as it has become a leading cause of morbidity and mortality in these patients (Triant *et al.*, 2007; Hsue *et al.*, 2008; Knudsen *et al.*, 2012).

With the implementation of cART in 1996, HIV-infection has changed from a lethal to a chronic disease in properly medicated patients (Deeks, 2009). The number of people receiving cART has tripled in the last five years and has reached in 2012 to 9.7 million in low- and middle-income countries. That total represents 65% of the global target of 15 million people set for 2015 (WHO, 2013).

Currently, there are more than twenty ARV drugs available, distributed by seven classes, according to their mechanism of action in the several steps of the HIV-life cycle **(Fig 1.1)**. These drugs are used in combination of three, two of which are nucleoside or nucleotide reverse transcriptase inhibitors (NtRTIs) plus one of the following options: a non-nucleoside reverse transcriptase inhibitor (NNRTI), one protease inhibitor (PI) boosted with ritonavir, or one integrase inhibitor (INI). Occasionally, these regimens could be altered if there is any presence of virological failure, side effects, pregnancy and co-infections (EACS, 2011; DGS, 2012). The choice of cART and the optimal time for its start for each patient should be in accordance with several parameters, such as clinical manifestations, the number of CD4 lymphocytes (<350 cell/mm^3), the viral load and the presence of co-morbidities.

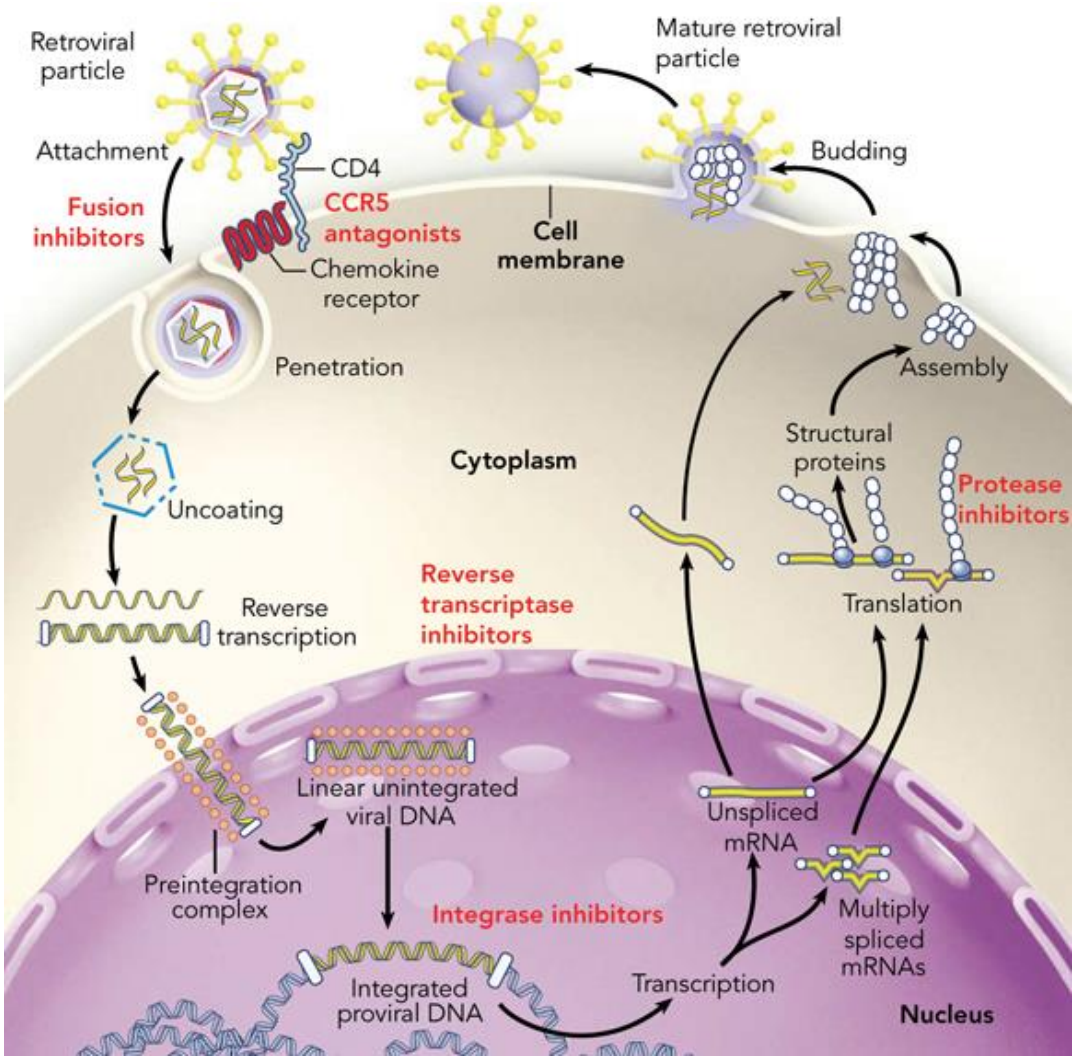

Figure 1.1 Schematic representation of the HIV-life cycle main steps and the targets of the several classes of antiretroviral drugs (Chen *et al.*, 2007). Mechanism of action, through the different phases of HIV-life cycle, of the different antiretroviral drugs classes: nucleoside reverse transcriptase inhibitors (NRTIs), nucleotide reverse transcriptase inhibitors (NtRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PI), fusion inhibitors (FI), co-receptors inhibitors (CRIs) and integrase inhibitors (INIs) (De Clercq, 2009).

The ARV drugs are a very dynamic therapeutic group since, drug availability, drug combination and also the criteria for the beginning and managing of cART has widely varied throughout the last two decades. Currently, the national guidelines state that the first-line cART should include a first generation NNRTI, nevirapine (NVP) or efavirenz (EFV) (DGS, 2012). Successful cART is associated with dramatic decreases in AIDS-defining conditions (Thompson *et al.*, 2010) and an increase in lifespan and quality of life of HIV-infected patients. Nevertheless, this clinical condition still faces several obstacles due to its chronic treatment, such as the adherence to a daily administration schedule, the loss of therapeutic efficacy and the drug-associated toxicity (Caixas *et al.*, 2012).

Increased evidence supports the association between CVD and cART in HIV-infected patients. The *Data Collection on Anti-HIV Drugs* (DAD) study showed that overall, cART was associated with a 26% relative increase in the rate of myocardial infarction (MI), *per* year of exposure during the first four to six years of use (The DAD Study Group, 2003). In HIV-infected patients, lipid abnormalities are very common (Grunfeld *et al.*, 1992), which as consequence, can lead to further development of CVD. Before treatment, HIV-infection results in substantial decreases in serum total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL) levels (Grunfeld *et al.*, 1992; Riddler *et al.*, 2003), and an increase in triglycerides (TG) levels (Fernández-Miranda *et al.*, 1998). Though not always is possible to see hypertriglyceridemia in early stages of the infection (Grunfeld *et al.*, 1992; Zangerle *et al.*, 1994), low levels of HDL are present in all its stages (Grunfeld *et al.*, 1992; Zangerle *et al.*, 1994; Fernández-Miranda *et al.*, 1998). The *Multicenter AIDS Cohort Study* (MACS) is one of the best reports that show that the reduction of HDL and LDL levels is associated with the infection (Riddler *et al.*, 2003), as it is the only study that has access to the patient's lipidic profile before the infection, post-infection without cART and after cART initiation. The first two stages were characterized by low levels of TC (-30 mg/dL), LDL (-22 mg/dL) and HDL (-12 mg/dL). Subsequent cART initiation was associated with increases in TG, TC and LDL but little change in HDL levels (Riddler *et al.*, 2003).

Both controlled and uncontrolled HIV-infection are associated with the risk of developing CVD. In treated HIV-infected patients, several metabolic complications have been associated with cART (Grinspoon and Carr, 2005), including dyslipidemia, insulin resistance and overt diabetes mellitus, all well-known risk factors for the development of CVD (Carr *et al.*, 1999; Vergis *et al.*, 2001; The DAD Study Group, 2003).

The introduction of cART has improved both morbidity and mortality rates of HIV-infected patients (Palella Jr *et al.*, 1998). Hence, it was expected that the successful control of HIV-infection would also reduce the risk of coronary artery disease (CAD) associated with the condition. However, the reported data suggests the opposite: despite treatment or possibly because of it, HIV-infection is associated with an increased risk of development of atherosclerosis (Hsue *et al.*, 2004) and at least a threefold of the risk of CAD (Hsue *et al.*, 2004; de Saint Martin *et al.*, 2006). Moreover, both MI (Guaraldi *et al.*, 2010) and asymptomatic ischaemic disease (Calza *et al.*, 2005) are also common conditions in HIV-infected patients. As so, cardiovascular complications are rapidly becoming one of the prevalent causes of morbidity and mortality in HIV-infected patients (Varriale *et al.*, 2004), and though the relative contribution of HIV-infection itself and adverse effects of cART is not quite clear, two consistent trends have emerged. Firstly, the risk of CVD is higher in untreated than treated HIV infection, probably because inflammation is increased on the first case (Kuller *et al.*, 2008). Secondly, some ARVs have direct effects that can potentiate the development of CVD. For instance, prolonged exposure to PIs is associated with hyperlipidaemia, insulin resistance and a higher rate of CVD events (The DAD Study Group, 2007). Moreover, abacavir (ABC), a commonly used nucleoside reverse transcriptase inhibitor (NRTI), seems to increase the risk of heart disease (Sabin *et al.*, 2008), perhaps because of its pro-inflammatory effect induced by toxic metabolites formation (Grilo *et al.*, 2013).

Nevertheless, HIV-infection *per se* is connected with dyslipidaemia and, particularly in advanced states, it has been shown to be an independent risk factor for adverse lipid parameters (El-Sadr *et al.*, 2005; Batuca *et al.*, 2012).

1.2 High-density lipoprotein in HIV-infection: the influence of combined antiretroviral therapy

Among the several factors influencing CVD, HDL is the most powerful independent predictor. The HDL protective effects have first been attributed to the ability to promote cellular cholesterol efflux from peripheral cells to the liver for excretion, a process known as reverse cholesterol transport (RCT) (Précourt *et al.*, 2011), which is essential for preventing foam cell formation and consequent atherosclerosis (Tohyama *et al.*, 2009). HDL is also involved in the inhibition of oxidation, inflammation, activation of the endothelium and coagulation or platelet aggregation (van Leuven *et al.*, 2007).

Additionally, HDL has also several other features that able it to be used for the assessment of the risk for developing CVD. For instance, on a methodological view, HDL can be an effective biomarker due to its easy quantification. Moreover, from an epidemiologic perspective, this lipoprotein has also been shown to be crucial for the development of many cardiovascular events in the non-HIV population. Large prospective studies such as the *Framingham Heart* study, the *Prospective Cardiovascular Münster* (PROCAM) study, the *Helsinki Heart* study and the *Lipid Research Clinics Prevalence Mortality* study have shown that the risk of CAD is reduced by 2-5% for every 0.025 mmol/L increase in plasma HDL levels (Abbott *et al.*, 1988; Wilson *et al.*, 1988; Frick *et al.*, 1990; Jacobs *et al.*, 1990; Assmann and Schulte, 1992; Assmann, 2001; Clotet *et al.*, 2003). From a pharmacological point of view, till this time, few drugs have the capacity to increase HDL levels, all of them showing practically no clinical benefits, as the increase in HDL concentration is really low (Birjmohun *et al.*, 2005; Filippatos and Elisaf, 2013). All the attempts to develop new drugs with HDL boosting effect have failed. Intensive efforts are in progress to develop new drugs that not only increases HDL quantity but also its quality (Tall *et al.*, 2007; Filippatos and Elisaf, 2013).

In HIV-patients, HDL metabolism is affected (Shor-Posner *et al.*, 1993; The DAD Study Group, 2007). However, in contrast to the atherogenic lipoprotein profile observed in patients treated with other HIV drugs such as PIs (Riddler *et al.*, 2003), several studies have demonstrated that treatment with NNRTIs such as NVP and EFV, increase plasma levels of HDL cholesterol by up 49% (van der Valk *et al.*, 2001; Clotet *et al.*, 2003; Tebas *et al.*, 2004; van Leth *et al.*, 2004; Fisac *et al.*, 2005; Young *et al.*, 2005; Pereira *et al.*, 2006), through an unknown mechanism. Moreover, NVP increases HDL and improves TC/HDL ratio apparently more than does EFV (van Leth *et al.*, 2004; Fisac *et al.*, 2005). NVP-containing cART may therefore contribute to reducing the risk of CVD in patients with HIV-infection, as well as it has been shown to have a protective role in pre-diabetic patients, behaving as a CVD-friendly drug (Srivanich *et al.*, 2010).

Nowadays, the information given by HDL quantity has to be complemented with quality assessment (Tall *et al.*, 2007; Filippatos and Elisaf, 2013). In this context, the enzyme paraoxonase-1 (PON1) has been implicated to play a pivotal role in the antioxidant protective functions of HDL, as it is found closely bound to this lipoprotein particle (Lusis, 2000; Gaidukov *et al.*, 2006).

1.3 The paraoxonase family in HIV-infection. The effect of combined antiretroviral therapy on PON1 activities

The PON enzyme family comprises three members, namely PON1, PON2 and PON3, that exhibits antioxidative properties mainly in the blood circulation. Their corresponding genes appears to have arisen from a duplication event of a common evolutionary ancestral, as they share considerable structural homology and are located adjacent to each other on chromosome 7q21-22 (Primo-Parmo *et al.*, 1996).

The three PON enzymes have different cell and tissues distributions, as well as different regulatory mechanisms. In the human body, PON1 and PON3 are found associated to HDL in circulation (Furlong, 2008). Regarding PON2, it is an intracellular enzyme, which is not detectable in serum, but is expressed in many tissues including brain, liver, kidney and testis (Ng *et al.*, 2001).

The different tissue distributions suggest distinct physiological roles for each of them, though they remain largely unknown (Draganov, 2007). Nevertheless, all three enzymes are able to reduce LDL oxidation (Aviram and Rosenblat, 2004). Moreover, PON2 is also able to reduce cellular oxidative stress and prevents apoptosis in vascular endothelial cells (Horke *et al.*, 2007). Among the three enzymes, PON1 is the best well known and characterized family member, and much of our understanding of PON enzymes is derived from the studies involving PON1 proteins.

In 1946, Abraham Mazur, who reported the presence of an enzyme in animal tissue able to hydrolyze organophosphate compounds (Mazur, 1946), led to the initial identification of PON1 (Aldridge, 1953a, b). PON1 is a calcium-dependent serum A-esterase enzyme, which protects LDL from oxidative modification by hydrolyzing lipid peroxides, thus exerting antioxidant and antiatherogenic effects (Mackness *et al.*, 1993). This glycoprotein of 354 amino acids, with a molecular mass of 43-45 kDa, is expressed in a variety of tissues (Marsillach *et al.*, 2008), but it seems probably that the liver is the main source of serum PON1. This organ is reported to have the highest PON1 gene expression and is also where a great part of HDL is synthesized and secreted into the circulation (Camps *et al.*, 2009).

In 1991, Mackness and co-authors did the first approximation for the identification of the physiological role of PON1, by showing that the enzyme prevents the generation of lipoperoxides during the process of LDL oxidation (Mackness *et al.*, 1991a). Subsequent studies reached the conclusion that PON1 protects LDL and HDL from lipid peroxidation by degrading oxidized cholesteryl esters and oxidized phospholipids contained in oxidized lipoproteins (Mackness *et al.*, 1993; Navab *et al.*, 1996; Aviram *et al.*, 1999). On the other hand, PON1 is inactivated by oxidized lipids, as shown by

Aviram and co-authors in an i*n vitro* incubation of PON1 with oxidized palmitoyl arachidonoyl phosphatidylcholine, lysophosphatidylcholine and oxidized cholesteryl arachidonate (Aviram *et al.*, 1999). PON1 was also shown to be able to hydrolyze hydrogen peroxide, a potentially important oxidative stress mediator in atherosclerosis (Aviram *et al.*, 1998). The effects of PON1 overexpression were also investigated in mice. Oda and co-authors (2002) generated a mouse model of a 5-fold increase in PON1 expression specifically in the liver. They found that PON1 was redistributed to HDL vehicles in the circulation. However, increased PON1 content in HDL did not alter HDL composition or properties, except that they were more protected from lipid peroxidation (Oda *et al.*, 2002).

Numerous factors are known to influence PON1 status **(Fig 1.2)**, including diet and life style habits (Deakin and James, 2004; Aviram *et al.*, 2005; Costa *et al.*, 2005b). Also, PON1 polymorphisms and its association with lipid metabolism, CVD and ischemic stroke have been documented in a considerable number of studies (McElveen *et al.*, 1986; Mackness *et al.*, 1991b; Garin *et al.*, 1997; Voetsch *et al.*, 2002). Epidemiological and molecular studies have identified that there are two major common functional genetic polymorphisms in the coding region of the PON1 gene due to glutamine or arginine at position 192 (Q192R) and leucine or methionine at position 55 (L55M) (Adkins *et al.*, 1993; Humbert *et al.*, 1993). The L55M polymorphism is located in the N-terminal side of PON1 gene, which plays a role in the binding of PON1 to HDL. Moreover, it also affects the enzyme concentration (Adkins *et al.*, 1993) with the M allele causing a decrease in protein stability (Leviev *et al.*, 2001). On the other hand, the Q192R polymorphism does not affect PON1 protein concentration (Leviev and James, 2000), although it is responsible for a striking substrate specific difference in the hydrolytic activities of the enzyme (Adkins *et al.*, 1993; Humbert *et al.*, 1993; Leviev *et al.*, 2001), especially in what regards the Q allele (Davies *et al.*, 1996; Mutch *et al.*, 2007). Furthermore, four additional polymorphisms in the promoter region of the PON1 gene has also been reported, namely C-107T, A-162G, G-824A and G-907C. These polymorphisms are reported to affect the expression and thus the serum concentration of the enzyme (Leviev and James, 2000). The C-107T polymorphism has been the most important genetic determinant of PON levels (Brophy *et al.*, 2001; Deakin *et al.*, 2003), influencing the gene expression, with the T allele reducing PON1 levels. This polymorphism contributes around 22-25% of variation in PON1 expression in caucasian adults (Leviev and James, 2000; Brophy *et al.*, 2001). However, PON1 allele frequencies show great variations between different populations (Rojas-Garcia *et al.*, 2005) and ethnic groups (Brophy *et al.*, 2001; Ginsberg *et al.*, 2009).

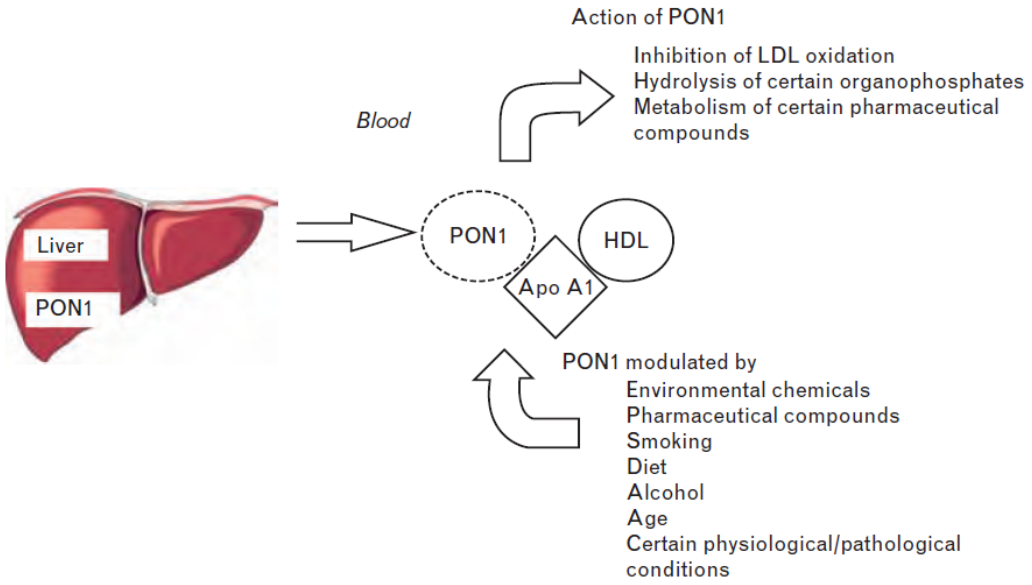

Figure 1.2 Biological effects of PON1 and its modulation (Macharia *et al.*, 2012). (PON1 - paraoxonase-1; Apo A1 – Apolipoprotein A-1; HDL – high-density lipoprotein)

The PON1 enzyme is considered as a human body endogenous free-radical scavenging system and has three main activities identified that could possibly explain its antioxidant and anti-inflammatory potential, which are paraoxonase (POase), arylesterase (AREase) and lactonase (LACase) activities.

Paraoxonase Activity

The enzyme was firstly found to have POase activity, reflecting its ability to catalyze the hydrolysis of paraoxon, an insecticide that gave rise to the family name, hence protecting against xenobiotic toxicity (Costa *et al.*, 2005a). The POase activity is not shared by the three enzymes of the PON family, as it is only confined to PON1.

Several reports using paraoxon as substrate demonstrated that there is a high inter-individual variability in PON1 POase activity (Humbert *et al.*, 1993), considering part of this variability due to the polymorphisms found for the PON1 gene. Regarding the Q192R polymorphism, the Q allele has been reported to have lower POase activity than the R allele (Humbert *et al.*, 1993; Davies *et al.*, 1996; Mackness *et al.*, 1997; Li *et al.*, 2000). For the L55M polymorphism, the L allele is correlated with higher POase activity and mRNA levels than the M allele (Leviev *et al.*, 1997; Li *et al.*, 2000). Moreover, PON1 C-107T polymorphism also has influence on the POase activity. For instance, the homozygotes for the T allele have, on average, 33-45 % lower POase activity as adults (Leviev and James, 2000; Brophy *et al.*, 2001) and 63 % lower as neonates (Chen *et al.*, 2003), relatively to the C homozygotes. Overall, genetic factors, including polymorphisms, were found to explain more than 60 % of phenotypic variance in PON1 POase activity, while demographic environmental factors accounted for only 1-6 % of changes and metabolic covariates for 4-19 % (Rainwater *et al.*, 2009).

The ability of PON1 to hydrolyze paraoxon was employed as a method to measure PON1 activity in several species and tissues. However, despite most of the studies exploring the role of PON1 in

disease used the POase activity as a biomarker for the enzyme status (Pereira *et al.*, 2009; Soyoral *et al.*, 2011), this activity does not reflect the real physiological role of PON1. Thus, it is critical to start looking at its remaining activities, AREase and LACase, which have been proved to be more physiological (Rosenblat *et al.*, 2006).

Arylesterase Activity

The detoxification of lipid peroxides by PON1 is possible via its AREase activity, being phenyl acetate one of its best substrates. Due to this feature, this activity is thought to be the one which best reflects the antioxidant capacity of PON1 (Rosenblat *et al.*, 2006). Hence, PON1 AREase activity seems to be involved on the reduction of the magnitude of LDL oxidation. It was found that components of oxidized LDL (ox-LDL) displayed the potency to strongly inactivate the activity. However, the presence of antioxidants, such as flavonoids, quercetin and glabridin during LDL oxidation, attenuated the loss of PON1 AREase activity (Aviram *et al.*, 1999). On the other hand, the activity declined rapidly in HDL following treatment with 3-morpholinosydnonimie, which generates peroxynitrite, a powerful oxidant (Ahmed *et al.*, 2001). PON2 does not have this activity and although PON3 also has it, it is very limited.

Regarding PON1 polymorphisms influence in the enzyme AREase activity, there has been some controversy. Initially, it was proposed that, in contrast with the POase activity, the measurement of the AREase activity of PON1 was not influenced by genetic polymorphisms (Eckerson *et al.*, 1983). Nevertheless, in 2001, Brophy and co-authors reported that the highest AREase activity was reported in individuals with the 192QQ genotype (Brophy *et al.*, 2001). Additionally, in a more recent study by Rainwater and co-authors (2009), the highest AREase activity was found in 192RR and 55LL individuals (Rainwater *et al.*, 2009).

Lactonase Activity

PON1 LACase activity was the latest to be discovered and protects against homocysteine thiolactone (HcyTL) toxicity (Jakubowski, 2000). Moreover, this activity is also involved in the metabolism of certain drugs, including the activation of the aquinolone antibiotic NM394, by the hydrolysis of the unsaturated cyclic carbonate prodrug prulifloxacin (Tougou *et al.*, 1998). The LACase activity of PON1 has also been used in the development of locally acting glucocorticoid drugs, which undergo rapid hydrolysis and inactivation when they reach the circulation (Biggadike *et al.*, 2000). More recently, PON1 LACase activity has been linked to the efficacy of clopidogrel (Camps *et al.*, 2011), an antithrombotic drug used to prevent CAD. The prevailing notion is that the hydrolytic activity towards lactones is the native activity of PON1 – a view supported by structure-activity studies indicating that lactones are the preferred substrate of PON1 (Harel *et al.*, 2004). Moreover, as the enzyme play a role in anti-inflammatory and antioxidant response, many oxidized metabolites of polyunsaturated fatty acids are structurally similar to lactones (Draganov *et al.*, 2005). The LACase activity is believed to have been conserved throughout the evolution of the enzyme, since it is shared

by all three members of the PON family. In fact, the term lactonase has been suggested as more fitting for the PON family as PON2 and PON3 lack any notable POase activity (Ng *et al.*, 2005).

Although The PON1 natural-substrates are uncertain, HcyTL is hydrolyzed to homocysteine (Hcy) by PON1 LACase activity (Mackness *et al.*, 1996; Draganov, 2007), and is a well-known risk factor for the development of CVD (Jakubowski, 2000; Clarke *et al.*, 2007). HcyTL is formed in all cell types, resulting from an error-editing of the met-tRNA synthetase when there is excess of Hcy. The interaction of HcyTL with proteins leads to protein homocysteinylation and loss of function (Jakubowski *et al.*, 2000). Therefore, detoxification of HcyTL is crucial, which in turn, is possible by the LACase activity of PON1 (Domagala *et al.*, 2006), contributing to its cardioprotective role. Regarding the polymorphisms modulation, the highest LACase activity was detected in 192QQ and also in 55LL individuals (Brophy *et al.*, 2001; Rainwater *et al.*, 2009).

Concerning HIV-infection, it is likely that PON1 contributes to the beneficial effects of higher HDL levels in HIV-infected patients. However, few or no data is available about the influence of HIV-infection and cART on PON1 activities, which can further reflect the quality of HDL on these patients. Nevertheless, the majority of the studies reporting PON1 activities in HIV-infected patients concern the POase activity. Usually, the patients have lower serum PON1 POase activity and higher PON1 concentration than the general population, and the activity is inversely correlated with the concentration of ox-LDL (Parra *et al.*, 2007). Both activity and concentration is thought to be influenced by HIV-infection, the alterations in HDL composition and the immunological state of the patients. Furthermore, a positive association was found between serum PON1 concentration and active viral replication (Parra *et al.*, 2007), which further suggests that PON1 could play a beneficial role in protecting patients from HIV-infection. This activity was also found to be significantly higher in patients treated with EFV than in patients without cART (naïve) (Pereira *et al.*, 2006). Another study through the POase activity reported that the enzyme appeared to be a marker for metabolic syndrome in HIV-population (Bobin-Dubigeon *et al.*, 2013). For the LACase activity, no differences were found between healthy and HIV-infected subjects regardless of cART use (Djeghader *et al.*, 2012).

1.4 Nevirapine two-faces: an high-density lipoprotein booster VS an hepatotoxic drug

Several studies reported that NVP raises HDL levels, resulting in an improvement of the atherogenic index of the HIV-infected patients (van der Valk *et al.*, 2001; van Leth *et al.*, 2004). The drug has been associated to an HDL increase up to 49%, which represents a more pronounced effect than the obtained with the currently available HDL-raising drugs, and is thought to be due to the stimulation of the apolipoprotein A-1 (Apo A-1) production (van Leth *et al.*, 2004; Sankatsing *et al.*, 2007; Franssen *et al.*, 2009).

NVP was the first NNRTI approved by the US Food and Drug Administration (FDA), in 1996, for the treatment of HIV type-1 infection, as part of cART (FDA, 1996). The drug acts by non-competitive inhibition of HIV-1 reverse transcriptase (Sweetman, 2008), and still is the most prescribed drug of its class, partly due to its low cost (Ades *et al.*, 2000; Lockman *et al.*, 2007). Furthermore, NVP has many features that can make it prone for its use in several conditions. The favorable metabolic profile conferred by the drug (Ruiz *et al.*, 2001; Clotet *et al.*, 2003; Srivanich *et al.*, 2010) turns it suitable for use in patients with diabetes, dyslipidemia or metabolic syndrome comorbidities. Moreover, the low incidence of adverse drug reactions in the central nervous system (Medrano *et al.*, 2008), in opposition to the other first-line NNRTI EFV, also allows its use in the context of psychiatric disorders or addiction to narcotic drugs. NVP is also highly efficient on the prevention of mother-to-child transmission of the HIV-1 infection, with the drug being commonly prescribed to pregnant women and their children (Ades *et al.*, 2000; Medrano *et al.*, 2008; Sweetman, 2008). Several studies have shown that NVP-based cART is capable of increasing plasma levels of HDL and that this effect is greater than the one expected from simply suppressing HIV alone. Hence, a NVP-based regimen could potentially reduce the cardiovascular risk for HIV-infected patients (van der Valk *et al.*, 2001; van Leth *et al.*, 2004; Fisac *et al.*, 2005).

While NVP is being monitored safely for long-term use in patients who are able to tolerate the initial regimen, the FDA has issued a black box warning to include information on hepatotoxicity associated with long-term use, and recommends against starting female patients on NVP if their $CD4^+$ count is >250 cells/µL and for male patients if their $CD4^+$ cell count is >400 cells/µL (Clotet, 2008).

Hence, the use of NVP has been associated with adverse toxicity reactions such as idiosyncratic hepatotoxicity and cutaneous hypersensitivity (Taiwo, 2006; De Lazzari *et al.*, 2008; Medrano *et al.*, 2008). These concerns arose following case reports of liver failure in individuals on post-exposure prophylaxis (Johnson and Baraboutis, 2000) and in asymptomatic HIV-infected patients with well-preserved immunity, administered NVP-containing first line cART (Cattelan *et al.*, 1999; Stern *et al.*, 2003). Clinically, most patients on NVP present with erythematous rashes often accompanied by fever, and in some cases with internal organ involvement (Pollard *et al.*, 1998). Nevertheless, a two week period of low dose treatment reduces the risk of rash (Montaner *et al.*, 2003), which is currently being applied as guideline.

However the mechanism that underlays this drug toxicity is still uncertain, several *in vitro* (Antunes *et al.*, 2008; Antunes *et al.*, 2010a; Antunes *et al.*, 2010b), in animal models (Shenton *et al.*, 2003; Chen *et al.*, 2008) and in man (Caixas *et al.*, 2012; Marinho *et al.*, 2013; Meng *et al.*, 2013; Sharma *et al.*, 2013) approaches have suggested that the bioactivation of the phase-I NVP metabolite 12-hydroxy-Nevirapine (12-OH-NVP), to reactive electrophiles, such as 12-sulfoxi-nevirapine (12-sulfoxy-NVP) is involved (Antunes *et al.*, 2008; Chen *et al.*, 2008; Antunes *et al.*, 2010a; Antunes *et al.*, 2010b; Caixas *et al.*, 2012; Pereira *et al.*, 2012).

Nevertheless, the less atherogenic lipid profile conferred by NVP could potentially make it a suitable component in first-line regimens for HIV-positive patients with multiple risk factors for cardiovascular events (van Leth *et al.*, 2004). Moreover, in second-line regimens the PI component

could be substituted for NVP in order to improve PI-initiated lipid abnormalities (Gil *et al.*, 2004; Fisac *et al.*, 2005).

1.5 Objectives

What is known?

1. CVD is currently the number one cause of mortality and morbidity in HIV-infected patients.
2. Low HDL levels are considered the most negative predictor of CVD.
3. NVP surely works as an HDL-modulator and its positive effect on HDL is greater than the available drugs with HDL booster properties.
4. Beyond HDL quantity, its quality is also relevant.
5. PON1 enzyme is responsible for the HDL antioxidant function.
6. PON1 is an enzyme synthesized by the liver and has three main functions: paraoxonase, arylesterase and lactonase.
7. NVP upon biotransformation gives rise to several phase-I metabolites.
8. The bioactivation of the phase-I metabolite 12-OH-NVP is a plausible mechanism underlying NVP hepatotoxicity.

What is needed to know?

1. What is the individual contribution of NVP and each one of its phase-I metabolites on PON1 activities?
2. Is PON1 a protective player against NVP toxicity?

Working hypothesis:

NVP is known to increase HDL levels (Sankatsing *et al.*, 2007), and can be considered a good alternative against the development of CVD or even in patients already with the condition. The current work aims at explore this HDL-booster effect provided by NVP as well as analyzing its quality, where PON1 is a major player.

However, NVP is associated with hepatotoxicity and skin rash (Yuan *et al.*, 2011), and the bioactivation of its phase-I metabolite 12-OH-NVP, through the formation of protein adducts, is a plausible mechanism underlying this toxicity (Caixas *et al.*, 2012) **(Fig. 1.3)**. These toxic effects can be potentiated due to the decrease in the antioxidant defenses presented in HIV-infection. As PON1 is an antioxidant enzyme, we hypothesize that may be it could also have a role on this context.

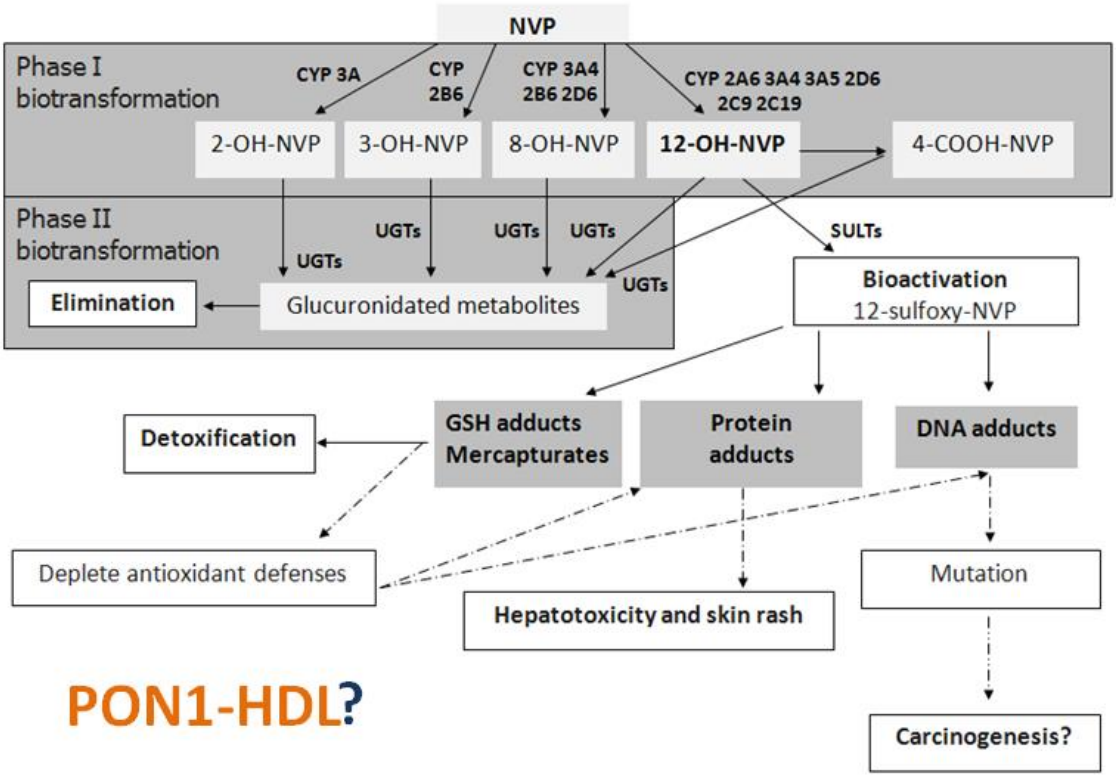

Figure 1.3 Nevirapine biotransformation, disposition and proposed bioactivation pathways (Marinho *et al*., 2013) (adapted). NVP is metabolized into its phase-I metabolites, by several isoforms of cytochrome P450 (CYP450), which are 2-hydroxy-nevirapine (2-OH-NVP), 3-hydroxy-nevirapine (3-OH-NVP), 8-hydroxy-nevirapine (8-OH-NVP) and 12-OH-NVP. This last metabolite is further oxidized by CYP450, giving rise to 4-carboxy-nevirapine (4-COOH-NVP). The UDP-glucoronosyltranferase (UGT) also represents a major pathway of NVP elimination. The bioactivation of 12-OH-NVP by SULTs can generate 12-sulfoxi-nevirapine (12-sulfoxy-NVP), which is a reactive metabolite that can bind to proteins and deoxyribonucleic acid (DNA).

PON1 is part of the endogenous detoxification system **(Fig 1.4)**. On one hand the enzyme is capable of detoxifying several endogenous toxic compounds (*e.g.* oxidized lipids and HcyTL) that can further lead to toxicity. On the other hand, PON1 enzyme can also contribute to the production of glutathione (GSH), another known antioxidant. As so, our work hypothesizes is that PON1 could have a protective role against the toxicity inherited to the use of NVP.

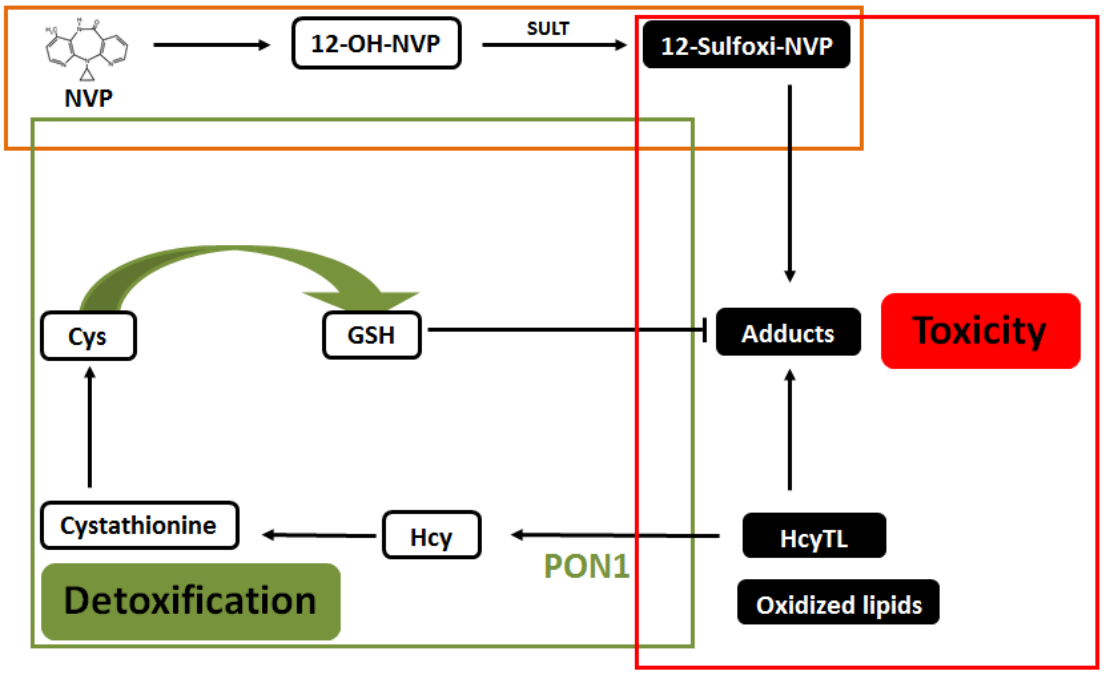

Figure 1.4 Work hypothesis. (PON1 - paraoxonase-1; HcyTL - homocysteine thiolactone; Hcy - homocysteine;; Cys – cysteine; Cys-Gly – cysteine-glycine; GSH – glutathione)

On this regard, the present study is aimed at (Fig. 1.5):

1. Develop and validate new methods for the quantification of the AREase and LACase activities, suitable for application in human blood.
2. Quantify the AREase, LACase and POase activities in HIV-infected patients under NVP-containing cART.
3. To explore the effect of chronic exposure of NVP and its phase-I metabolites on the three PON1 activities.

It is generally expected to give new insights on the mechanisms underlying NVP effects on HDL properties and to also give evidence for a plausible protective role of PON1 in the NVP-toxicity as well as give support to a rational prescription of lipid friendly cART in HIV-infected patients with or at increased of CVD development.

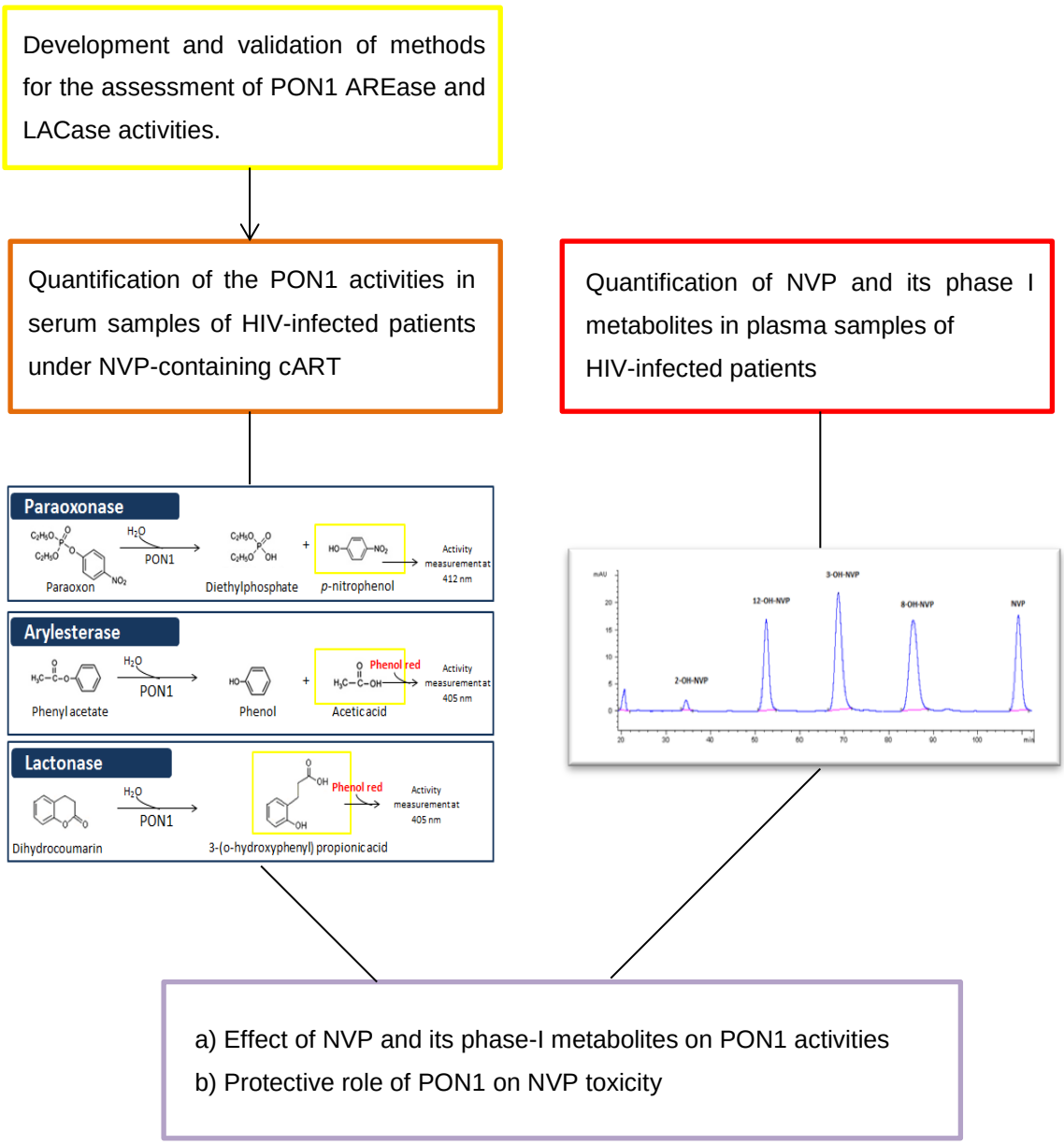

Figure 1.5 Graphic summary of the work plan. (PON – paraoxonase-1; AREase – arylesterase; LACase – lactonase; HIV – human immunodeficiency virus; NVP – nevirapine; cART – combined antiretroviral therapy; 2-OH-NVP – 2-hydroxy-nevirapine; 3-OH-NVP – 3-hydroxy-nevirapine; 8-OH-NVP – 8-hydroxy-nevirapine; 12-OH-NVP – 12-hydroxy-nevirapine)

2. Materials and Methods

2.1 Inclusion of patients, clinical data gathering and blood sampling

A group of healthy volunteers were included for the definition in method validation of the blood sampling conditions for the PON1 activities assessment. Blood samples were collected by venipuncture, at the *Centro Hospitalar de Lisboa Central, EPE*.

The current work was conducted in accordance with the Declaration of Helsinki. The study protocol received prior approval from the Ethics Committees of *Centro Hospitalar de Lisboa Central, EPE* (process number 32-CHLC) and *Hospital Prof. Doutor Fernando Fonseca, EPE* (process number CA 21/2011), and was also approved by the National Committees for Data Protection (process number 6567/2009). All patients gave their written informed consent and adherence was controlled by the clinicians. All patients were adults with documented HIV-infection, who had received continuous treatment with NVP-containing cART regimens (400 mg once daily) for more than 1 month, regardless of the past therapeutic history. Exclusion criteria were defined as being less than 18 years of age, having AIDS-defining conditions, and compliance issues. The following clinical data were gathered for each patient: age, sex, ethnicity, weight and height, time on NVP, viral load, CD4 T-cells count, and lipid parameters (*e.g.* HDL, LDL, TG and TC). Blood samples were collected by venipuncture.

2.2 PON1 activities assessment

2.2.1 Arylesterase activity of PON1

In order to assess PON1 AREase activity, a new method was developed and validated.

i. Rational

Although being considered a non-physiological activity, the measurement of the POase activity of PON1 enzyme using paraoxon as substrate has been the most widely used standardized method for the assessment of its status (Pereira *et al.*, 2009; Soyoral *et al.*, 2011). Therefore, the study of the AREase activity of PON1, which best reflects its antioxidative role, can potentially give additional information.

Despite the methods already available for this purpose (Beltowski *et al.*, 2002; Naderi *et al.*, 2011), they do not fulfill our aims of easy application in clinical setting. The rational for the method developed herein is taking advantage of the acetic acid production by the hydrolysis of phenyl acetate, a substrate of the AREase activity of PON1 **(Fig 2.1)**. The acetic acid formation can be monitored by the color variation of the titration with phenol red reagent. Briefly, a molecule of phenyl acetate is hydrolyzed into phenol and acetic acid. Hence, the acetic acid is produced in stoichiometric amounts

to the substrate hydrolysis. This reaction can be monitored spectrophotometrically, at 405 nm, by the color change of the phenol red reagent.

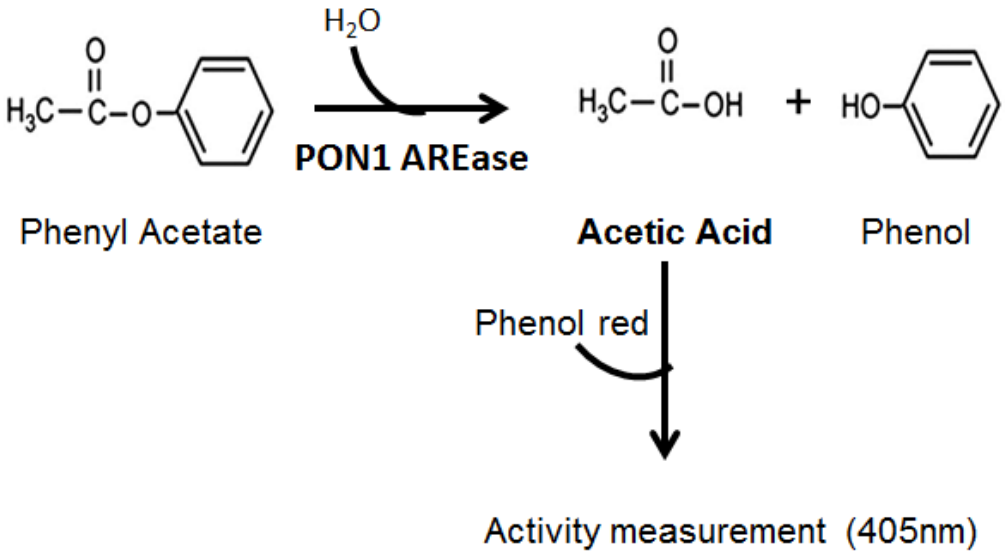

Figure 2.1 Method rational: hydrolysis of phenyl acetate by paraoxonase-1 and its monitoring for the assessment of the arylesterase activity. (AREase – Arylesterase; PON1 - paraoxonase-1)

ii. Standards preparation for the calibration curve

Stock solutions

The stock solutions were prepared by adding the appropriate amount of acetic acid (*M&B Laboratory Reagents*) and phenol (*Fluka*) in freshly prepared HEPES (*Roth*) buffer (2.0 mM, at pH 8.0), containing $CaCl_2$ (*BDH Chemicals Ltd Pool England*) (1.0 mM) and albumin from bovine serum (BSA) (*Roth*) (0.005%). Ideally, these solutions should be used right after its preparation and should be prepared in tubes covered in aluminum, since phenol is a photoreactive compound.

Standards preparation

For the preparation of the standards, the stock solutions were diluted in physiological serum in order to obtain six standards. Furthermore, a standard only with physiological serum and HEPES buffer (blank sample) was also prepared in order to correct the non-enzymatic hydrolysis of phenyl acetate.

iii. Standard operating procedure

The AREase activity was obtained by measuring the extent of the hydrolysis of phenyl acetate using a spectrophotometric method adapted for a 96-well microplate. Serum or plasma samples were diluted in the proportion of 1:5 in physiological serum and 10 µL were added *per* well. The samples and the previous prepared standards were incubated at 37 °C, during 10 minutes. Subsequently, 190 µL of freshly prepared HEPES buffer (2.0 mM, at pH 8.0) containing $CaCl_2$ (1.0 mM), BSA (0.005%), phenol red (*Fluka)* (106 µM) and 5.0 mM phenyl acetate (*Fluka*) (5.0 mM) were added to each well. The absorbance at 405 nm was measured on a microplate reader (*Biotrack II* plate reader, *Amersham Biosciences*). The activity was directly obtained from the calibration curve and expressed as kU/L, defined as the amount of enzyme producing 1 mM of acetic acid per minute. All samples/standards were analyzed in triplicate.

iv. Method validation

The validation criteria were defined according to guidebooks, regarding the validation of bioanalytical methods (Shah *et al.*, 1992; González and Herrador, 2007; EMA, 2011). For all validation purposes, each sample was analyzed in triplicate.

Linearity

Three calibration curves were prepared from different stock solutions and using six standards within the concentration range: 5.50 mM (lower limit of quantification, LLOQ) to 26.21 mM (higher limit of quantification, HLOQ). The calibration curves were constructed to explore the linearity of the method. Also, the slopes and Y-intercept (Y0) of the curves where compared in order to access reproducibility.

Lower limit of quantification

In order to validate the LLOQ, six samples with a concentration of 5.50 mM were analyzed for the accuracy and the intra-assay and the inter-assay precisions.

Accuracy

To study the accuracy of the method, six samples from the LLOQ and the HLOQ as well as 2 quality control samples (QC1 and QC2) in between the concentration range (12.58 mM and 16.78 mM) were analyzed. The accuracy was calculated according with the equation (1) and expressed in percentage (%)

$$\text{Accuracy (\%)} = \frac{\text{obtained concentration}}{\text{exact concentration}} \times 100 \qquad (1)$$

Precision

Intra-assay precision

The intra-assay precision was evaluated by analyzing six aliquots of the LLOQ, QC1, QC2 and HLOQ. These aliquots were analyzed on the same run.

The intra-assay precision was obtained by subtracting the variation coefficients (CV) of the analyzed aliquots, according with the equation (2). The calculation of the intra-assay precision was performed assuming that its value would ideally be 100%. Samples obtained from a healthy volunteer were also analyzed.

$$\text{Intra-assay precision (\%)} = 100 - CV \quad (2)$$

Inter-assay precision

For the study of this parameter, the same samples described in the previous sub-section were analyzed, albeit these analyses were performed in different runs. The inter-assay precision was calculated using the equation (3). Samples obtained from a healthy volunteer were also analyzed.

$$\text{Inter-assay precision (\%)} = 100 - \text{Inter-assay CV} \quad (3)$$

2.2.2 Lactonase activity of PON1

In the subsequent sections it is described the steps used to develop and validate a method to assess PON1 LACase activity.

i. Rational

The study of the LACase activity of PON1 also gives additional and relevant information than only using the POase activity for the assessment of PON1 status. Moreover, as the LACase activity is thought to be the primary activity of PON1, the interest is increased (Harel *et al.*, 2004). The development of new techniques for the quantification of this activity is of crucial importance, as this is the activity known to detoxify innumerous toxic lactones, including HcyTL, a major player in CVD (Jakubowski, 2000).

Several methods have already been proposed for the assessment of the LACase activity of PON1 (Billecke *et al.*, 2000; Gaidukov and Tawfik, 2005; Rock *et al.*, 2008), though all of them have inherited disadvantages in what concerns its clinical application. The method herein proposed is based on the production of 3-(o-hydroxyphenyl) proprionic acid (o-HPPA) when the lactone, dihydrocoumarin (DHC), is hydrolyzed by PON1 LACase activity **(Fig. 2.2)**. This reaction can be monitored by the color change from red to yellow of the titration with phenol red reagent. Briefly, a molecule of DHC is

hydrolyzed into a molecule of o-HPPA. Hence, the acid is produced in stoichiometric amounts to the amount of substrate hydrolysis. This reaction can be monitored spectrophotometrically, at 405 nm, by the color change of the phenol red reagent.

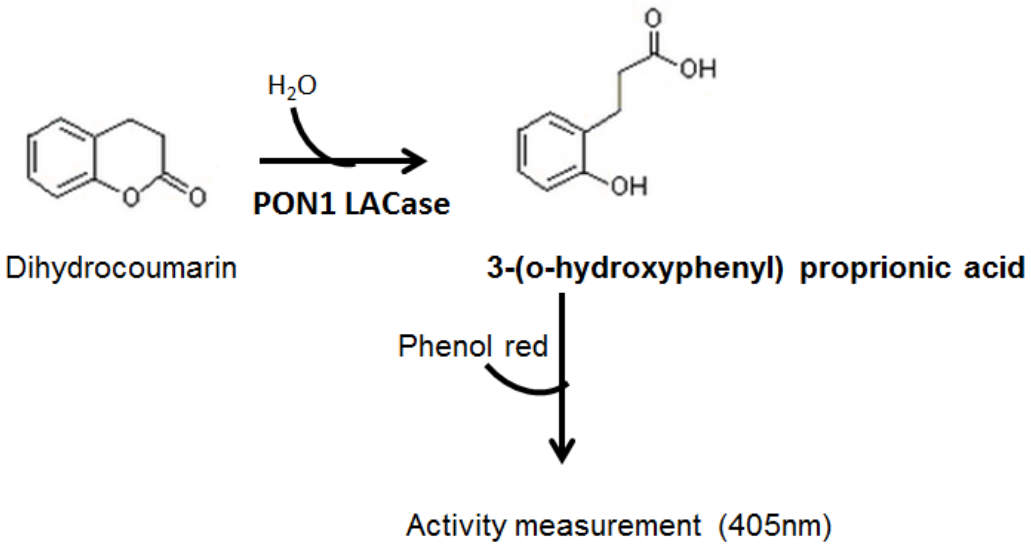

Figure 2.2 Method rational: hydrolysis of dihydrocoumarin by paraoxonase-1 and its monitoring for the assessment of the lactonase activity. (LACase – lactonase; PON1 - paraoxonase-1)

ii. Standards preparation for the calibration curve

Stock solutions

The stock solutions were prepared, by adding the appropriate amount of o-HPPA (*Sigma-Aldrich*) in freshly prepared HEPES buffer (2.0 mM, pH 8.0), containing $CaCl_2$ (1.0 mM) and BSA (0.005%).

Standards preparation

The standards were prepared with the same procedure used for the method for the AREase activity assessment.

iii. Standards operating procedure

The LACase activity was obtained by measuring the extent of the hydrolysis of DHC, using a spectrophotometric method adapted for a 96-well microplate. Serum or plasma samples were diluted in the proportion of 1:5 in physiological serum and then 10 µL were added to each well. The samples and also the previous prepared standards were incubated a 37 ºC, during 10 minutes, where upon 190 µL of freshly prepared HEPES buffer (2.0 mM, at pH 8.0) containing $CaCl_2$ (1.0 mM), BSA

(0.005%), phenol red (106 µM) and DHC (1.0 mM) (*Sigma-Aldrich*) were added *per* well. Then, after 1 minute of incubation at room temperature, the absorbance at 405 nm was measured on a microplate reader. The activity was directly obtained from the calibration curve and expressed as kU/L, which is defined as the amount of enzyme producing 1 mM of o-HPPA per minute. All samples/standards were analyzed in triplicate.

iv. Method validation

For the validation of this method, the same guidelines were used as for the method for the AREase activity assessment.

Linearity

The same procedure was applied, as it was for the method developed to quantify the AREase activity of PON1. The concentrations from the calibration curve ranged from 1.29 mM (LLOQ) to 10.24 (HLOQ).

Lower limit of quantification

The same procedure was applied, as it was for the method for the AREase activity assessment method, using the concentration of 1.29 mM as the LLOQ.

Accuracy

The same procedure was applied, as it was for the AREase activity quantification method. The QC1 and QC2 concentrations were 4.92 mM and 6.55 mM, respectively.

Precision

Intra-assay and inter-assay precisions

The same procedure was applied, as it was for the method to monitor the AREase activity.

2.2.3 Paraoxonase activity of PON1

The POase activity was assessed through the quantification of *p*-nitrophenol formation, as previously described by Batuca *et al*. (Batuca *et al.*, 2007). Briefly, paraoxon (1.0 mM) (Sigma-Aldrich) freshly prepared in 290 µL of 50 mM glycine buffer containing 1 mM CaCl2 (pH 10.5) was incubated with 10 µL of sample, at 37 °C, for 10 min, in 96 well plates (Polysorp). *P*-nitrophenol formation was

monitored at 412 nm and the activity was expressed as µmol *p*-nitrophenol, per mL of serum, per minute.

2.2.4 Blood sampling conditions definition

A group of five healthy volunteers were included for the definition of blood sampling conditions for the development and validation of the PON1 activities methods. Blood samples were collected from five healthy volunteers by venipuncture. Three types of samples were obtained: a) blood collected without anticoagulants, b) blood collected with lithium heparin and c) blood collected with ethylenediaminetetra-acetic acid (EDTA). Serum or plasma were aliquoted after centrifugation and then stored at -80 °C until analysis.

2.3 Effect of chronic exposure of nevirapine on PON1 activities in HIV-infected patients

2.3.1 Quantification of nevirapine and its phase-I metabolites in plasma of HIV-infected patients

The extraction and quantification of NVP and its phase-I metabolites was performed as previously described (Marinho *et al.*, 2013). Briefly, the analytes were extracted from plasma previously heated at 60 ºC for 60 minutes for viral inactivation, with dichloromethane (*VWR*, Radnor, PA). NVP was obtained from *Cipla* (Maharashtra, India) and the 2-OH, 3-OH, 8-OH, and 12-OH-NVP metabolites were synthesized at *Instituto Superior Técnico*, as already described (Grozinger *et al.*, 2000; Antunes *et al.*, 2011).

High-performance liquid chromatography (HPLC) analyses was performed on an Agilent 1100 Series system (*Agilent Technologies*, Santa Clara, CA) using a reversed-phase *Luna* C18 (2) column (250 mm × 4.6 mm; 5 µm; *Phenomenex*, Torrance, CA). The column temperature was 40 ºC, the injection volume was 100 µL, and ultraviolet (UV) absorbance was monitored at 254 nm. For each analyte, the LLOQ of the method was 10 ng/mL.

2.3.2 Quantification of PON1 activities

The three activities of PON1 were quantified, using the procedures already described in the previous sections, in serum samples of the selected HIV-infected population.

2.4 Statistical analysis

Statistical analysis was performed using *GraphPad Prism*® version 5.0 (Motulsky, 2007). Data was expressed as mean ± standard error of the mean (SEM), median [interquartile range, IQR] or frequencies (%), whenever applicable. The test of *Pearson* or the test of *Spearman* were used to explore correlations. Comparisons among groups were performed using *One-way ANOVA*, *Student t*-test or *Mann Whitney U* test, whenever applicable. The *F*-test was used to explore differences between the slopes and the elevations of the calibration curves in the validation of the methods. Variability among data was expressed in variation coefficient (CV), expressed as %.

3. Results

3.1 Development and validation of PON1 activities methods

3.1.1 Arylesterase activity of PON1

i. Linearity

The r^2 of the 3 calibration curves was 0.997 ± 0.003. There were no differences between the slopes and the elevations of the calibration curves.

ii. Lower limit of quantification

The accuracy and precision of the LLOQ were calculated in the next sub-sections and are presented in **Table 3.1**.

iii. Accuracy

The accuracy values obtained for the QC1, QC2 and HLOQ were between 90% and 103% **(Table 3.1)**.

iv. Precision

Intra-assay precision

The values obtained for the intra-assay precision were higher than 94% **(Table 3.1)**.

The AREase activity of the serum sample from the healthy volunteer was 115 kU/L, and the intra-assay precision was 97%.

Inter-assay precision

The inter-assay precision for all the samples evaluated was higher than 92% **(Table 3.1)**.

Table 3.1 Accuracy, intra-assay precision and inter-assay precision of the method for the arylesterase activity quantification.

Standard (mM)	Accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)
LLOQ (5.50)	90	94	92
QC1(12.58)	103	96	96
QC2 (16.78)	102	95	96
HLOQ (26.21)	100	98	97

LLOQ – lower limit of quantification; QC1 – quality control 1; QC2 – quality control 2; HLOQ - higher limit of quantification.

3.1.2 Lactonase activity of PON1

i. Linearity

The r^2 of the 3 calibration curves was 0.999 ± 0.0002 and there were no differences between the slopes and the elevations of the calibration curves.

ii. Lower limit of quantification

The accuracy and precision of the LLOQ are presented in **Table 3.2**.

iii. Accuracy

The accuracy values obtained for the QC1, QC2 and HLOQ were between 94% and 102% **(Table 3.2)**.

iv. Precision

Intra-assay precision

The values obtained for the intra-assay precision were higher than 93% **(Table 3.2)**. The serum LACase activity of the healthy volunteer was 17 kU/L, and the intra-assay precision was 94%.

Inter-assay precision

The inter-assay precision was higher than 92% for all the controls tested **(Table 3.2)**.

Table 3.2 Accuracy, intra-assay precision and inter-assay precision of the method for the lactonase activity quantification.

Standard (mM)	Accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)
LLOQ (1.29)	98	93	92
QC1(4.92)	102	96	94
QC2 (6.55)	97	98	97
HLOQ (10.24)	94	96	96

LLOQ – lower limit of quantification; QC1 – quality control 1; QC2 – quality control 2; HLOQ - higher limit of quantification.

3.2 Blood sampling conditions definition

In the current study, the serum, heparinized plasma and EDTA-plasma samples obtained from five healthy volunteers, were used to quantify the AREase, LACase and POase activities of PON1, in order to optimize and validate the blood sampling. The five volunteers were all caucasians, with ages between 22 and 27 years old and four were female.

3.2.1 Quantification of the PON1 activities

The different PON1 activities of the three samples tested are presented on **Table 3.3**. For the assessment of the effect of the anticoagulants used, the value of each activity obtained in serum samples was considered as 100%. The three activities were not influenced by heparin, while EDTA strongly inhibited all of them. Moreover, the male volunteer had the lowest AREase (47 kU/L for serum, 53 kU/L for heparinized plasma and 34 kU/L for EDTA-plasma) and LACase (9 kU/L for serum, 8 kU/L for heparinized plasma and 7 kU/L for EDTA-plasma samples) activities, though its POase activity had the highest values on the three samples (298 U/L for serum, 270 U/L for heparinized plasma and 148 U/L for EDTA-plasma samples).

There was a direct correlation between each one of the PON1 activities of serum and heparinized plasma (*Pearson* r=0.999, p<0.0001 for the AREase activity; *Pearson* r=0.992, p<0.0001 for the LACase activity and *Pearson* r=0.972, p=0.006 for the POase activity). However, this association failed to be proved for EDTA samples.

Table 3.3 Blood sampling conditions and PON1 activities.

		Sample		
	Parameter (Unit)	**Serum**	**Heparinized plasma**	**EDTA plasma**
AREase	**Activity (kU/L)**	106 ± 34	107 ± 32	43 ± 11
	p-value*	-	NS	<0.05
	Inhibition (%)	-	NS	55 ± 17
LACase	**Activity (kU/L)**	17 ± 5	14 ± 4	10 ± 2
	p-value*	-	NS	<0.05
	Inhibition (%)	-	NS	38 ± 14
POase	**Activity (kU/L)**	243 ± 41	228 ± 37	152 ± 61
	p-value*	-	NS	<0.05
	Inhibition (%)	-	NS	38 ± 19

NS: non-significant; **One-way ANOVA with Bonferroni post test for multiple comparisons.* (AREase – arylesterase; LACase – lactonase; POase – paraoxonase; EDTA - ethylenediaminetetra-acetic acid)

3.2.2 Evaluation of the association among the different PON1 activities in the different conditions tested

The POase activity was not related to the AREase or LACase activity in any of the different conditions tested. The same result was found regarding EDTA inhibition. Although, a strong association was found between the AREase and LACase activities in serum (Pearson r=0.950, p=0.014) and heparinized plasma samples (Pearson r=0.955, p=0.012), but not in EDTA samples.

3.3 Effect of chronic exposure of nevirapine on PON1 activities in HIV-infected patients

3.3.1 Anthropometric and clinical data of the included patients

A total of fifty-four HIV-infected patients (30 men) were included in this study. Thirty-two patients were Caucasian, twenty-one were African and 1 was Indian. Only 2 patients had detectable viral load. The patient's anthropometric and clinical data are shown in **Table 3.4**.

Table 3.4 Anthropometric and clinical data from the included patients.

Parameter (Unit)	Value
N	54
Proportion of men (%)	56
Proportion of non-Caucasians (%)	41
Age (years) [a]	46 [39-55]
Body weight (kg) [b,]	69 ± 2
Height (m) [b,]	1.68 ± 0.01
BMI (kg/m^2) [b,]	25 ± 1
Time on NVP (months) [a]	42 [23-84]
HDL (mg/dL) [a]	60 [47-68]
LDL (mg/dL) [a, c]	124 [77-154]
TG (mg/dL) [a]	105 [68-139]
TC (mg/dL) [a]	202 [170-221])
CD4 T-cells count (cell/mm^3) [a]	538 [378-680]

[a] Median[IQR]; [b] Mean ± SEM; [c] only 15 patients had available data for this parameter (N – number; BMI – body mass index; NVP – nevirapine; HDL – high-density lipoprotein; LDL – low-density lipoprotein; TG – triglyceride; TC – total cholesterol; CD4 – cluster of differentiation 4) (ideal values: HDL 42 - 88 mg/dL; LDL < 130 mg; TG: 40 – 150 mg/dL; TC <200 mg/dL)

3.3.2 Quantification of nevirapine and its phase-I metabolites

NVP and its phase-I metabolites concentrations are presented in **Table 3.5**. All patients had 8-OH-NVP levels below the quantification limit of the HPLC method.

Table 3.5 Nevirapine and its phase-I metabolites concentrations.

Analytes	Concentrations [a] (ng/mL)
NVP [a]	4,137 [3,388-4,991]
2-OH-NVP [a, b]	55.0 [24.2-106.8]
3-OH-NVP [a]	25.5 [17.6-35.7]
12-OH-NVP [a]	371.0 [250.7-513.7]

[a] Median[IQR]; [b] for 2-OH-NVP, only 29 patients had quantifiable concentrations. (NVP – Nevirapine; 2-OH-NVP – 2-hydroxy-nevirapine; 3-OH-NVP – 3-hydroxy-nevirapine; 12-OH-NVP - 12-hydroxy-nevirapine)

3.3.3 Influence of anthropometric and clinical data of patients on nevirapine and its metabolites concentrations

The influence of body weight and body mass index (BMI) as well as time on NVP-containing cART on the concentrations of NVP and its phase-I metabolites was assessed and is represented in **Table 3.6**.

Table 3.6 Influence of body weight, body mass index and time on nevirapine-containing combined antiretroviral therapy on nevirapine and its phase-I metabolites.

Parameter (Unit)	ng/mL			
	NVP	2-OH-NVP	3-OH-NVP	12-OH-NVP
Body weight (kg)	NS	NS	↓ r=-0.482, p<0.001 [a]	NS
BMI (kg/m^2)	↑ r=0.295, p=0.042 [b]	↓ r=-0.423, p=0.044 [a]	↓ r=-0.290, p=0.046 [a]	NS
Time on NVP (months)	NS	NS	NS	NS

[a] *Spearman* correlation; [b] *Pearson* correlation; NS – non significant (NVP – nevirapine; 2-OH-NVP – 2-hydroxy-nevirapine; 3-OH-NVP – 3-hydroxy-nevirapine; 12-OH-NVP – 12-hydroxy-nevirapine; BMI – body mass index; NVP - nevirapine)

Though both body weight and BMI might have an influence on nevirapine and its phase-I metabolites, time on NVP seemed to have no influence on them.

3.3.4 Assessment of the possible relations among the three activities of PON1 in HIV-infected patients

The activities of PON1 enzyme were quantified in all included patients and its mean values are presented in **Table 3.7**. A positive correlation was found between the AREase and POase activities (*Spearman* r=0.335, p=0.014) and also between the AREase and LACase activities (*Spearman* r=0.773, p<0.0001) **(Fig. 3.1)**. Even though there was a relation between the AREase and POase activities, the relation between the AREase and LACase activities was stronger. No such correlation was found between the POase and LACase activities **(Table 3.8)**.

Table 3.7 PON1 activities in serum samples.

PON1 activities	Value
AREase (kU/L) [a]	80 ± 35
LACase (kU/L) [a]	14 ± 8
POase (U/L) [a]	211 ± 61

[a] Mean ± SEM (PON1 – paraoxonase-1; AREase – arylesterase; LACase – lactonase; POase – paraoxonase)

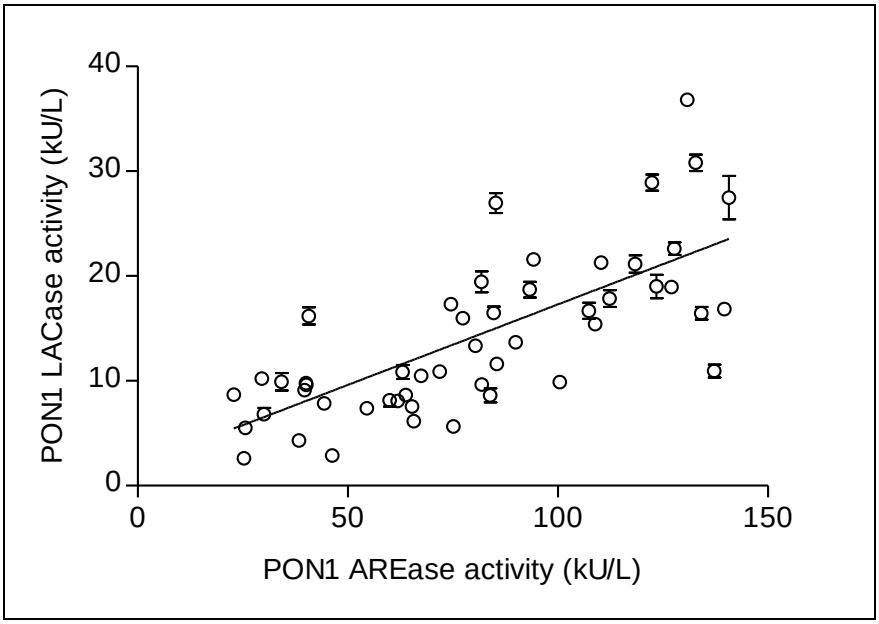

Figure 3.1 Correlation between PON1 AREase and LACase activities in serum samples of HIV-infected patients. (Spearman r=0.773, p<0.0001)

Table 3.8 Relations between the three activities of PON1.

	AREase (kU/L)	LACase (kU/L)
POase (U/L)	↑ r=0.335, 0.014 [a]	NS
AREase (kU/L)	NA	↑ r=0.773, p<0.0001 [a]

[a] *Spearman* correlation; NS – non significant; NA – not applicable (POase – paraoxonase; AREase – arylesterase; LACase – lactonase)

3.3.5 Association between the anthropometric and clinical data from the included patients and PON1 activities

The three assessed PON1 activities were associated with clinical data of the patients. The obtained results are presented in **Table 3.9**.

Regarding PON1 AREase activity, no significant sex differences were found. The activity was slightly correlated with age (*Spearman* r=-0.366, p=0.007), body weight (Pearson r=0.369, p=0.008) and BMI (*Pearson* r=0.337, p=0.017).

PON1 AREase activity was also positively correlated with HDL (*Spearman* r=0.326, p=0.022), TG (*Pearson* r=0.501, p=0.0002), TC (*Spearman* r=0.302, p=0.033) and negatively correlated with time on NVP treatment (*Pearson* r=-0.402, p=0.003).

For the LACase activity, no differences were found between women and men. On the other hand, a negative correlation was found between the activity and ageing (*Spearman* r=-0.293, p=0.039) and time on NVP-treatment (*Spearman* r=-0.294, p=0.038). Positive correlations were found for TG (*Pearson* r=0.531, p=0.0001), TC (*Spearman* r=0.321, p=0.026) and CD4 T-cells count (*Pearson* r=0.301, p=0.036).

As for the POase activity, a higher activity was found among women in comparison with men (p=0.032), but no effects of age were found. The activity was found to decrease with the increment of body weight (*Spearman* r=-0.289, p=0.042), though no correlation was found with BMI. Regarding clinical data, no correlation was found between the activity and LDL, TC and TG concentrations as well as with time on NVP treatment and CD4 T-cells count. A positive correlation was found between the activity and HDL concentration (*Spearman* r=0.290, p=0.044).

Table 3.9 Possible relations between the anthropometric and clinical data and PON1 activities

Parameter (Unit)	AREase (kU/L)	LACase (kU/L)	POase (U/L)
Gender	NS	NS	p=0.032 [c]
Ethnicity	NS	NS	NS
Age (Years)	↓ r=-0.366, p=0.007 [b]	↓ r=-0.293, p=0.039 [a]	NS
Weight (kg)	↑ r=0.369, p=0.008 [b]	NS	↓ r=-0.289, p=0.042 [a]
BMI (kg/m^2)	↑ r=0.337, p=0.017 [b]	NS	NS
Time on NVP (months)	↓ r=-0.402, p=0.003 [b]	↓ r=-0.294, p=0.038 [a]	NS
CD4 T-cells ($cell/mm^3$)	NS	↑ r=0.301, p=0.036 [b]	NS
HDL (mg/dL)	↑ r=0.326, p=0.022 [a]	NS	↑ r=0.290, p=0.044 [a]
LDL (mg/dL)	NS	NS	NS
TG (mg/dL)	↑ r=0.501, p<0.001 [b]	↑ r=0.531, p<0.001 [b]	NS
TC (mg/dL)	↑ r=0.302, p=0.033 [a]	↑ r=0.321, p=0.026 [a]	NS

[a] *Spearman* correlation; [b] *Pearson* correlation; [c] *Mann Whitney U* test; NS – non significant (AREase – arylesterase; LACase – lactonase; POase – paraoxonase; BMI – body mass index; NVP – nevirapine; CD4 - cluster of differentiation 4; HDL – high-density lipoprotein; LDL – low-density lipoprotein; TG – triglycerides; TC – total cholesterol)

3.3.6 Relationship between the assessed analytes and PON1 activities

The results obtained for the association between the PON1 activities and the concentrations of NVP and its phase-I metabolites are presented in **Table 3.10**.

The metabolite 3-OH-NVP seemed to have no influence, whilst 12-OH-NVP was the metabolite with the highest negative influence on the three PON1 activities (*Spearman* r=-0.305, p=0.031 for the AREase activity, *Spearman* r=-0.342, p=0.019 for the LACase activity and

Spearman r=-0.284, p=0.044 for the POase activity). Moreover, the effect of both metabolites 2-OH and 12-OH-NVP was more pronounced than the effect of NVP by itself.

As the LACase activity was only influenced by 12-OH-NVP, we thought to study the proportions of this metabolite. In fact, we found a high variability on 12-OH-NVP proportions, and patients who had lower formation of this metabolite had higher AREase and LACase activities (*Pearson* r=0.452, p=0.001 for the AREase activity, and *Pearson* r=0.292, p=0.044 for the LACase activity). Moreover, no differences were found between patients who had 2-OH-NVP concentrations above or below the limit of quantification on the three activities.

NVP or its phase-I metabolites were not correlated with HDL levels. Since 3-OH-NVP was the only analyte correlated with body weight, the relations between the three activities and 3-OH-NVP concentrations adjusted for body weight were also explored. However, no correlation was found.

Table 3.10 Correlations found between the analytes concentrations and PON1 activities.

Parameters (Unit)	AREase (kU/L)	LACase (kU/L)	POase (U/L)
NVP (ng/mL)	↓ r=-0.294, p=0.036 [a]	NS	↓ r=-0.290, p=0.037 [a]
2-OH-NVP (ng/mL)	↓ r=-0.461, p=0.023 [a]	NS	↓ r=-0.450, p=0.028 [b]
3-OH-NVP (ng/mL)	NS	NS	NS
12-OH-NVP (ng/mL)	↓ r=-0.305, p=0.031 [a]	↓ r=-0.342, p=0.019 [a]	↓ r=-0.284, p=0.044 [a]
NVP:12-OH-NVP	↑ r=0.452, p=0.001 [b]	↑ r=0.292, p=0.044 [b]	NS

[a] *Spearman* correlation; [b] *Pearson* correlation; NS – non significant (AREase – arylesterase; LACase – lactonase; POase – paraoxonase; NVP – nevirapine; 2-OH-NVP – 2-hydroxy-nevirapine; 3-OH-NVP – 3-hydroxy-nevirapine; 12-OH-NVP – 12-hydroxy-nevirapine)

4. Discussion

For the assessment of the quality of HDL in HIV-infection, a two-part study was herein undertaken. The first part was regarded to the development of new methods for the assessment of the AREase and LACase activities of PON1.

The development of microplate-based methods has allowed a high-throughput measurement of PON1 activity using paraoxon as substrate (Beltowski *et al.*, 2002; Naderi *et al.*, 2011). The same can be applied for the other identified activities of PON1, namely the AREase and LACase activities, avoiding in turn the use of higher amounts of both biological sample and remaining reagents.

In the present study, two simple, fast and inexpensive methods suitable for the measurement of the AREase and LACase activities of PON1 enzyme in human blood were developed and validated. These methods are capable of measuring the two activities in several samples simultaneously, using a very small amount of biological fluid and remaining solutions, which is a major advantage especially for research purposes, where the volume of the samples available is a serious limitation. Furthermore, as the assays are not performed at an UV, but at a visible spectrum range (405 nm), the use of quartz microplates is not required. The enzymatic kinetic can be performed in a spectrophotometer available at any research/clinical facility.

Regarding the AREase activity, the current available methods for its assessment monitor the formation of phenol at an UV range. However, the quantification of acetic acid instead of phenol allows the use of simple titration based-methods that can be monitored at a visible range, thus being unnecessary the use of sophisticated and expensive spectrophotometers, which might not be available in clinical routine labs. The enzymatic assay herein described is based on a method initially proposed by Sharp and Rosenberry (1982) for the measurement of the kinetic properties of acetylcholinesterase with its physiological substrate, acetylcholine (Sharp and Rosenberry, 1982). As in the hydrolysis of acetylcholine, the hydrolysis of phenyl acetate by PON1 produces acetic acid in stoichiometric amounts to the substrate degradation. By including a pH indicator dye such as phenol red, the color change resulting from the production of acetic acid can be monitored spectrophotometrically in the visible range and be directly related with the AREase activity. However, the use of phenol, which in turn is toxic and photoreactive, can be set as the major drawback of the proposed method. The use of toxic substrates is a handicap of PON1 measurement, as paraoxon is also toxic, requiring the use of appropriate safety precautions, such as wearing mask and gloves to protect against accidental contact or inhalation of the toxic fumes (Pereira *et al.*, 2009).

The enzymatic assay here proposed for the LACase activity of PON1 was also based on the same phenol red assay of Sharp and Rosenberry (Sharp and Rosenberry, 1982), modified later by Billecke and co-authors, in 1999 (Billecke *et al.*, 1999; Billecke *et al.*, 2000). Herein, the fact that the hydrolysis of DHC also produces an acid, the o-HPPA, was taken as an advantage. Likewise, the o-HPPA is produced in stoichiometric amounts to the degradation of DHC, which can be monitored spectrophotometrically in the visible range.

The levels obtained for the AREase and LACase activities in the serum samples of the healthy volunteers were found to be similar to the ones already obtained in other studies, which for the AREase activity had monitored phenol formation (Hernández *et al.*, 2009). These methods have applicability in plasma and serum samples, and since PON1 enzyme is associated with circulating HDL, the blood would be the main fluid of interest. However, these methods could possibly be adapted, with suitable changes, for other sample types, such as cell culture supernatants and even cerebrospinal fluids, further showing its applicability for clinical and research proposes.

An aspect there is sometimes mistreated is the blood sampling conditions, which for the case of PON1 activities assessment is very imperative. The enzyme activities measured in plasma are often lower than those measured in serum samples, due mainly to the ability of fibrin clots to retain a certain amount of water, resulting in a higher concentration of analytes in serum (Mackness, 1998b; Guthold *et al.*, 2004). Although, we showed that both methods are suitable for serum and heparinized plasma samples. To the best of our knowledge, this relationship was only previously demonstrated for the POase activity (Ferre *et al.*, 2005; Araoud *et al.*, 2011). Whereas serum is the preferred sample for the measurement of the POase activity, previous reports classified the effect of lithium-heparin on POase activity measurement as negligible (Ferre *et al.*, 2005). Several groups have reported studies on the POase activity in lithium-heparin-treated samples, and the results were lower but consistent with those obtained in serum samples (Jarvik *et al.*, 2002; Martín-Campos *et al.*, 2002). In the present study, the three activities measured in heparinized plasma were consistent with the ones obtained in serum samples, showing that the lithium-heparin-treatment has no significant influence on the AREase and LACase besides the POase activities of PON1 enzyme.

On the other hand, the present data showed that EDTA-containing samples should be completely avoided. The three activities of PON1 require calcium for their function (Kuo and La Du, 1998; Billecke *et al.*, 2000). The use of the anticoagulant EDTA is well known as unsuitable for the POase activity assay (Erdös and Boggs, 1961; Mackness, 1998a). In fact, EDTA-plasma samples had lower POase activity than serum samples by 38%, and was consistent with previous published studies using patients samples, with a mean inhibition of 41% (Araoud *et al.*, 2011). Moreover, a similar inhibition percentage was also found in studies using the purified PON1 enzyme (Golmanesh *et al.*, 2008). This decrease on activity was not dependent on the activity *per se*, nor related to the activity in serum or heparinized plasma samples. Given that PON1 requires calcium for both activity and stability, the presence of calcium chelators (*e.g.* EDTA and citrate) as anticoagulants is expected to inhibit its activities. Therefore, the inhibition of PON1 by EDTA has been reported for the measurement of the activities of the enzyme using a variety of substrates (Erdös and Boggs, 1961). Herein it was for the first time found that this inhibition is even more pronounced for the AREase activity, and for the LACase activity has a similar effect to the one found for the POase activity. As such, in studies where PON1 is involved, the blood collection conditions should be carefully defined and the use of plasma samples collected with EDTA should be avoided.

Lastly, when exploring the possible relations between the three different PON1 activities, the POase activity was not related with the AREase and LACase activities in the three different blood sampling conditions tested. The AREase and LACase activities were highly correlated. Despite data are conflicting on this issue (Nevin *et al.*, 1996; Camuzcuoglu *et al.*, 2009; Camuzcuoglu *et al.*, 2011), structure-activity studies might give some clues for this evidence. Firstly, a histidine dyad composed of His^{115} and His^{134} was suggested to be directly involved in the catalytic mechanism of PON1 for both ester (*e.g.* phenyl acetate) and phosphotriester (*e.g*. paraoxon) hydrolysis. Mutagenesis experiments support this mechanism although it was later found that these mutants were probably misfolded and, therefore, inactive (Harel *et al.*, 2004), undermining these results. Subsequently, Khersonsky and Tawfik (2006) showed, by site-directed mutagenesis, that the LACase and AREase activities were both mediated by the His^{115}-His^{134} dyad and that notably, the POase activity, which is a promiscuous activity of PON1, is mediated by other residues (Khersonsky and Tawfik, 2006). This evidence might explain the absence of relation between the POase activity and the two other activities and also the relation found between the AREase and LACase activities.

On the second part of this study the developed methods were applied into a cohort of HIV-infected patients that were under NVP-containing cART for at least one month. Besides its low cost and the efficient prevention of mother-to-child HIV-1 transmission, NVP has consistently been linked with a better lipidic profile in comparison with other antiretroviral drugs (van der Valk *et al.*, 2001; van Leth *et al.*, 2004). NVP has also been widely used in the simplification of PI-containing therapy (Harris, 2003), and the risk of major toxicities in this setting seems to be lower than in naïve patients (Mocroft *et al.*, 2007). Moreover, NVP was found to have a protective role on pre-diabetes (Srivanich *et al.*, 2010).

In the present study, no correlation was found between NVP concentrations and body weight. Several published studies, though not all of them (De Matt *et al.*, 2002), support the idea that body weight has no effect on NVP concentration (Kappelhoff *et al.*, 2005; Stöhr *et al.*, 2008; Marinho *et al.*, 2013). Additionally, only the concentrations of 3-OH-NVP were negatively correlated with body weight. The concentrations of 2-OH-NVP and 3-OH-NVP decreased with the increment of BMI, while NVP levels increased. As NVP is a highly lipophilic drug (Mirochnick *et al.*, 2000), it distributes readily across tissues and might probably accumulate on the less perfused tissues such as the fat tissue, further leading to an increase in its concentration. Nevertheless, NVP concentrations were previously reported to be reduced with higher BMI levels (Autar *et al.*, 2005). As for the PON1 activities, previous reports but not all of them (Abbott *et al.*, 1995; Veiga *et al.*, 2011), have shown a decrease of serum PON1 in obese patients (Ferretti *et al.*, 2005; Bajnok *et al.*, 2007; Bajnok *et al.*, 2008; Ozenoglu *et al.*, 2008; Ferretti *et al.*, 2010; Aslan *et al.*, 2011; Ferretti *et al.*, 2012a). In this study, the POase activity was decreased in patients with higher body weight, whilst the AREase activity was positively correlated with both body weight and BMI. A possible explanation for these results is the fact that only five patients had BMI levels higher than 30 kg/m^2, the number from which above a

person is considered to be obese (WHO, 1995), with the higher value observed of 33 kg/m^2, further suggesting that the selected population was not considered obese at all. However, no relation was found between PON1 and NVP and its phase-I metabolites concentrations when adjusting by body weight and BMI.

NVP has the capacity to increase HDL levels, in a way that the current available drugs for this purpose cannot: fibrates and statins have been associated with modest HDL changes (Birjmohun *et al.*, 2005) and niacin showed a moderate increase in HDL levels, but with no clinical benefits (Filippatos and Elisaf, 2013). The ability of NVP to increase HDL levels is indisputable, but the question of its quality is also a debatable issue.

Regarding the relation between the three PON1 activities assessed in HIV-patients, they were different from the ones found for the healthy volunteers. The POase activity was positively correlated with the AREase but not with the LACase activity. Moreover, the AREase and LACase activities were highly correlated. The relation found between the POase and AREase activities is consistent with previous reported studies in other pathological conditions (Camuzcuoglu *et al.*, 2009; Camuzcuoglu *et al.*, 2011; Ferretti *et al.*, 2012b). Also, a similar relation was found for the POase and LACase activities (Ferretti *et al.*, 2012b; Sztanek *et al.*, 2012). As so, it is possible to assume that there are different relations between the activities of PON1 in healthy and in pathological states, which can be probably due to an unknown mechanism that could change the structure of PON1 and, consequently, its activities. Moreover, the results of PON1 activities may depend on the methods used as well as the disease studied, hence the necessity to explore all its activities and standardize substrate and methods to explore them as possible disease biomarkers (Parra *et al.*, 2010b).

The fact that PON1 enzyme activities and levels vary broadly in humans (Costa *et al.*, 2005b) needs to be taken into account. Several differences were found between the different assessed activities when correlating them with anthropometric and clinical data from the HIV population. For instance, regarding sex differences, though the AREase and LACase activities were also higher in women than in men, only the POase activity was found to be statistically significant. Results from previous human and animals studies show that females have higher PON1 activity than males (Mueller *et al.*, 1983; Kleemola *et al.*, 2002; Rainwater *et al.*, 2005; Birjmohun *et al.*, 2009; Eom *et al.*, 2011). This higher levels are thought to be caused, for instance, by oral contraceptives intake (Rainwater *et al.*, 2009), which are mainly composed by estrogens. Though among the HIV population, the values of HDL are reported to be higher in women (El-Sadr *et al.*, 2005), the fact that NVP is as well more toxic on them (Marinho *et al.*, 2013) can be an excuse for the results found for the AREase and LACase activities, which are considered more physiological activities than the POase activity.

It is already known that serum PON1 levels and activities vary widely among diverse ethnic populations (Mohamed Ali and Chia, 2008), due mainly to genotypic differences (Sanghera *et al.*, 1997; Sanghera *et al.*, 1998; Poh and Muniandy, 2007), with the caucasian population

having lower values of the POase activity (Pereira *et al.*, 2009). However, on this study no differences were found between the ethnic groups, and yet nearly half population was non-caucasian. On the other hand, ageing was correlated with a decrease in both the AREase and LACase activities, which in turn was not observed in the POase activity. The negative effect of ageing on PON1 activities has been consistently reported (Jarvik *et al.*, 2002; Seres *et al.*, 2004; Cherki *et al.*, 2007; Ayotte *et al.*, 2011) and has been attributed to an increase in oxidative stress in the elderly population (Jaouad *et al.*, 2006). As so, this effect is expected to be more pronounced in the AREase and LACase activities as they are responsible for the detoxification of endogenous toxic compounds, oxidized lipids and HcyTL, respectively, which are known to be linked to this condition (Sentí *et al.*, 2003; Ferretti *et al.*, 2005; Rosenblat *et al.*, 2006; Mendes *et al.*, 2010; Bharathselvi *et al.*, 2013).

In what concerns the lipid parameters, the positive association between the AREase and POase activities and HDL was expected since PON1 is associated with this lipoprotein in the circulation (Sorenson *et al.*, 1999). This relation has been reported in other studies involving HIV-infected patients (Parra *et al.*, 2010b) healthy subjects (Ferre *et al.*, 2003; Boesch-Saadatmandi *et al.*, 2010), smokers (Mouhamed *et al.*, 2010) and also in clinical conditions where the lipidic profile is altered, including familial hypercholesterolemia (van Himbergen *et al.*, 2005). Though the lack of association between the LACase activity and HDL levels was a surprise, it was already reported (Gugliucci *et al.*, 2011). A possible explanation could be the fact that since HDL metabolism is impaired in HIV-infection, its relation with the three activities of PON1 can also be affected.

On the other hand, no correlation was found between the activities and LDL levels, though the inverse was already reported (Kotani *et al.*, 2009). In our study, only fifteen patients had reported LDL levels, which can probably undermine the nonexistence of such correlation. No further associations between the POase activity and other lipid parameters were observed, which was consistent with previous studies (Sumegová *et al.*, 2007; Toy *et al.*, 2009). Correlations between TG and TC were found for both AREase and LACase activities. These correlations could be expected, as these activities are considered the physiological ones. However, the fact that the mean TG levels among the population were not extremely high needs to be taken into account.

A negative correlation between the AREase and LACase activities and time on NVP-containing cART was found, which, to the best of our knowledge, was never reported. As the initiation of NVP needs to be carefully monitored with CD4 T-cell count (Leith *et al.*, 2005), the effect of this parameter on PON1 activities was also explored, though only the LACase activity was related with it. Relations between CD4 T-cell count and the POase activity were previously described by our group (Pereira *et al.*, 2009) and others (Parra *et al.*, 2007), though the studied populations were under different cART regimens, which can induce differences on the these parameter. This positive relation between CD4 T-cell count and the LACase activity further indicates lower PON1 activities is associated with a more severe infection.

Though NVP was already found to raise HDL levels in HIV-infected patients, through an increase in Apo A-1 expression (Fisac *et al.*, 2005), no such correlation was found in this study. However, as there was no access to the HDL baseline values, it is impossible to know if there was any kind of increment on its concentration since then. Our group has previously show that EFV only increased HDL levels in patients who had baseline value of HDL lower than 40 mg/dL (Pereira *et al.*, 2006). In terms of raising the HDL quality, there was also some controversy, as the AREase and POase activities decreased with the increment of NVP, while no correlation was found with the LACase activity. These results can further demonstrate that although NVP could raise HDL in terms of quantity, its quality, as far as it concerns its antioxidant potential, could be quiet doubtful, supporting the current idea that HDL function is defective in this population (Parra *et al.*, 2007; Daminelli *et al.*, 2008; Pereira *et al.*, 2009).

The association between HIV-infection and HDL metabolism is already known. The depletion of the cholesterol efflux reduces the production of the viral particles (Ono and Freed, 2001). In macrophages, the HIV-1 negative regulatory factor (Nef) accessory protein inhibits the cholesterol efflux via ATP-binding cassette transporter (ABCA1) (Mujawar *et al.*, 2006). Moreover, in HIV-infected patients, the concentrations and activities of both lecithin-cholesterol acyltransferase (LCAT) and cholesterylester transfer protein (CETP) are increased, which can divert the latter steps of RCT from delivery of cholesterol directly to the liver (Rose *et al.*, 2008). Though the liver, through LDL receptors, will take up the majority of transferred cholesterol, the amount of cholesterol available for modification and uptake by extrahepatic tissues will also increase proportionally. If this situation persists over a long period of time, it can potentiate the development of atherosclerosis. These changes in HDL can be reflected on the activities and function of PON1 (Rose *et al.*, 2008).

Regarding NVP metabolism, there are many potential reactive metabolites inherited to the drug. In both humans and rats, the major metabolic pathways of NVP involve 2-, 3-, and 12-hydroxylation (Riska *et al.*, 1999a; Riska *et al.*, 1999b). The first two are para-positions to a nitrogen atom, and further oxidation can lead to quinoneimine type reactive metabolites, whilst 12-OH-NVP has the potential to be sulfated (*e.g.* 12-sulfoxy-NVP) followed by loss of sulfate to form a reactive quinone methide (Spahn-Langguth and Benet, 1992). Though the associations between the NVP and its phase-I metabolites and PON1 activities were never reported, several assumptions can be made. Firstly, the metabolite 3-OH-NVP seems to have no influence at all on the activities of PON1. On the other hand, 12-OH-NVP was found to reduce all the activities. Moreover, 2-OH-NVP concentrations were also found to reduce the AREase and POase activities. It was also possible to see that 2-OH and 12-OH-NVP had a more exacerbated toxic effect on the activities than NVP by itself, further supporting the idea that NVP is toxic upon its biotransformation (Caixas *et al.*, 2012). When analyzing the absence of relation between NVP concentrations and the LACase activity, it was thought that this could be due to the variability in 12-OH-NVP proportions among patients and also because it is the major NVP metabolite. In

fact, we found a 35% of variability on the proportions of the metabolite (data not shown), and patients who had lower 12-OH-NVP formation had higher LACase and AREase activities. As so, the negative correlation found between the activities and 12-OH-NVP concentrations and proportions was already expected, as this metabolite has been consistently associated with adverse-NVP reactions (Antunes *et al.*, 2008; Chen *et al.*, 2008; Antunes *et al.*, 2010a; Antunes *et al.*, 2010b; Caixas *et al.*, 2012; Pereira *et al.*, 2012; Marinho *et al.*, 2013; Meng *et al.*, 2013; Sharma *et al.*, 2013).

The study of PON1 in HIV-infection is still limited. Few studies are available, and the majority of them refers to the POase activity (Parra *et al.*, 2007; Daminelli *et al.*, 2008; Pereira *et al.*, 2009), which, as already mentioned, could not totally reflect the real physiological capacity of the enzyme. Moreover, most studies have inherited disadvantages, which can undermine their results, such as the inclusion of both patients treated for the first time and with cART experience and with different co-infections (*e.g.* hepatitis C). For instance, there were two studies describing that HIV-infected patients had lower PON1 POase activity than HIV-negative individuals, regardless of cART (Parra *et al.*, 2007; Daminelli *et al.*, 2008). A cross-sectional study on the POase activity in HIV-infected patients, concluded that EFV-containing cART increased the activity (Pereira *et al.*, 2009). Recently, a study was published concluding that PON1 appeared to be a marker for the metabolic syndrome on HIV-infected patients (Bobin-Dubigeon *et al.*, 2013). Till this time, every study performed with PON1 had never been done correlations with the drugs or even its metabolites concentrations. For the LACase activity, there is one report regarding the DING proteins, which have been described as possessing anti-HIV activity (Berna *et al.*, 2009). Apparently, these proteins share with PON1 a relation with the human phosphate binding protein (HPBP). Though not directly related with the study, they reported that there were no differences on the LACase activity assessed in HIV-infected patients and in healthy volunteers (Djeghader *et al.*, 2012). However, it is needed to take into account that more than half patients were co-infected with hepatitis C. In 2010, Parra and colleagues also reported the methodological constraints in interpreting serum PON1 activity measurements. Both POase and LACase activities were higher in control subjects than in HIV-infected patients. They also concluded that the results of clinical studies on PON1 may vary depending on the methods used (Parra *et al.*, 2010b). For the AREase activity, the information is even scarcer. Only one abstract from an oral presentation was found, reporting that the levels of AREase activity were lower than the value for healthy controls, further suggesting that immunosuppression in HIV-infected patients could affect PON1 and its subsequently antioxidant effect (Tungsiripat *et al.*, 2012).

In summary, two reproducible, reliable and suitable methods for the monitoring of the AREase and LACase activities of PON1 in human blood are here described. The methods allow the assessment of these activities on a large number of samples in a short period of time, which is especially advantageous for clinical application. As far as we know, this study reports, for the

first time, the effect of different blood sampling collection on the AREase and LACase activities. Currently, there are no new updates on what regards the substrates available for the measurement of the AREase activity. In what regards the LACase activity, HcyTL, which is the alleged physiological substrate of PON1, has only recently become available for purchase. However, concerning has been rising on whether or not this naturally endogenous substrate is appropriate for the measurement of PON1 enzyme activity (Yilmaz, 2012). This only could be verified by building up a new method using this substrate, which is currently underway.

On the other hand, herein is also reported for the first time the effect of NVP and its phase-I metabolites on the activities of PON1. Though NVP is known as an HDL booster, in this study we saw a negative effect of the drug on PON1 activities, which might be due to the effect of its biotransformation products and not a particularly effect of the drug itself. It was clearly observed that the metabolite 3-OH-NVP had no influence on the activities of PON1, while 12-OH-NVP metabolite could be responsible for the noted decrease of the activities, once again proving the negative effects of this particular metabolite. Furthermore, it is plausible to assume that PON1 could have a potential role on the detoxification of the toxicity state conferred by the bioactivation of the 12-OH-NVP metabolite, mainly because the enzyme has the capability to detoxify HcyTL, which can further led to adduct formation. On the other hand, as PON1 also contributes to the production of GSH, which is another antioxidant known to have an effect on adducts (Chung *et al.*, 2005) **(Fig 1.4)**. Further studies are needed in order to clearly unveil the mechanisms involved on this protective function, but the study of the three known PON1 activities and the effect of different adducts-producing ARVs *per se* and in combination, namely other NNRTIs (EFV) or ABC, by probably an *in vitro* incubation, could be important in order to assess if the enzyme can, in fact, have a protective role against this toxicity.

On the other hand, the field for developing new drugs with HDL-booster effects is an appealing area, as the current efforts have failed. For instance, torcetrapib, a CETP inhibitor, showed a unacceptable safety profile (Tall *et al.*, 2007). Moreover, dalcetrapib, another CETP inhibitor, seems to have no influence on cardiovascular outcome (Filippatos and Elisaf, 2013).

Along with the study of PON1 in HIV-infection and CVD and with the background acquired, the candidate attempted to study this enzyme in brain tumorigenesis process (**Fig. 4.1**), a project that is currently under review for a scholarship for the candidate, funded by *Liga Portuguesa Contra o Cancro* and *Pfizer Laboratories*. Briefly, it is possible to draw a square using oxidative stress, antioxidant thiols, lipid peroxidation and carcinogenic processes as vertices, and the role of PON1 on the dynamic of this cycle remains to be explored.

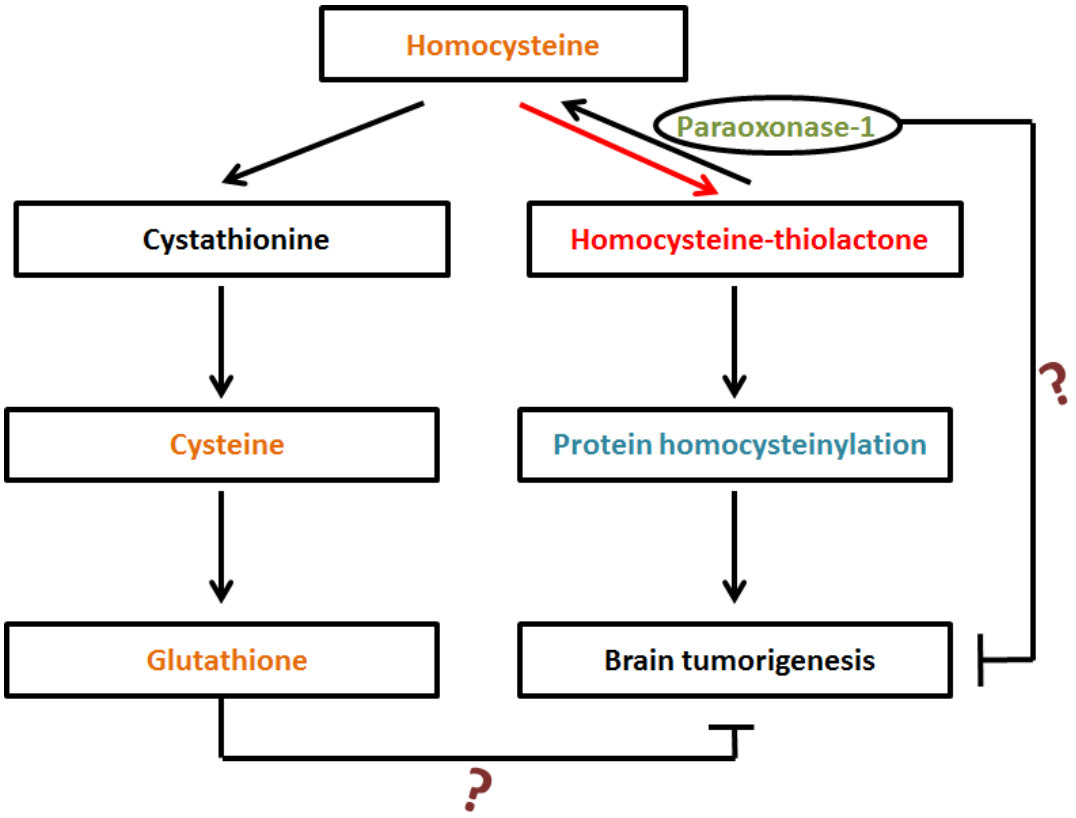

Figure 4.1 Schematic representation of the biochemical interactions where PON1 can be a player in brain tumorigenesis.

5. References

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